British Journal of Pharmacology

February 1991

Volume 102 Number 2 pages 287–550

Br. J. Pharmacol. (1991), **102**, 289–296 © Macmillan Press Ltd, 1991

(+)-Isradipine but not (−)-Bay-K-8644 exhibits voltage-dependent effects on cat adrenal catecholamine release

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1 Catecholamine release from cat adrenal glands perfused at a high rate (4 ml min^{-1}) at 37°C with polarizing (1.2 or 5.9 mM K^+) or depolarizing (17.7, 35, 59 or 118 mM K^+) solutions, was triggered by 5 or 10 s pulses of Ca^{2+} (0.5 or 2.5 mM) in the presence of various concentrations of K^+.

2 In polarized glands, secretion was greater the higher the K^+ concentration present during the secretory K^+/Ca^{2+} test pulse. Thus, in 17.7 mM K^+, catecholamine released was 162 ± 27 ng per pulse, while in 118 mM K^+ secretion rose to 1839 ± 98 ng per pulse. In depolarized glands, secretion reached a peak of around 1000 ng per pulse in 35–59 mM K^+; in 118 mM K^+, secretion did not increase further, suggesting that voltage changes are implicated in the control of the secretory process.

3 Blockade of secretion by increased concentrations of (+)-isradipine was much more manifest in polarized glands. The higher the degree of depolarization was (35, 59 or 118 mM K^+), the lower the IC_{50}s were. So, the ratios between the IC_{50}s in polarized and depolarized glands rose from 3.92 in 35 mM K^+ to 26.7 in 118 mM K^+.

4 In contrast, the Ca^{2+} channel activator (−)-Bay K 8644 potentiated catecholamine release evoked by K^+/Ca^{2+} pulses equally well in polarized or depolarized glands. The ratios between EC_{50}s in polarized or depolarized glands were, respectively, 0.30, 0.59 and 0.69 for 17.7, 35 and 118 mM K^+.

5 In simultaneous experiments, the two enantiomers of Bay K 8644 exhibited opposite effects on secretion. (+)-Bay K 8644 (a Ca^{2+} channel blocker) inhibited secretion better in depolarized than in polarized glands, whilst (−)-Bay K 8644 potentiated secretion in a voltage-independent manner.

6 Potentiation of secretion by (−)-Bay K 8644 was concentration-dependent from 10^{-8} to 10^{-6} M. At 10^{-5} M, such potentiation largely disappeared in both polarized and depolarized glands. However, this dual effect of (−)-Bay K 8644 was better seen in depolarizing conditions, suggesting that using the same enantiomer, the voltage-dependence is only seen when blockade of secretion dominates.

7 In the presence of increasing concentrations of (−)-Bay K 8644 (3×10^{-9}, 3×10^{-8} and 3×10^{-7} M), the concentration-response curves for (+)-isradipine to inhibit secretion were displaced to the right. However, a Schild plot of (dose ratio − 1) against (−)-Bay K 8644 concentrations gave a slope of 0.6, suggesting that the interactions between (+)-isradipine and (−)-Bay K 8644 were non-competitive in nature. The pA_2 for (−)-Bay K 8644 was 9.13.

8 Overall, the results suggest that potentiation of secretion by (−)-Bay K 8644 (a voltage-independent phenomenon), and blockade by (+)-isradipine or (+)-Bay K 8644 (a voltage-dependent phenomenon) might be exerted through binding of the dihydropyridines activators and blockers to separate sites on chromaffin cell L-type Ca^{2+} channels.

Introduction

Voltage-dependence of 1,4-dihydropyridine (DHP) derivatives has been established chiefly on the basis of their effects on Ca^{2+} current kinetics (Lee & Tsien, 1983; Sanguinetti & Kass, 1984a,b; Bean, 1984; Uehara & Hume, 1985; Gurney *et al.*, 1985; Sanguinetti *et al.*, 1986; Brown *et al.*, 1986; Rane *et al.*, 1987). Though this approach is important in establishing the mechanism of action of DHPs on the L-type Ca^{2+} channel kinetics, information on how the cell membrane potential might condition the pharmacological effects of these drugs in various tissues is lacking. Functional studies are, therefore, clearly needed.

For various reasons, the perfused cat adrenal gland constitutes a unique model to approach such a problem (see review by Artalejo *et al.*, 1988a). $^{45}Ca^{2+}$ uptake into, and catecholamine release from its medullary tissue are highly sensitive to DHP. In addition, secretion can be triggered by brief (5–10 s) pulses of Ca^{2+}-plus K^+-containing solutions; in this manner, little time is allowed for DHPs to change their equilibrium receptor binding reached during the pre-pulse perfusion period with various K^+ concentrations in the absence of Ca^{2+}. Using these protocols, we have demonstrated previously that under depolarizing conditions, [^{3}H]-nitrendipine binding to adrenal medullary tissue is markedly increased; this is accompanied by an enhanced blockade of $^{45}Ca^{2+}$ uptake into and catecholamine release from cat adrenal glands (Artalejo *et al.*, 1988b). The IC_{50} for nitrendipine to block Ca^{2+}-evoked secretion was 214 nM in polarized glands and only 0.99 nM in depolarized glands; so, the DHP was 215 fold more potent in depolarizing conditions (López *et al.*, 1989).

The question arises as to whether the DHP Ca^{2+} channel activator Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) also exhibits voltage-dependence in its well established potentiating effects of secretion in the cat adrenal gland (García *et al.*, 1984; Montiel *et al.*, 1984). Here, we have studied the effects of (−)-Bay K 8644 on Ca^{2+}-evoked catecholamine release from cat adrenal glands perfused with various K^+ concentrations to achieve different degrees of depolarization of their medullary chromaffin cells. We selected the pure (−)-enantiomer of Bay K 8644 since its (+)-enantiomer exhibits Ca^{2+} channel-blocking effects (Franckowiak *et al.*, 1985); in this manner, we expected to obtain clearer results. To contrast the voltage-independence of the activator, we selected the pure enantiomer DHP blocker (+)-isradipine (PN200-110), which

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is a potent inhibitor of K^+-evoked secretion in the cat adrenal gland (Gandía et al., 1987).

Methods

Cats of either sex weighing 2.5–4 kg were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.). Both adrenal glands were dissected and prepared for retrograde perfusion as described by García et al. (1980). Glands were perfused at a rate of 4 ml min^{-1}, at 37°C with Krebs-HEPES solution pH 7.4, continuously bubbled with pure O_2 and having the following composition (mM): NaCl 144, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10 and glucose 11. Glands were initially perfused with this solution for 60 min to allow their equilibration with the experimental conditions. Modified solutions will be abbreviated with the mM concentrations and the symbol of the ions affected; for instance, a solution 5.9 K^+/0 Ca^{2+} means a Krebs-HEPES solution lacking Ca^{2+} and containing 5.9 mM K^+.

Experimental design

To study the blocking effects of (+)-isradipine on Ca^{2+}-evoked catecholamine release from polarized or depolarized glands, the following protocol was selected (Figure 1). After 1 h equilibration the polarized gland was continuously perfused with a 5.9 K^+/0 Ca^{2+} solution and at 15 min intervals, secretion was tested by perfusing during 5 s 35 K^+/2.5 Ca^{2+}, 59 K^+/2.5 Ca^{2+} or 118 K^+/2.5 Ca^{2+}; each test pulse contained the same K^+ concentration as in the depolarizing conditions of the contralateral gland. The depolarized gland was perfused continuously with 35 K^+/0 Ca^{2+}, 59 K^+/0 Ca^{2+} or 118 K^+/0 Ca^{2+} and at 15 min intervals secretion was tested by reintroducing 2.5 mM Ca^{2+} during 5 s.

In the case of (−)-Bay K 8644, the following protocol was used (Figure 4). After 1 h, two glands from the same cat were stimulated to secrete catecholamines with a fourth-step pattern of perfusing solutions: (1st) both glands were perfused for 15 min with 1.2 K^+/0 Ca^{2+}; (2nd) one gland was perfused for an additional 10 min with this solution (polarized gland) and the contralateral with varying solutions containing high K^+ concentrations (17.7, 35 or 118 mM) but no Ca^{2+}; (3rd) 10 s with 17.7 K^+/0.5 Ca^{2+}, or 35 K^+/0.5 Ca^{2+} or 118 K^+/0.5 Ca^{2+} (secretion test pulses, respectively, for the glands previously depolarized with 17.7 K^+/0 Ca^{2+}, 35 K^+/0 Ca^{2+} or 118 K^+/0 Ca^{2+}); and (4th) 50 s back to 1.2 K^+/0 Ca^{2+} solution (polarized glands) or their respective 17.7 K^+/0 Ca^{2+}, 35 K^+/0 Ca^{2+} or 118 K^+/0 Ca^{2+} solutions (depolarized glands), to collect the secreted catecholamines in 15 s periods. These stimulation cycles were repeated 8 times in each pair of glands at 25 min intervals.

Control glands were exposed to vehicle only (ethanol). (+)-PN200-110, (+)-Bay K 8644 or (−)-Bay K 8644 were tested by adding increasing concentrations (10^{-10}–10^{-5} M) during steps 2, 3 and 4 of each stimulation cycle. DHP were dissolved in ethanol; appropriate dilutions were performed in perfusing solutions. All experiments were performed under sodium light.

Catecholamine assay and quantitation of results

Catecholamines present in each collection sample (media were acidified with perchloric acid to 0.05 N) were measured fluorometrically according to Shellenberger & Gordon (1971). Data were expressed as μg of total catecholamine release per Ca^{2+} test pulse and are means ± s.e.mean. To calculate the concentrations of drugs blocking the secretory process by 50%, the sigmoid inhibition curves were converted into straight lines by plotting values in the ordinates as log (Y/100 − Y); the intercepts with the abscissae (Y = 0) gave the IC_{50}s.

Drugs and chemicals

(+)-Bay K 8644 and (−)-Bay K 8644 were a kind gift of Prof. F. Hoffmeister, Bayer A.G., Wuppertal, F.R.G. (+)-PN200-110 ((+)-isradipine) was kindly provided by Dr R.P. Hof, Sandoz, Basel, Switzerland. All other chemicals were reagent grade.

Results

Catecholamine release evoked by brief pulses of calcium plus potassium

The amount of catecholamine secreted into the perfusion fluid, upon challenging the glands with brief applications of K^+/Ca^{2+} pulses is shown in Figure 2. Catecholamines collected in each challenge depended on two variables: the K^+ concentration present in the test solution containing

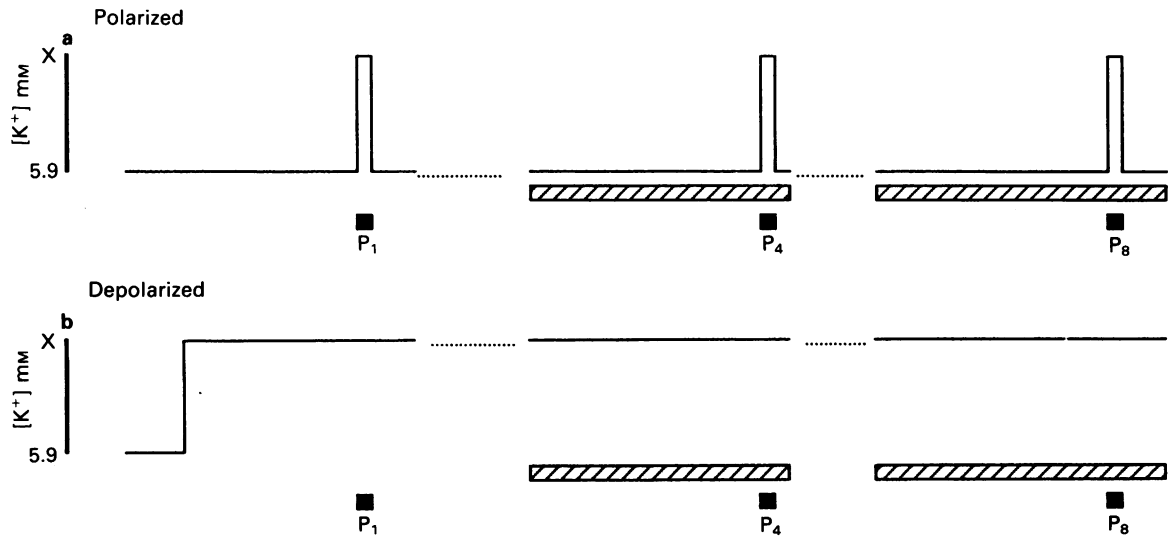


Figure 1 Diagram of the protocol used to study voltage-dependence of blockade of secretion by (+)-isradipine in the perfused cat adrenal gland. The polarized gland (a) is continuously perfused with Krebs solution containing 0 Ca^{2+}/5.9 mM K^+ and the depolarized gland (b) is perfused with a solution containing 0 Ca^{2+} and X K^+ (X = 35, 59 or 118 mM). Every 15 min both glands are challenged with a secretory pulse of 2.5 mM Ca^{2+} 5 s^{-1} (■) and X mM K^+ (the same concentration as the one used in the depolarized gland) for 5 s. The total amount of K^+/Ca^{2+} pulses per experiment are eight: from P_1 to P_8. For a given concentration-response curve, the drug is perfused from the fourth pulse (P_4) onwards, when responses were stabilized. Hatched bar indicates presence of drug.

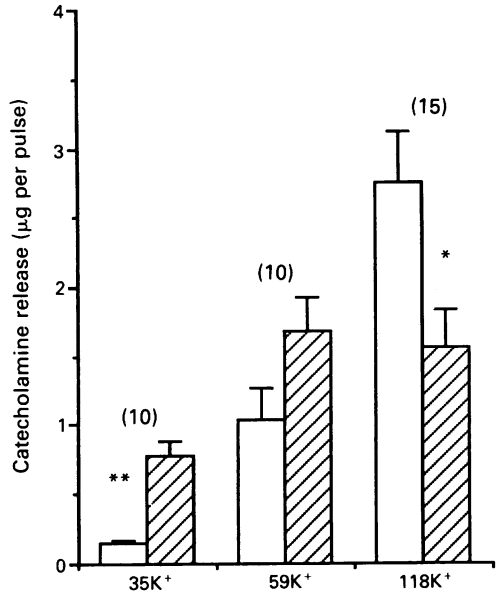


Figure 2 Catecholamine released by K^+/Ca^{2+} pulses from cat adrenal glands which were polarized (open columns) or depolarized (hatched columns) before the application of such pulses (see Figure 1). Pairs of glands from the same animal were taken for each individual experiment. One was perfused continuously with Krebs solution lacking Ca^{2+} and containing 5.9 mM K^+ (polarized glands); the contralateral, with a solution also deprived of Ca^{2+} but containing 35, 59 or 118 mM K^+. At 15 min intervals, these solutions were changed during 5 s to another containing 2.5 mM Ca^{2+} and 35, 59 or 118 mM K^+. In each pair of glands, a single K^+ concentration was tested, both at the depolarizing solution in the absence of Ca^{2+} and during the K^+/Ca^{2+} test pulse. Data were pooled from 8–12 pairs of glands; they represent means ± s.e.mean of the number of K^+/Ca^{2+} pulses shown in parentheses. * $P < 0.05$; ** $P < 0.01$, paired comparisons.

2.5 mM Ca^{2+} and whether chromaffin cells were depolarized or polarized at the moment of application of the K^+/Ca^{2+} pulse. When 35 mM K^+ was used, secretion was substantially poorer under polarizing, than under depolarizing conditions. In 59 mM K^+, the secretory response was slightly, but significantly smaller in polarized than in depolarized glands; on the contrary, in 118 mM K^+, a 50% decrease of catecholamine release was observed in depolarized conditions.

The blocking effects of (+)-isradipine on catecholamine release from polarized and depolarized glands

(+)-Isradipine blocked the secretory responses to K^+/Ca^{2+} pulses in a concentration-dependent manner (Figure 3). In the case of 35 and 59 mM K^+, the inhibition was complete at 30 nM; in 118 mM K^+, full blockade could not be achieved in polarized glands. The sensitivity of the secretory response to (+)-isradipine was substantially higher in depolarized glands; thus, the IC_{50} was at the subnanomolar level at the three K^+ concentrations tested. In contrast, the IC_{50} for the drug in polarized glands was always above nanomolar concentrations and increased from 2.36 nM in 35 mM K^+ to 12 nM in 118 mM K^+. IC_{50}s in depolarized glands were significantly lower than in polarized organs and similar at the three concentrations of K^+ tested (Table 1).

Potentiation of secretion by (−)-Bay K 8644 in polarized and depolarized glands

To study the effects of (−)-Bay K 8644 on catecholamine release, some modifications of the protocol used for (+)-isradipine were introduced (Figure 4). A lower K^+ concentration was selected (17.7 mM) in addition to 35 and 118 mM; Ca^{2+} present in the solutions used to test secretion was only 0.5 mM. These conditions were more suitable for demonstration of the

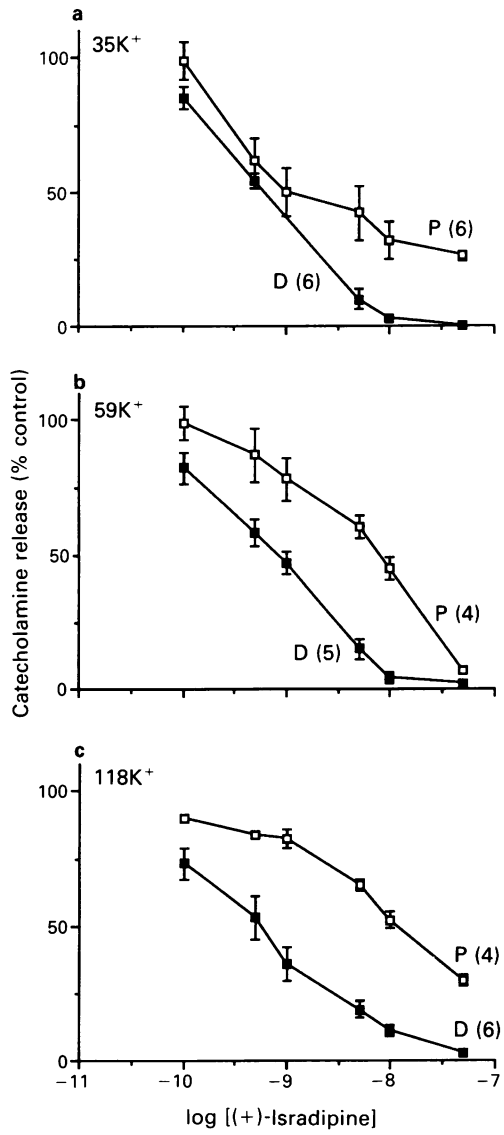


Figure 3 Concentration-response curves for (+)-isradipine to block secretion of catecholamines from polarized (P) and depolarized (D) glands. The secretory responses of pairs of glands were tested upon application of K^+/Ca^{2+} pulses, as described in the legend to Figure 1. Eight test pulses were applied to each gland with solutions containing 2.5 mM Ca^{2+} and 35 (a), 59 (b) or 118 (c) mM K^+, secretion was tested in the absence of drug. From P_4 to P_8, secretion was evoked after perfusion for 15 min with increasing concentrations (0.1–30 nM) of (+)-isradipine (abscissae). Each drug concentration was present for 15 min before and during the corresponding K^+/Ca^{2+} pulse; note that before each pulse, (+)-isradipine was perfused 15 min in 0 Ca^{2+}-Krebs solution containing 5.9 (polarized) or 35, 59 and 118 mM K^+ (depolarized). Data on catecholamine release (ordinates) were expressed as a % of the secretion obtained in P_3 (pulse before the introduction of the lowest drug concentration, once the secretory response stabilized). They represent the means of the number of experiments shown in parentheses; s.e.mean shown by vertical bars.

Table 1 IC_{50}s for (+)-isradipine to block catecholamine secretion evoked by K^+/Ca^{2+} pulses in polarized and depolarized glands

K^+ (mM)	*Polarized*	*Depolarized*	*Ratio*
35	2.36	0.60*	3.92
59	8.70	0.64*	13.7
118	12	0.45*	26.7

Data, expressed in nM, were calculated by plotting concentrations of (+)-isradipine versus log Y/100 − Y.
* $P < 0.01$, compared with polarized glands.

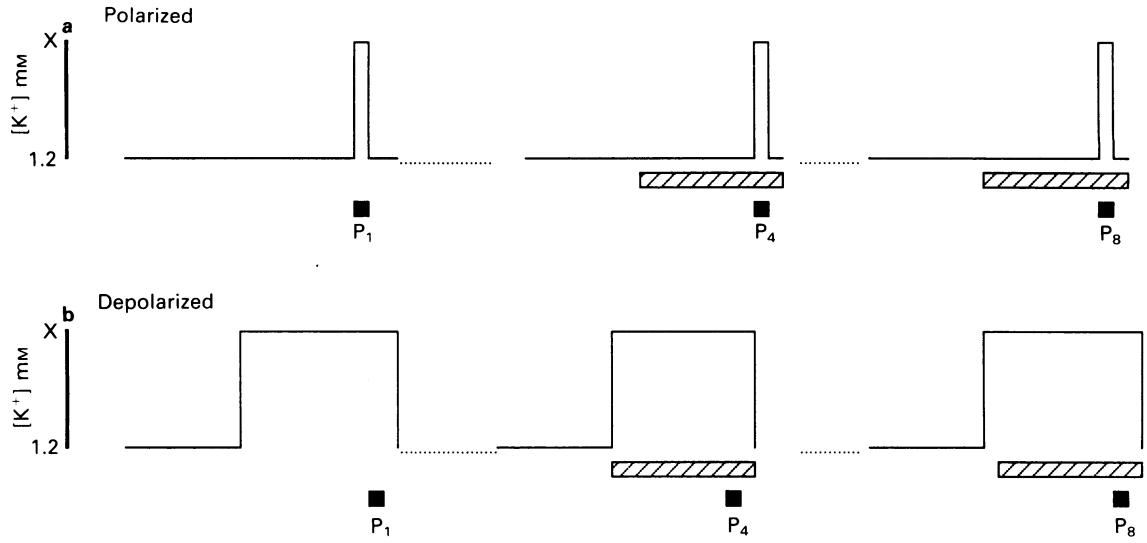


Figure 4 Protocol used to study voltage-independent potentiation of secretion by (−)-Bay K 8644. After 1 h equilibration of the glands they are continuously perfused with a Krebs solution containing $0\,Ca^{2+}/1.2\,K^+$. The difference between the polarized (a) and the depolarized (b) gland is that the latter is perfused with a solution containing $0\,Ca^{2+}$ and X K^+ (X = 17.7, 35 or 118 mM) 10 min before the secretory pulse which contains $0.5\,Ca^{2+}$ and the same concentration of K^+ used to pre-depolarize the glands for 10 s. The secretory test pulse (Ca^{2+} pulse, 0.5 mM $5\,s^{-1}$, ■) is the same in polarized and depolarized glands. Glands are stimulated for secretion eight times ($P_1 \ldots P_4 \ldots P_8$). For concentration-response curves, (−)-Bay K 8644 is perfused, at increasing concentrations, from the fourth pulse (P_4) onwards. Hatched bar indicates presence of drug.

potentiation by Bay K 8644 of catecholamine release from cat adrenal glands (García *et al.*, 1984; Ladona *et al.*, 1987).

In general, secretion of catecholamines in control conditions was lower than that obtained with the protocol to study the blocking effects of (+)-isradipine (compare Figures 2 and 5) which may be due to the lower Ca^{2+} concentration used in these experiments. At 17.7 mM K^+, secretion was significantly higher in depolarized glands; the reverse was true for the 118 mM K^+ concentration. However, at 35 mM K^+, secretion was similar in both polarized and depolarized glands (Figure 5).

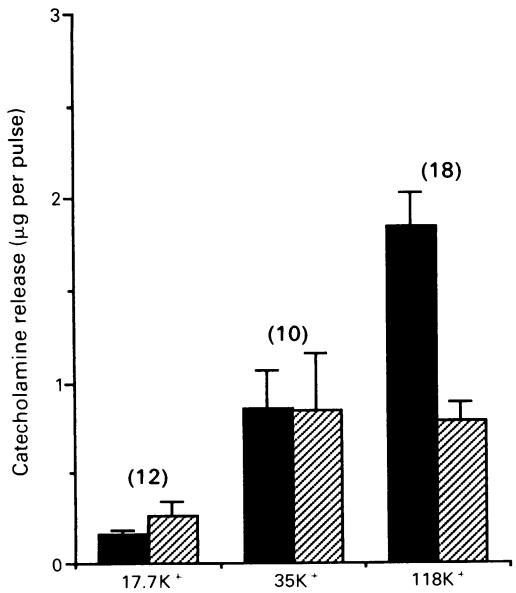


Figure 5 Catecholamine release evoked by K^+/Ca^{2+} pulses from polarized (solid columns) or depolarized (hatched columns) glands. The protocol (see Figure 4) was essentially similar to the one shown in Figure 1 but here a lower K^+ concentration was used (17.7 mM) and the pulses were given with 0.5 mM Ca^{2+} for 10 s. Data are expressed in total ng of catecholamines released in each pulse (ordinate scale); they represent means of the number of experiments shown in parentheses; vertical bars show s.e.mean.

(−)-Bay K 8644 potentiated secretion evoked by K^+/Ca^{2+} pulses both in polarized and depolarized glands. Potentiation was concentration-dependent from 10^{-9} to 10^{-6} M; however, a marked decrease of the potentiation of catecholamine release was observed at 10^{-5} M in all cases, except in polarized glands using 17.7 mM K^+ in the test pulse (Figure 6). In general, the minor potentiating effect seen with 10 μM (−)-Bay K 8644 was more clearly observed in depolarized glands, suggesting that at high concentrations, a Ca^{2+} channel blocking component of the DHP might exhibit voltage-dependence. At 17.7 mM K^+, the absolute amounts of catecholamine released were significantly higher in depolarized glands; the opposite was true at 118 mM K^+, where secretion was higher in polarized glands. In 35 mM K^+, catecholamine released was very similar under polarizing or depolarizing conditions.

When the absolute values of secretion are normalized as % of the maximum increase in release obtained in each individual gland, differences between polarized and depolarized organs were minimal (Figure 7). A slightly higher secretory response was seen in polarized glands, only at 10^{-7} M (−)-Bay K 8644. The EC_{50}s for potentiation of secretion, calculated from the logarithmic transformation of the data in Figure 7, are listed in Table 2; there were no differences between polarized and depolarized glands.

Figure 8 shows the ratios of the IC_{50} for (+)-isradipine or the EC_{50} for (−)-Bay K 8644 obtained in polarized and depolarized glands. It is clear that with the DHP blocker, the ratio polarized/depolarized increased linearly with increasing K^+ concentrations. This contrasts sharply with the situation with the DHP activator; the ratios for the EC_{50} in polarized versus depolarized glands were close to unity and similar at the three K^+ concentrations studied.

Table 2 EC_{50}s for (−)-Bay K 8644 to potentiate catecholamine release evoked by K^+/Ca^{2+} pulses in polarized and depolarized glands

K^+ (mM)	*Polarized*	*Depolarized*	*Ratio*
17.7	26.4	87.0	0.30
35	12.6	21.3	0.59
118	27.5	39.6	0.69

EC_{50}s expressed in nM, were calculated through the logarithmic transformation of data in Figure 7.

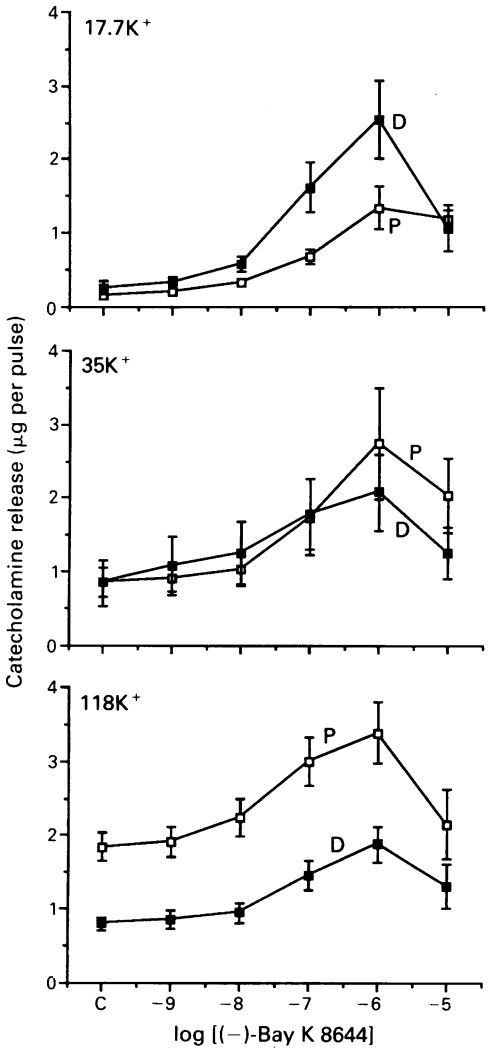


Figure 6 Catecholamine release evoked by K^+/Ca^{2+} pulses in glands previously perfused with Ca^{2+}-free solutions containing 1.2 mM K^+ (P, □, polarized) or 17.7, 35 or 118 mM K^+ (D, ■, depolarized). The experimental protocol was similar to that in Figure 3 but here, increasing concentrations of (−)-Bay K 8644 instead of (+)-isradipine, were used. Secretory responses were tested by introducing solutions containing 0.5 mM Ca^{2+} and the corresponding concentration of K^+ (17.7, 35 or 118 mM) at 15 min intervals. Data are expressed in ng of total catecholamines secreted in each pulse (ordinates).

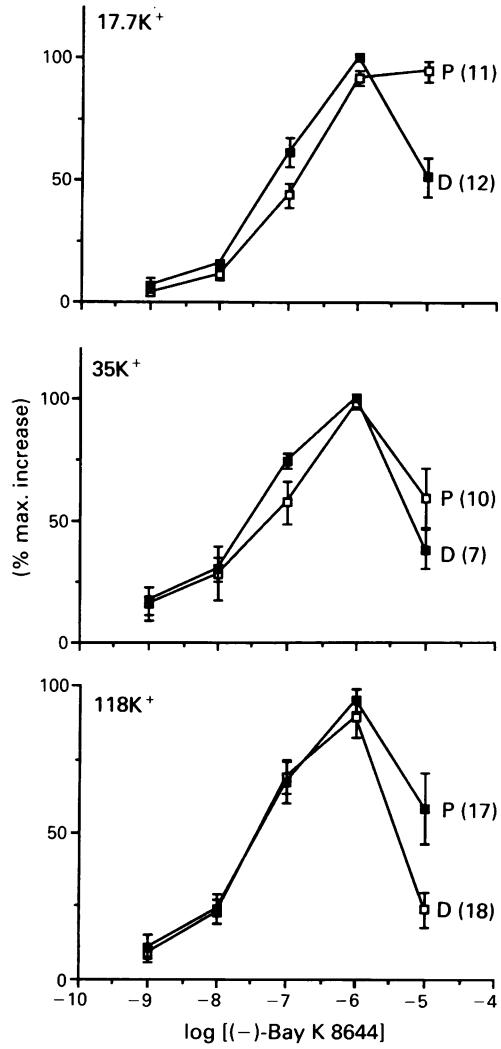


Figure 7 Normalized values of catecholamine release evoked by K^+/Ca^2 pulses in polarized (P) or depolarized (D) glands, in the presence of increasing concentrations of (−)-Bay K 8644. Data were calculated by expressing the secretion values in Figure 6 as % of the maximum increase of secretion obtained in each individual gland. Values are means with s.e.mean shown by vertical bars.

Effects of the two enantiomers of Bay K 8644 on catecholamine release from polarized and depolarized glands

(+)-Bay K 8644 blocked secretion in response to pulses of 118 mM K^+ plus 0.5 mM Ca^{2+} in a concentration-dependent manner (Figure 9). The drug was more efficacious in depolarized glands (perfusion with a solution containing 118 mM K^+ but no Ca^{2+}) than in polarized glands (perfusion with a solution containing 1.2 mM K^+ but no Ca^{2+}). However, it was substantially less potent than (+)-isradipine; IC_{50}s were 3000 and 300 nM in polarized and depolarized glands respectively, compared with 12 and 0.45 nM for (+)-isradipine.

As expected, the (−)-enantiomer also potentiated secretion in a concentration-dependent manner. However, its potencies were similar in polarized and depolarized glands, a finding that contrasts once more with the behaviour of the blockers (Figure 9).

Interactions of (+)-isradipine and (−)-Bay K 8644

To study interactions between (+)-isradipine and (−)-Bay K 8644, adrenals were continuously perfused with 5.9 K^+/0 Ca^{2+} and secretion was tested by applying 10 s pulses of 35 K^+/0.5 Ca^{2+} at 30 min intervals. A fixed concentration of (−)-Bay K 8644 was present from the second secretory pulse and once the secretory response stabilized, increasing concentrations of (+)-isradipine were added.

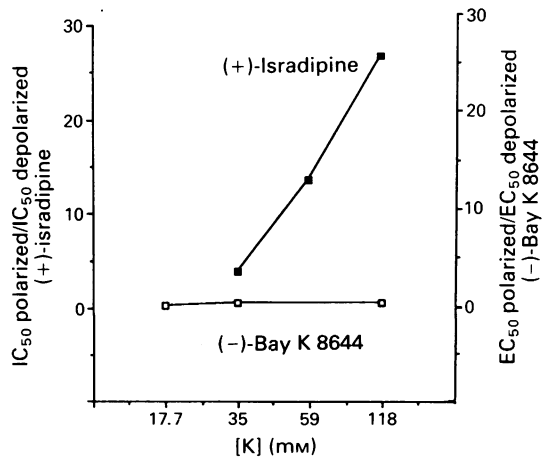


Figure 8 Ratios between IC_{50} and EC_{50} for (+)-isradipine (■) and (−)-Bay K 8644 (□) respectively, obtained in polarized and depolarized glands. The lines were drawn from data taken from Tables 1 and 2.

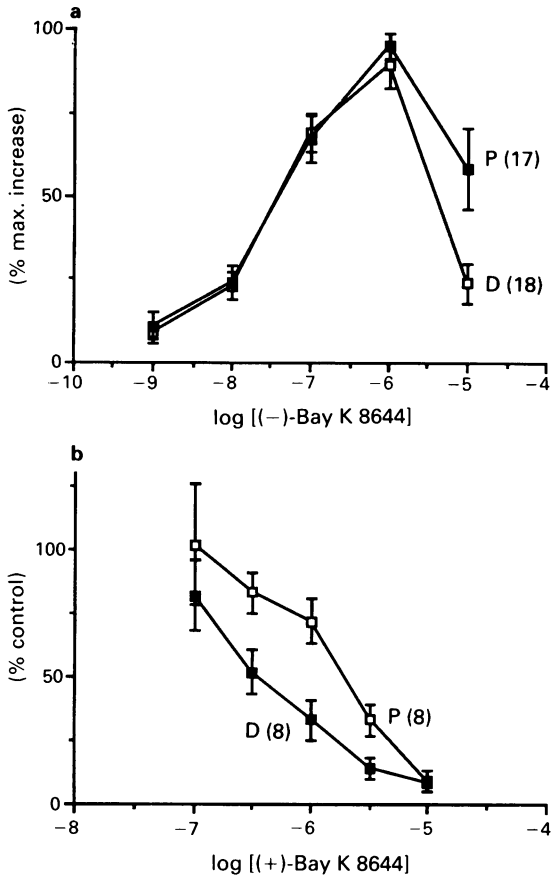


Figure 9 Potentiation by (−)-Bay K 8644 (a) and blockade by (+)-Bay K 8644 (b) of catecholamine release responses to K^+/Ca^{2+} pulses. Polarized glands (P) were perfused with 1.2 mM K^+ and depolarized glands (D) with 118 mM K^+, both with no Ca^{2+}. Secretion was evoked by applying pulses of a solution containing Ca^{2+} (0.5 mM) and K^+ (118 mM). Otherwise, the experimental protocol was similar to that in Figure 4. Data are means of the number of pairs of glands shown in parentheses; s.e.mean shown by vertical bars.

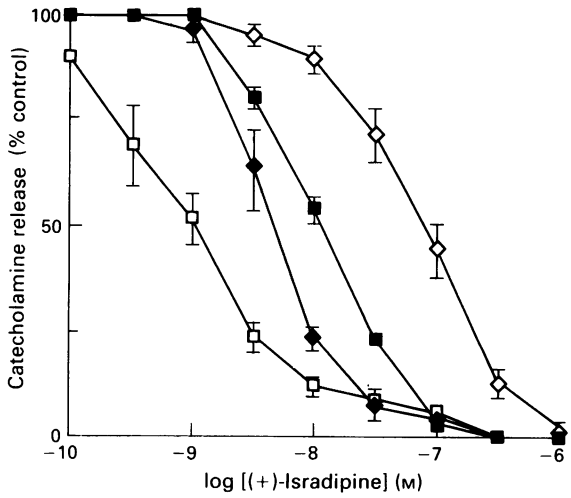


Figure 10 Displacement to the right by (−)-Bay K 8644 of the concentration-response curves to inhibit catecholamine release by (+)-isradipine. Adrenals were perfused with 5.9 K^+/0 Ca^{2+}. At 30 min intervals, secretion was tested by applying 10 s pulses of 35 K^+/0.5 Ca^{2+}. (−)-Bay K 8644 was present in a given gland at a fixed concentration (□ control; ◆ 3×10^{-9} M; ■ 3×10^{-8} M; ◇ 3×10^{-7} M), it was applied from the P_2 secretory pulse until the end of the experiment; once the secretory response in the presence of (−)-Bay K 8644 stabilized, increasing concentrations of (+)-isradipine were added to test its blocking effects on secretion. Data were normalized to % of the initial secretion obtained in the presence of (−)-Bay K 8644; they were plotted as % of blockade exerted by each concentration of (+)-isradipine (ordinate scale). Data are the means of 4–6 glands for each concentration of (−)-Bay K 8644; vertical bars shown s.e.mean.

Figure 10 shows concentration-response curves for (+)-isradipine in blocking Ca^{2+}-evoked catecholamine release from depolarized adrenal glands, in the absence (control), and the presence of increasing concentrations of (−)-Bay K 8644. (−)-Bay K 8644 displaced the (+)-isradipine concentration-response curves to the right. Thus, IC_{50}s for (+)-isradipine over block secretion were 4.2, 11.4 and 88 nM, respectively in the presence of 3, 30 and 300 nM (−)-Bay K 8644. The calculated pA_2 value for (−)-Bay K 8644 was 9.13 ± 0.48 and the slope of the straight line from the Schild plot was 0.6.

Discussion

The main conclusion emanating from these experiments is the voltage-independence of (−)-Bay K 8644 in enhancing Ca^{2+}-evoked catecholamine release from perfused cat adrenal glands; this finding contrasts with the strong voltage-dependence of the blocking effects of (+)-isradipine. Though in the cat adrenal gland, voltage-dependent effects of another DHP blocker, nitrendipine, are already known (Artalejo *et al.*, 1988b; López *et al.*, 1989) this paper compares the voltage-dependent effects of the channel antagonists with the voltage-independent effects of the activators, using pure enantiomers.

There is a great body of evidence suggesting that cat adrenal medullary cells contain a DHP receptor coupled to an L-type Ca^{2+} channel to control its function and through it, the secretory process (Artalejo *et al.*, 1988a). One would expect that if (+)-isradipine and (−)-Bay K 8644 were acting on the same locus on this receptor, their binding affinities to such sites should be equally changed on varying the membrane potential at different K^+ concentrations. Certainly, this was not the case, as shown in Figure 6; while the ratios IC_{50} polarized/IC_{50} depolarized increased 30 fold with increasing K^+ concentrations in the case of the blocker, the ratios EC_{50} polarized/EC_{50} depolarized were close to unity at the three K^+ concentrations tested with the activator.

Many DHP derivatives are known to block (nifedipine, nimodipine, nitrendipine, isradipine, niludipine, nisoldipine, felodipine) or enhance (Bay K 8644, CGP28392, YC-170, H160/51, Sandoz 202-791) contractile or secretory mechanisms (see reviews by Artalejo *et al.*, 1988a; Tsien *et al.*, 1988; Kostyuk, 1989; Triggle & Rampe, 1989). However, the question still remains whether activators and blockers act on the same receptor at the Ca^{2+} channel to facilitate its opening or closing, respectively. Most of these derivatives are racemates; sometimes the mixture is formed by two blocking agents exhibiting eudismic ratios of 100 or more, as in the case of (+)- and (−)-isradipine in the cat adrenal, whereas in other cases the racemates are formed by enantiomers with opposite effects, such as (+)-202-791 (activator) and (−)-202-791 (blocker) (Fonteríz *et al.*, 1987). This is also true for Bay K 8644, the (+)-enantiomer behaving as a weak blocker and the (−)-enantiomer as a potent activator (Franckowiak *et al.*, 1985).

Various features suggest that dihydropyridine Ca^{2+} channel blockers and activators might be acting at different receptor sites to control the kinetics of L-type Ca^{2+} channels:

First, while the blockers exhibit a strong voltage-dependence in their effects on Ca^{2+} currents (Sanguinetti & Kass, 1984a; Bean, 1984) and secretion (López *et al.*, 1989), the activators show a complex pattern of voltage-dependence (Sanguinetti & Kass, 1984b; Kamp *et al.*, 1989) or no voltage-dependence at all (this paper).

Secondly, the interactions of (+)-isradipine and (−)-Bay K 8644 on the cat adrenal secretory mechanism are non-competitive (Figure 10). This finding is in line with others in the literature. For instance, at certain concentrations, nitrendipine increases (rather than blocks) the cardiac inotropic effects of Bay K 8644 (Schwartz *et al.*, 1984), its contractile actions on

pig coronary arteries (Dubé *et al.*, 1985b) and enhances Ca^{2+} influx (Miwa & Schwartz, 1987). In addition, nimodipine also enhances the contractile effects of Bay K 8644 on coronary arteries (Dubé *et al.*, 1985a). On the other hand, (+)-Bay K 8644 blocks (−)-Bay K 8644-evoked contractions of guinea-pig ileal strips at low concentrations, but at higher concentrations the contractions are restored (Jurkiewicz, A., Jurkiewicz, N.H. & García, A.G., unpublished results). These findings are not compatible with simple competitive interactions of DHP activators and blockers on the same binding site.

Finally, in displacing the binding of [^{3}H]-nimodipine to brain cortical membranes (Bellemann & Franckowiak, 1985), [^{3}H]-isradipine to chick cardiac membranes (Maan & Hosey, 1987) and [^{3}H]-isradipine to adrenal medullary membranes (Castillo *et al.*, 1989) various DHP antagonists exhibit subnanomolar K_i values, while the agonists have receptor affinities one to two orders of magnitude lower. Affinities of (+)- and (−)-Bay K 8644 were only slightly different in brain membranes (K_i for the (−)-enantiomer to displace [^{3}H]-nimodipine binding was 25 nM and for (+)-Bay K 8644, 65 nM). In cardiac membranes, the K_i for (+)-Bay K 8644 to displace (+)-[^{3}H]-isradipine binding was 28.3 nM, while a K_i of 7.4 nM was found for the (−)-enantiomer. In adrenal medulla plasma membranes, (+)-Bay K 8644 displaced [^{3}H]-isradipine with a K_i of 22 nM and (−)-Bay K 8644 with a K_i of 26.5 nM. In contrast to this poor stereoselectivity for the enantiomers of Bay K 8644, the enantiomers of isradipine exhibit strong chiral effects, i.e. 100 fold difference between K_i for the (+)- and (−)-enantiomers in adrenal medulla (Castillo *et al.*, 1989) and 200 fold in the heart (Maan & Hosey, 1987).

In this framework, our data could be explained as follows: (+)-isradipine and (−)-Bay K 8644 act on chromaffin cells to decrease or enhance, respectively, their secretory activity, by acting on two separate sites (receptors?) on L-type Ca^{2+} channels. These channels control the entry of extracellular Ca^{2+}. Parallelisms between [^{3}H]-nitrendipine binding and blockade of $^{45}Ca^{2+}$ uptake and secretion strongly support this idea (Artalejo *et al.*, 1986). In addition to DHP receptors, these channels (and the secretory process) might be regulated by oscillations of the membrane potential, since 'conditioning' depolarizing pre-pulses block Ca^{2+} uptake into and secretion from the cat adrenal medulla chromaffin cells (Garrido *et al.*, 1990). In line with this view is the fact that in the present paper, polarized glands secrete more catecholamines the higher the K^+ concentrations present during the secretory pulse; this was not the case in depolarized glands where the higher K^+ concentrations evoked secretory responses similar to those obtained with lower concentrations of the cation.

Supported in part by grants from Fundación Ramón Areces, FISS (N° 89/0638), CICYT (N° PB87-0093-C03-01) and Plan Concertado de Investigación Alter-U.A.M. We thank Mrs M.C. Molinos for typing the manuscript. P.M. and L.G. are fellows of F.I.S.S., Spain.

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(Received April 3, 1990
Revised September 13, 1990
Accepted September 19, 1990)

Br. J. Pharmacol. (1991), **102**, 297–304 © Macmillan Press Ltd, 1991

New, long-acting, potent bradykinin antagonists

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1 Three new bradykinin (BK) antagonists, D-Arg^0-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound I), D-Arg^0-Hyp^3-D-Tic^7-Oic^8-BK (compound II), and Arg(Tos)^1-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound III), were tested against the effects of BK in 9 bioassay preparations including visceral smooth muscles, vasoconstriction, plasma protein extravasation, release of prostaglandin E_2, bronchoconstriction, and stimulation of afferent C-fibre nociceptors. In some of these tests the effects of the new compounds were compared with those of the antagonist D-Arg^0-Hyp^2-Thi^{5,8}-D-Phe^7-BK (compound IV), described by Stewart & Vavrek (1987).

2 For all bioassays the general rank order of potency of the compounds was found to be I > II > III ⩾ IV. The new antagonists were long-acting; in some bioassays their effects outlasted the duration of the experiment.

3 The inhibitory effects of the new BK antagonists were specific for BK; actions of noradrenaline, angiotensin II, acetylcholine or histamine were unaffected by the antagonists. They did not stimulate the release of histamine or prostaglandins. An agonistic effect was observed only with very high concentrations of compounds I and II in the plasma protein extravasation test.

4 The long duration of action of the new BK antagonists is probably due to a high and long-lasting affinity to the BK receptors. A high resistance of the antagonists to enzymatic destruction may be another reason.

5 The new BK antagonists will be valuable tools for the investigation of the pathophysiological role of BK. In addition they may offer a potential for therapeutic applications.

Introduction

Following the synthesis of the first bradykinin (BK) antagonists (Regoli & Barabé, 1980) and the discovery of novel potent BK antagonists of broad spectrum by Vavrek & Stewart (1985), a number of further antagonists have been synthesized and are being explored at present from different angles. Although the specificity and high potency of these antagonists has been unequivocally established (Regoli *et al.*, 1986; Griesbacher & Lembeck, 1987; Schachter *et al.*, 1987; Steranka *et al.*, 1988; Francel *et al.*, 1989; Griesbacher *et al.*, 1989), their susceptibility to fast enzymatic degradation limited their usefulness (Griesbacher *et al.*, 1989). Now, new analogues of BK have been synthesized in which unusual amino acids have been inserted with the intention of making the antagonists more resistent to enzymatic cleavage. In the present investigation the BK antagonistic potency and duration of action of the following 3 new compounds were tested: D-Arg^0-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound I), D-Arg^0-Hyp^3-D-Tic^7-Oic^8-BK (compound II), and Arg(Tos)^1-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound III). They were compared with the BK antagonist D-Arg^0-Hyp^2-Thi^{5,8}-D-Phe^7-BK (compound IV) described earlier (Stewart & Vavrek, 1987; Francel *et al.*, 1989; Linz *et al.*, 1990).

In vitro and *in vivo* tests were carried out in several BK bioassay systems which had been used in our previous work on BK antagonists (Griesbacher & Lembeck, 1987; Griesbacher *et al.*, 1989). In addition, the BK-induced longitudinal contraction of the guinea-pig ileum (*in vitro*), and plasma protein extravasation in internal organs of the rat and bronchoconstriction in guinea-pigs (*in vivo*) were used.

Methods

Isolated visceral smooth muscle preparations

Rat uterus (in oestrous) Female Sprague-Dawley rats were injected with diethylstilboestrol (100 μg kg^{-1}, s.c.) 16 h before the uterus was used. The distal parts of the uterine horns were suspended at 32°C in De Jalón solution containing atropine (0.35 μM) and indomethacin (2.8 μM). Isotonic contractions were recorded under a resting tension of 0.5 g. BK (1.6 nM) was added to the organ bath at 5 min intervals and left in the organ bath for 1 min. Compounds I (0.25–2.5 nM), II (0.25–7.5 nM), III (0.75–25 nM), or IV (75–750 nM) were added to the bath 4 min before the 3rd dose of BK; thus, they were in contact with the tissue for a total period of 5 min.

Rat duodenum The proximal part (1.5 cm) of the duodenum of female Sprague-Dawley rats was suspended at 32°C in De Jalón solution containing atropine (0.35 μM) and indomethacin (2.8 μM). A resting tension of 2 g was applied to the tissue and isotonic relaxations in response to BK (3 nM) were recorded. Compounds I (1.25–5 nM), II (1.25–5 nM), III (1.25–50 nM), or IV (4–500 nM) were added 4 min before a subsequent addition of BK.

Guinea-pig ileum The ileum (1.5 cm) was suspended in Tyrode solution at 32°C. Isotonic contractions were recorded under a resting tension of 0.5 g. BK (73 nM) was applied repeatedly at intervals of 3 min. One min after each addition of BK the bath solution was replaced. Compounds I (3–100 nM), II (1–100 nM), III (3–300 nM), or IV (100–10000 nM) were added to the organ bath 2 min before the following dose of BK.

In all tissues a concentration-response curve to BK was established at the beginning of each experiment in order to insure that the test doses of BK used were submaximal. After the addition of the antagonists, the BK additions were continued for a period of 30 min to monitor the recovery of the BK response.

Vasoconstriction in the isolated perfused ear of the rabbit

Rabbits of either sex were killed by an i.v. injection of an overdose of pentobarbitone sodium. The auricular arteries of both ears were cannulated and the ears were then separated from the head as described earlier (Griesbacher & Lembeck, 1987). The ears were perfused with Tyrode solution (37°C) and the

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perfusion pressure was adjusted to give a flow rate of 5 ml min^{-1}. Then, the perfusion pressure (30–60 cmH_2O) was kept constant. Vasoconstriction was measured by recording the venous outflow with an electronic drop interval timer. BK (100 pmol) was injected into the arterial inflow cannula at intervals of 30 min. The antagonists were infused during the 5 min period preceding the third injection of BK, the subsequent doses of BK were injected as before. On several occasions angiotensin II (30 pmol) or noradrenaline (100 pmol) were injected between the BK injections to test the specificity of the antagonists.

Release of prostaglandin E_2 from the rabbit ear

Rabbit isolated ears were prepared as described above. Perfusion with Tyrode solution at 37°C was carried out at a constant flow rate of 1.6 ml min^{-1} using a roller pump for at least 30 min before the tests were started. The BK antagonists (final concentration 20 nM) were then added to the perfusion medium of one ear for a period of 5 min. In each experiment a corresponding volume of a 154 mM solution of NaCl was infused into the contralateral control ear. Five min after the end of these infusions, BK (1 nmol) was injected into the arterial inflow cannula of each ear. The venous outflow was collected in 5 min fractions before and after the BK injections and stored at −20°C. The prostaglandin E_2 (PGE_2) content of these fractions was estimated by radioimmunoassay (Juan, 1977).

Plasma protein extravasation in rats

Female Sprague-Dawley rats (150–200 g) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, s.c.). A jugular vein was cannulated and Evans blue dye (20 mg kg^{-1}) was injected i.v. to stain plasma proteins. Compound I or II (500 nmol kg^{-1}) was given i.v. 5 min later, followed by BK (500 nmol kg^{-1}) injected i.v. 3, 10 or 30 min after the BK antagonist. In 2 control groups no BK antagonists were used: in one of them BK was injected, in the second group physiological saline was used instead of BK. At the end of the experiments, 5 min after the last injections, the thorax was opened and the rats were perfused via the aorta with 50 ml of physiological saline. The trachea, the urinary bladder, and the duodenum (proximal 1.5 cm) were dissected, weighed and incubated for 48 h in formamide at 50°C (Saria *et al.*, 1983). The amount of Evans blue extracted was measured photometrically at 620 nm. Plasma protein extravasation was expressed as ng Evans blue mg^{-1} wet weight.

In order to establish whether a BK antagonist increased plasma protein extravasation when given on its own, compounds I, II or III were injected i.a. at the 10 fold higher dose of 5 μmol kg^{-1} and the experiment terminated 5 min later. To test whether histamine, acting via H_1 receptors, or prostaglandin release contributed to the observed BK-induced plasma protein extravasation, animals were pretreated with mepyramine maleate (10 mg kg^{-1}, s.c.) or indomethacin (10 mg kg^{-1}, s.c.) 30 min before the beginning of the experiment.

Bronchoconstriction in guinea-pigs

Guinea-pigs of either sex (400–700 g body weight) were anaesthetized with urethane (25% w/v in physiological saline, 7.5 ml kg^{-1}, i.p.). The trachea was cannulated and the animals were put on artificial respiration (stroke volume 10 ml kg^{-1}, 65 strokes min^{-1}). As an index of bronchoconstriction the pulmonary inflation pressure (PIP) was measured in a side arm of the tracheal cannula with a Statham pressure transducer (Jin *et al.*, 1989). One carotid artery was cannulated to measure the arterial blood pressure. BK (1 nmol) was injected into a jugular vein at intervals of 10 min. The BK antagonists I (1 nmol) or II (1 nmol) or III (2 nmol) were injected i.v. 1 min before a subsequent injection of BK. Compound IV (100 nmol) was injected i.v. immediately before the test dose of BK. To test the specificity of the antagonists, experiments were carried out in which histamine (10 nmol) was injected instead of BK. The injection volume of BK, the BK antagonists, and histamine was 0.1 ml.

Rabbit iris sphincter muscle

To test the antagonists in a system where BK acts on peptidergic afferent C-fibre nociceptors, the isolated sphincter muscle of the rabbit was used. In this preparation the BK-induced contractions of the smooth muscles are induced by the release of tachykinins from primary afferent trigeminal neurones (Bynke *et al.*, 1983; Ueda *et al.*, 1984; Håkanson *et al.*, 1987). Rabbits of either sex were killed by an i.v. injection of an overdose of pentobarbitone. The eyes were enucleated and the iris sphincter muscles were prepared as described by Kern (1970). Isotonic contractions were recorded under a resting tension of 0.5 g. The BK antagonists were added to the organ bath (Krebs solution, 37°C) 5 min before cumulative concentration-response curves to BK were established (Griesbacher & Lembeck, 1987); an appropriate amount of physiological saline was added to the organ bath of control tissues.

Stimulation of nociceptors in the rabbit ear

Rabbits of either sex (3–6 kg) were anaesthetized with pentobarbitone sodium (45 mg kg^{-1}, i.v.). The arterial blood pressure in a carotid artery was recorded by means of a Statham pressure transducer on a Grass Model 7B Polygraph. To maintain anaesthesia small amounts of pentobarbitone sodium (6 mg) were injected into a jugular vein; the stability of blood pressure and the corneal reflex were used to monitor anaesthesia. The central ear artery was cannulated and the ear separated from the head with the exception of the auricular nerve, which remained connected to the head (Griesbacher & Lembeck, 1987). The ear was perfused with Tyrode solution under constant pressure at a flow rate of 5 ml min^{-1}. PGE_1 (final concentration 30 nM) was added to the Tyrode solution in order to augment the responses to BK and acetylcholine (ACh) (Juan & Lembeck, 1974). BK (100 pmol) and ACh (60 nmol) were injected alternately into the arterial inflow cannula at intervals of 15 min; 26 min after the second injection of BK compound I (final concentration 1.5 or 15 nM) or compound III (final concentration 5 or 50 nM) were infused into the arterial inflow cannula of the ear for 4 min. Immediately after the end of this infusion the next dose of BK was injected. The reflex falls in carotid blood pressure induced by BK and ACh were then monitored over a period of 1 h.

Substances

Bradykinin (BK) was obtained from Bachem, Switzerland. The BK antagonists D-Arg^0-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound I), D-Arg^0-Hyp^3-D-Tic^7-Oic^8-BK (compound II), Arg(Tos)^1-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound III), and D-Arg^0-Hyp^2-Thi^{5,8}-D-Phe^7-BK (compound IV) (Thi = β-(2-thienyl)-L-alanine; D-Tic = D-(1,2,3,4-tetrahydroisoquinolin-2-yl-carbonyl); Oic = L-[(3aS,7aS)-octahydroindol-2-yl-carbonyl]; Arg(Tos) = N^G-tosyl-L-arginine) were synthesized by solid phase peptide synthesis by Hoechst AG.

Further substances used were: acetylcholine chloride (Becker, Austria); atropine sulphate (Merck Sharp & Dohme, F.R.G.); diethylstilboestrol, histamine dihydrochloride (Serva Feinbiochemie, F.R.G.); Evans blue, indomethacin, noradrenaline hydrochloride, prostaglandin (PG) E_1 (Sigma, U.S.A.); mepyramine maleate (Smith Kline & French, U.K.); pentobarbitone sodium (Ceva, F.R.G.); PGE_2 antiserum (BioMakor, Israel); [^{3}H]-PGE_2 (Amersham International, U.K.).

All peptides were dissolved in physiological saline containing 1 g l^{-1} gelatine and 25 mg l^{-1} cialit (sodium 2-

ethylmercuriothio-benzoxazole-5-carboxylate, Asid-Institut GesmbH, F.R.G.) to prevent absorption to glass and bacterial growth. Diethylstilboestrol was dissolved in and diluted with olive oil. Indomethacin was dissolved in 2% Na_2CO_3 and diluted with phosphate buffer (20 mM KH_2PO_4 and 50 mM Na_2HPO_4). PGE_1 was dissolved in 50% ethanol. All other substances were made up in physiological saline.

The compositions of the physiological salt solutions were (in mM): De Jalón: NaCl 154.0, KCl 5.6, $CaCl_2$ 0.54, $MgCl_2$ 0.05, $NaHCO_3$ 11.9, D-glucose 3.0; Krebs: NaCl 118.0, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.17, NaH_2PO_4 25.0, D-glucose 10.1; Tyrode: NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.15, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, D-glucose 5.6; physiological saline: NaCl 154. All solutions used in *in vitro* experiments were gassed with a mixture of 95% O_2 and 5% CO_2.

Statistical analysis

In the smooth muscle preparations, the effects of BK before and after the addition of the antagonists were compared by the Quade test (Conover, 1980). To quantify the potency of the antagonists, the % inhibition, transformed to logits as a linearising transformation, was plotted against the negative logarithm of the antagonist concentrations and log-logit regression analysis was used to calculate IC_{50} values. Log-logit regression analysis was used to test the concentration-response curves to BK on the rabbit isolated iris sphincter muscle for non-identity and non-parallelism. Where the concentration-response curves in the absence and presence of an antagonist proved not to deviate significantly from parallelism, the horizontal displacement to the right was calculated (Geigy, 1980) to determine the concentration-ratio.

Vasoconstriction in rabbit isolated perfused ears was analyzed by performing multiple nonparametric comparisons (Zar, 1984) of the BK-induced effect in antagonist-treated ears compared with that in the saline-treated control ears. The Mann-Whitney U test was used to compare the magnitude of the release of PGE_2 from rabbit ears that had been perfused with an antagonist with the release of PGE_2 from control ears. Dunnett's t test was employed to compare results obtained in individual groups in the plasma protein extravasation test. The effects of the BK antagonists *in vivo* on BK-induced bronchoconstriction in guinea-pigs and on BK-induced nociceptor stimulation in the perfused rabbit ear were analyzed by comparing the effects of BK before and after the administration of the antagonists with the Quade test.

Values calculated by regression analysis are given with 95% confidence intervals. All other values are given as means ± s.e.mean.

Results

Isolated visceral smooth muscle preparations

All BK antagonists tested inhibited the contractile effects of BK on the rat uterus and on the guinea-pig ileum, as well as the relaxant effects of BK on the rat duodenum in a concentration-dependent manner. However, the concentrations of compound IV that were needed to inhibit effectively the actions of BK were about 2–3 orders of magnitude higher than those of compounds I, II and III (Table 1).

In addition, there was a difference in the duration of the action of the antagonists in these preparations (Figure 1 shows the results from the rat uterus). Compound IV reduced the effects of BK only while it was present in the organ bath; the next application of BK already produced a response as before the addition of compound IV. The new antagonists I, II and III inhibited BK for a much longer time, despite the repeated washing procedures after each addition of BK. A period of 30 min was required to restore the full potency of BK when these antagonists were used in concentrations which almost abolished the effect of the first dose of BK added to the organ bath while the antagonist was still present (see Figure 1).

Table 1 Inhibition of the bradykinin (BK)-induced contractions of the isolated rat uterus and the guinea-pig ileum, and of the BK-induced relaxations of the isolated rat duodenum

	$-log\ IC_{50}$		
Antagonist	*Rat uterus*	*Rat duodenum*	*Guinea-pig ileum*
I	9.3 (8.6–10.0)	8.7 (8.3–9.3)	8.1 (7.4–8.8)
II	9.2 (8.5–10.0)	8.5 (7.9–9.1)	7.9 (7.0–8.8)
III	8.4 (7.3–9.5)	8.1 (7.4–8.7)	7.8 (6.8–8.8)
IV	6.2 (5.3–7.1)	7.1 (5.3–8.8)	6.3 (5.5–7.1)

BK was applied to the tissues at concentrations that gave approximately 70% of the maximum effect. The inhibition by the BK antagonists was plotted against the antagonist concentration and $-\log IC_{50}$ values were calculated by log-logit regression analysis; 95% confidence intervals are given in parentheses. Each value was obtained from a total of 24–30 experiments.

Vasoconstriction in the rabbit isolated perfused ear

Intra-arterial injections of BK (100 pmol) at intervals of 30 min elicited, under control conditions, consistent decreases of the venous outflow between 60 and 70%. Short infusions (5 min) of the BK antagonists reduced the effect of subsequent injections of BK in a concentration-dependent manner (Figure 2). Compound I was the most potent antagonist: in a concentration of 5 nM it reduced the effects of BK significantly ($P < 0.05$); a concentration of 15 nM blocked the BK-induced vasoconstriction completely ($P < 0.001$). Three times higher concentrations of compound II and compound III were required to obtain comparable inhibitory effects. The inhibition of the BK-induced vasoconstriction by these antagonists persisted during the whole duration of the experiment, i.e. up to 3 h (see Figure 2). In 2 ears the antagonistic effect of compound I (15 nM) was monitored for a period of 6 h during which the responses to BK did not recover.

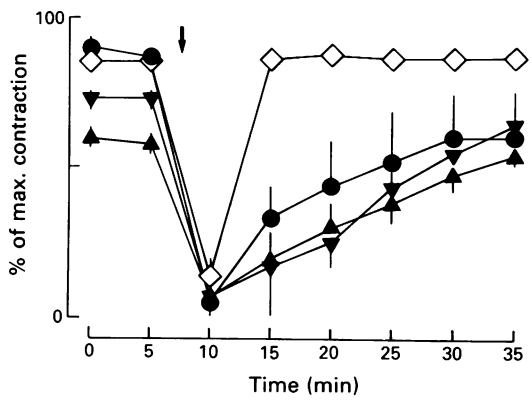


Figure 1 Inhibition of the effect of bradykinin (BK) on the rat uterus: BK (1.6 nM) was added to the organ bath at intervals of 5 min and the medium (De Jalón solution) was changed 1 min later. Ordinate scale: contractions of the uterus as % of the maximum BK-induced response. Abscissa scale: time in min. At the time indicated by the arrow, the BK antagonists compound I (2.5 nM, ●), compound II (2.5 nM, ▲), compound III (25 nM, ▼), or compound IV (2500 nM, ◇) were added. The effect of the subsequent dose of BK was, in all cases, nearly abolished ($P < 0.001$). The next response to BK was again normal when compound IV had been used; in the case of the other antagonists the BK response stayed significantly decreased for at least 25 min. Symbols represent mean values ($n = 6$); vertical lines show s.e.mean. Where no s.e.mean is given it was smaller than the symbol.

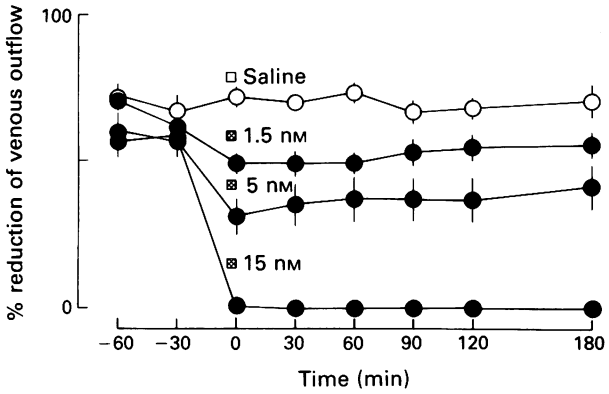


Figure 2 Rabbit isolated ears were perfused under constant pressure with Tyrode solution at an initial flow rate of 5 ml min^{-1}. Bradykinin (BK, 100 pmol) was injected i.a. at intervals of 30 min. The BK-induced vasoconstriction was quantified as % reduction of the venous outflow from the ear. Compound I was infused into the arterial inflow (final concentrations 1.5 nM, 5 nM, and 15 nM, ●) during a period of 5 min prior to the third injection of BK (cross-hatched squares); control animals (○) received a corresponding volume of physiological saline (open square). Subsequent effects of BK were either completely blocked (15 nM, $P < 0.001$) or partly inhibited (5 nM, $P < 0.05$) by compound I. Each antagonist concentration was tested in 5–7 ears. Symbols represent mean values; vertical lines show s.e.mean. Where no s.e.mean is given it was smaller than the symbol.

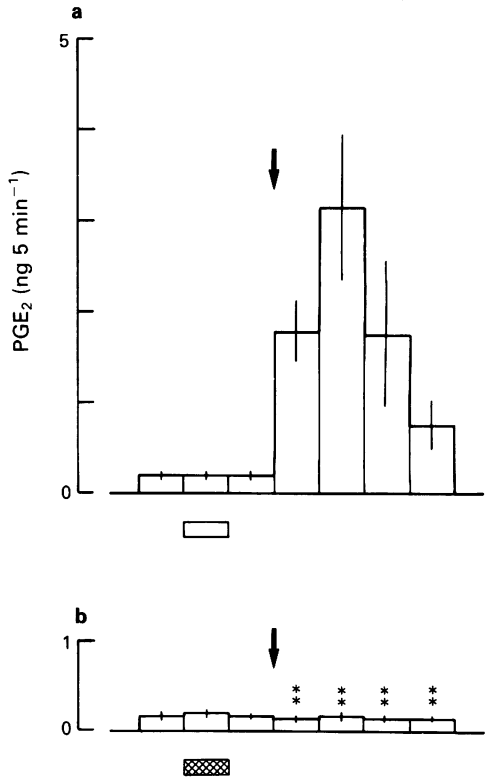


Figure 3 Release of prostaglandin E_2 (PGE_2, in ng 5 min^{-1}) from the rabbit isolated perfused ear (Tyrode solution, constant flow of 1.6 ml min^{-1}): bradykinin (1 nmol) was injected i.a. as a bolus (indicated by the arrow). Compound I (final concentration 20 nM) was present in the Tyrode solution for 5 min (indicated by cross-hatched bar below abscissa scale in b). In control ears a corresponding volume of physiological saline (indicated by open bar below abscissa scale, a) was infused. The PGE_2 content of the venous effluent, collected in 5 min fractions, was determined by radioimmunoassay. Columns represent mean values with s.e.mean shown by vertical lines; $n = 6$. Significance of difference from the corresponding fractions of control ears: ** $P < 0.01$.

Compound IV infused at concentrations of 50, 150, and 500 nM reduced, but did not abolish, BK-induced vasoconstriction; 30 min after the infusion the degree of vasoconstriction induced by BK was again the same as in the control ears.

The order of potency of the antagonists was I $\geqslant$ II $\approx$ III $\gg$ IV (Table 2). Vasoconstrictions elicited by i.a. injections of angiotensin II (30 pmol) or noradrenaline (100 pmol) were not affected by the infusions of the BK antagonists ($n = 4$; data not shown).

Release of prostaglandin E_2 from the rabbit ear

The basal PGE_2 release from rabbit isolated ears, perfused at a constant flow rate of 1.6 ml min^{-1}, was 0.2 ± 0.05 ng 5 min^{-1} ($n = 22$). Injection of BK (1 nmol, i.a.) caused a release of a further 8.4 ± 0.9 ng PGE_2 during a period of 20 min (compare Figure 3, upper panel). All antagonists were infused i.a. (final concentration 20 nM) over a period of 5 min. No increase of the PGE_2 content of the venous outflow was observed during these infusions. Five min after the infusion of compound I the PGE_2 release normally induced by an injection of 1 nmol BK was completely abolished (Figure 3b). Infusions of compound II or compound III also abolished the effect of BK ($n = 5$–6; results not shown). When compound IV was infused, the PGE_2 content of the venous outflow was not reduced; it amounted to 9.4 ± 1.7 ng during 20 min ($n = 6$) and did not differ from the values obtained in the corresponding control ears (10.1 ± 2.7 ng).

Table 2 Inhibition of bradykinin (BK)-induced vasoconstriction in the isolated perfused ear of the rabbit

Antagonist	$-log\ IC_{50}$
I	8.5 (7.9–9.1)
II	8.2 (7.1–9.1)
III	8.2 (7.5–9.0)
IV	6.9 (5.7–8.2)

BK (100 pmol) was injected into the arterial inflow cannula at intervals of 30 min. The antagonists were infused i.a. during the 5 min period preceding the third injection of BK. $-log\ IC_{50}$ values were calculated by log-logit regression analysis; 95% confidence intervals are given in parentheses. Each value was obtained from a total of 15–28 experiments.

Plasma protein extravasation in rats

Intravenous injections of BK (500 nmol kg^{-1}) in anaesthetized rats caused a significant increase ($P < 0.01$) in plasma protein extravasation over control levels in the trachea, the urinary bladder and the duodenum. It was most pronounced in the duodenum. When compound I (500 nmol kg^{-1}) or compound II (500 nmol kg^{-1}) had been given i.v. 3, 10, or 30 min before the BK injection, BK-induced plasma protein extravasation was completely abolished in all organs (Table 3). The effect of BK was totally unaffected by pretreatment with either mepyramine (10 mg kg^{-1}, s.c.) or indomethacin (10 mg kg^{-1}, s.c.).

Figure 4 shows plasma protein extravasation in the duodenum induced by i.v. injections of BK antagonists injected alone in doses 10 times higher than those used in the experiments above. Only compound I (5 μmol kg^{-1}) elicited plasma protein extravasation to an extent comparable to that induced by BK (500 nmol kg^{-1}). This effect was seen in duodenum, trachea and bladder. Compound II caused plasma protein extravasation only in the trachea, whereas compound III showed no agonist activity in any of the organs tested.

Bronchoconstriction in guinea-pigs

BK (1 nmol) injected i.v. into anaesthetized guinea-pigs at 10 min intervals elicited reproducible increases in PIP between

Table 3 Plasma protein extravasation in the duodenum, trachea and urinary bladder of anaesthetized rats (pretreated i.v. with Evans blue)

	Duodenum	*Trachea*	*Bladder*
Saline	37 ± 8	25 ± 7	10 ± 1
BK	140 ± 22**	90 ± 15**	74 ± 12**
BK + I			
3 min	52 ± 19	28 ± 5	14 ± 2
10 min	36 ± 5	30 ± 5	12 ± 2
30 min	26 ± 6	20 ± 3	10 ± 2
BK + II			
3 min	34 ± 4	30 ± 5	11 ± 2
10 min	34 ± 8	28 ± 4	18 ± 6
30 min	38 ± 8	18 ± 3	8 ± 1

Bradykinin (500 nmol kg^{-1}) was injected i.v. either alone (BK) or 3–30 min after the i.v. injection of compound I or II. Control animals were injected with physiological saline instead of BK. Evans blue content of the tissues (in ng mg^{-1} wet weight) was determined 5 min after the injection of BK or physiological saline. Means ± s.e.mean; $n = 4$–10. Significance of difference from saline controls: ** $P < 0.01$.

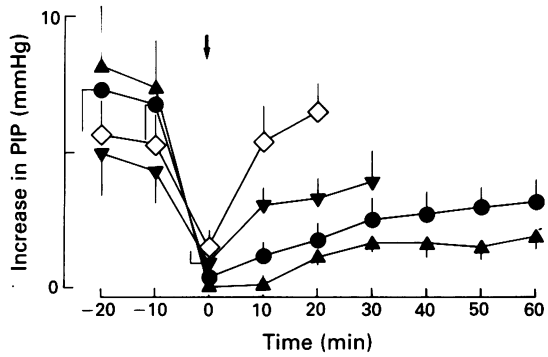


Figure 5 Changes of pulmonary inflation pressure (PIP, in mmHg) in anaesthetized guinea-pigs *in vivo*: bradykinin (BK, 1 nmol) was injected i.v. at intervals of 10 min. At the time indicated by the arrow an i.v. injection of compound I (1 nmol, ●), compound II (1 nmol, ▲), compound III (2 nmol, ▼), or compound IV (100 nmol, ◇) was made. The response to the next dose of BK was nearly abolished by all compounds ($P < 0.001$). The effects of subsequent 6 doses of BK were significantly ($P < 0.01$) inhibited by compounds I and II. Symbols represent mean values; vertical lines give s.e.mean. $n = 6$–9.

5 and 8 mmHg (Figure 5). The BK antagonists were injected i.v. 1 min before the third injection of BK. The response to this BK injection was completely blocked when compound I (1 nmol) or compound II (1 nmol) had been given; 60 min later the effect of BK was still reduced to 51% and 23%, respectively. Compound III (2 nmol) reduced BK-induced bronchoconstriction to 16% of the control value; 30 min later the BK response was back to 70% of its original value. To block BK-induced bronchoconstriction with compound IV, a dose of 100 nmol had to be given and the response to BK given 10 min later was already back to normal.

None of the antagonists had a significant effect on the increase in PIP induced by i.v. injections of histamine (10 nmol, $n = 5$).

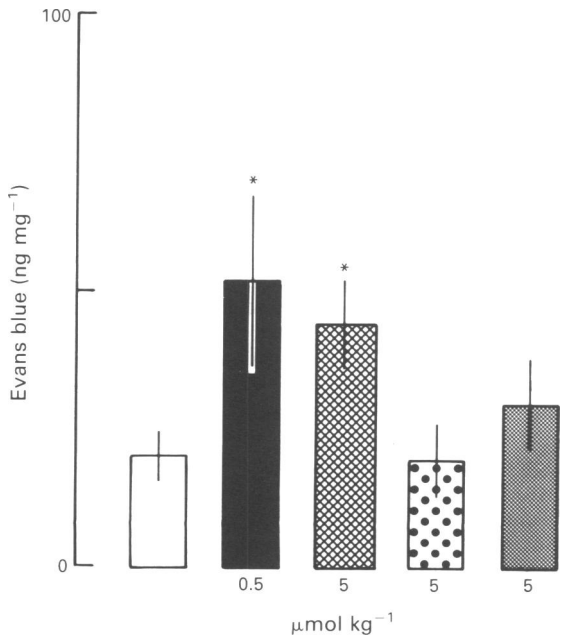


Figure 4 Plasma protein extravasation in the duodenum of anaesthetized rats (pretreated i.v. with Evans blue): column heights represent Evans blue content of the duodenum (in ng mg^{-1} wet weight) of rats 5 min after the i.v. injection of physiological saline (open columns), bradykinin (BK, solid column), or of the BK antagonists on their own (compound I: cross-hatched column; compound II: stippled column; compound III: shaded column). The doses of BK and the BK antagonists are given below the columns (in μmol kg^{-1}). Significance of difference from saline controls: * $P < 0.05$. Means with s.e.mean shown by vertical lines; $n = 6$.

Rabbit iris sphincter muscle

Cumulative concentration-response curves to BK were shifted to the right when the antagonists were present in the bathing solution of the preparations. The maximum contraction obtained with BK in the presence of the antagonists was not different from that seen with control tissues and was 70–90% of the contraction induced by a maximal concentration of carbachol (10 μM). The concentrations of the antagonists were chosen so as to give a rightward shift by approximately 0.5 to 1 log unit. The regression lines for the concentration-response relations proved not to differ significantly from parallelism. Therefore, concentration-ratios were calculated by regression analysis (Table 4). When Compound I was employed at the same concentration as compound II and compound III (5 nM) the highest BK concentration that could be used (5 μM) yielded only 42 ± 11% of the carbachol-induced maximum. Since no information on the upper regions of the concentration-response curve could be obtained, no regression line was calculated for this concentration of compound I. However, the data available suggest a shift of the concentration-response curve by 2–2.5 log units. Compound IV was about 1000 times less potent than compound I and about 100 times less potent than compounds II and III.

Stimulation of nociceptors in the rabbit ear

Basal systemic arterial blood pressure was 90–110 mmHg and remained constant throughout the course of the experiment.

Table 4 Bradykinin (BK)-induced contractions of the rabbit isolated iris sphincter muscle

Antagonist	*Concentration* [nM]	*Concentration-ratio for BK*
I	0.5	7.3 (5.3–10.0)
II	5	4.5 (3.8–5.5)
III	5	10.2 (7.7–13.5)
IV	500	4.2 (3.3–5.3)

Cumulative concentration-response curves to BK were established in the absence or presence of the BK antagonists. Log-logit regression analysis was used to calculate regression lines for the BK responses. All antagonists shifted the concentration-response curves to the right. No significant deviation from parallelism could be detected. The horizontal displacement of the regression lines was used to calculate concentration-ratios for BK with 95% confidence intervals (given in parentheses). For each antagonist 5–6 pairs of tissue preparations were used.

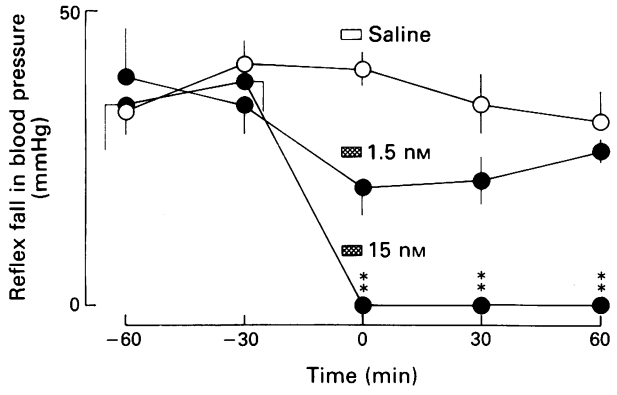


Figure 6 Nociceptor stimulation in the rabbit ear: bradykinin (BK, 100 pmol) was injected into the arterial inflow of isolated perfused ears (left in contact with the head only by the intact auricular nerve) at intervals of 30 min. The ensuing reflex falls in systemic blood pressure (in mmHg) were measured in a carotid artery. In two groups of rabbits, compound I (final concentrations 1.5 nM or 15 nM, ●) was added to the perfusion of the ears during a period of 5 min (cross-hatched bars) prior to the 3rd injection of BK. Control animals (○) received a corresponding volume of physiological saline (open bar). Symbols represent mean values, vertical lines show s.e.mean. For each concentration of compound I (0–15 nM) 3 animals were used. Significance of difference from values before the antagonist: ** $P < 0.01$.

Stimulation of paravascular nociceptors by injections of BK (100 pmol) at intervals of 30 min into the arterial inflow cannula of the 'reflex ear' preparation (Juan & Lembeck, 1974) caused short-lasting reflex falls in blood pressure by 35–40 mmHg (Figure 6). When compound I was present in the perfusion medium (final concentration 15 nM, $n = 3$) over a period of 5 min, the effect of subsequent injections of BK were completely abolished ($P < 0.01$); in a concentration of 1.5 nM compound I significantly attenuated ($P < 0.05$) the actions of BK. As with the results on vasoconstriction in the rabbit isolated perfused ear (see Figure 2), the antagonism was not reversible during the time of observation (1 h). Similar results were obtained when higher concentrations of compound III were used: a concentration of 5 nM reduced the BK-induced fall in blood pressure from 34 ± 6 mmHg to 12 ± 10 mmHg, and 50 nM abolished the effect of BK. The complete block of the effect of BK seen after compound III (50 nM) also lasted throughout the course of the experiment ($n = 3$). However, a reflex fall in blood pressure could still be elicited when much higher doses of BK (1 and 10 nmol) were injected. The nociceptor stimulation by ACh (60 nmol, not shown in Figure 6) was completely unaffected by compounds I or III. Compounds II and IV were not tested in this preparation.

Discussion

Isolated smooth muscles

The actions of four new BK antagonists were studied on BK-induced contractions of the guinea-pig ileum and the rat uterus. The former preparation has often been used in earlier investigations of this type of drugs (Vavrek & Stewart, 1985; Regoli *et al.*, 1986; Schachter *et al.*, 1987; Stewart & Vavrek, 1987; Steranka *et al.*, 1988), and the latter has been employed because of its high sensitivity to BK. In addition, the rat duodenum was used because it responds to BK with a relaxation by a mechanism which is not yet fully understood, but involves activation of BK receptors (Boschcov *et al.*, 1984; Griesbacher *et al.*, 1989; Pereira & Paiva, 1989; Altinkurt & Öztürk, 1990). The order of potency of the antagonists was similar with all three preparations: I ≈ II > III ⩾ IV (Table 1). Compound I was 100–1000 times more potent than compound IV.

Furthermore, a distinction between the BK antagonists regarding their duration of action was demonstrated on the rat uterus. Compound IV (2500 nM) caused a complete inhibition of the response to BK by this tissue when it was present in the organ bath. Much lower concentrations of the other antagonists (e.g. 2.5 nM for compounds I and II) showed an inhibitory effect for a much longer period of time. In spite of repeated changes of the bath fluid, the inhibition by compounds I, II and III did not fully disappear even 20–30 min after their washout, indicating that the new antagonists have a long duration of action (Figure 1).

Rabbit isolated perfused ear

This preparation has previously been shown to be useful for testing the inhibition of the BK-induced vasoconstriction, and the BK-induced release of PGE_2 by BK antagonists (Griesbacher & Lembeck, 1987). The reduction in venous outflow induced by BK results from venoconstriction (Guth *et al.*, 1966). To inhibit BK-induced venoconstriction the antagonists corresponded in potency to the rank order found in the isolated smooth muscle preparations: I > II ≈ III ⩾ IV (Table 2). The duration of action of the new compounds on the ear veins was remarkable (Figure 2). The complete inhibition of this BK response by compound I exceeded 3 h and in 2 preparations lasted longer than 6 h. This is in contrast to the effect of compound IV. Even at a concentration of 500 nM the inhibition was incomplete and terminated within 30 min. Thus, compounds I, II and III appear to have an unusually high affinity for the BK receptor and/or are resistent to enzymatic cleavage.

The antagonists I, II, III and IV (20 nM) did not evoke a release of PGE_2 by themselves, and thus did not act like BK agonists in this test. The BK-evoked PGE_2 release could be blocked completely by compound I (Figure 3); it is likely that such an effect could have been achieved by a concentration much lower than 20 nM. A complete inhibition of the BK-evoked PGE_2 release was also obtained with compounds II and III. Compound IV was inactive at this concentration. The related BK antagonist B4310 (Stewart & Vavrek, 1987) was found to inhibit the BK-induced PGE_2 release only at a concentration of 800 nM (Griesbacher & Lembeck, 1987). The mechanism involved in the BK-induced synthesis and release of PGE_2 is unknown. The present results suggest that the mode of action involves specific BK receptors.

Plasma protein extravasation

BK-induced plasma protein extravasation has usually been tested in the skin of rats, guinea-pigs and rabbits. In a previous study, the combined injection of BK and BK antagonists in rabbit skin demonstrated that this response can be inhibited (Griesbacher & Lembeck, 1987), but the method did not seem to be very reliable. Therefore, in the present study, plasma protein extravasation was measured in several internal organs following i.v. injection of BK (Saria *et al.*, 1983). Plasma protein extravasation induced by BK was most prominent in the duodenum, the urinary bladder and the trachea. This effect was unaffected by mepyramine or by indomethacin. Thus, the release of histamine to act on H_1 receptors, or prostaglandins is not involved in the plasma protein extravasation induced by BK. Compounds I and II blocked BK-induced plasma protein extravasation for at least 30 min (Table 3). When the antagonists I, II and III were injected i.v. alone in a dose that was 10 times as large as that required for a full antagonistic effect (and 10 times as large as the dose of BK employed in this test), only compound I caused plasma protein extravasation (Figure 4). This indicates an agonistic action of compound I only at extremely high doses.

Bronchoconstriction

Changes in pulmonary inflation pressure were used to assess the action of the new antagonists on BK-induced broncho-

constriction *in vivo*. All of the antagonists inhibited the BK-induced bronchoconstriction within 1 min and the order of potency was I ≈ II > III ⩾ IV. Similar differences were found when the durations of inhibition were compared. The effect of compound IV lasted for less than 10 min whereas an inhibitory effect of compounds I and II was still detectable after 60 min (Figure 5). The new antagonists, therefore, also have a high potency and long duration of action in the guinea-pig *in vivo*. The bronchoconstriction induced by histamine was not reduced by any of the four compounds, which demonstrates their specificity. No bronchoconstriction occurred after the injection of the antagonists on their own; thus, agonistic effects at the BK receptor in the bronchi, or release of histamine by the antagonists can be excluded.

Farmer *et al.* (1989) have reported that, in a similar experimental setup, a bradykinin analogue similar to compound IV (D-Arg-[Hyp^3-D-Phe^8]-BK) was only a very weak inhibitor of BK, and other related antagonists were virtually inactive. These findings, together with the results from binding studies, have been interpreted by suggesting a B_3 BK receptor subtype. It is therefore remarkable that our new compounds are very potent BK antagonists also in the guinea-pig airways *in vivo*.

Effects of bradykinin on afferent neurones

Two models of BK-induced stimulation of afferent neurones have been used to test the new BK antagonists. BK is the most potent stimulator known, of paravascular nociceptors in the 'rabbit reflex ear' preparation *in vivo* (Juan & Lembeck, 1974). In addition, the BK-induced contractions of the isolated iris sphincter muscle of the rabbit *in vitro* are due to the release of tachykinins from peripheral terminals of afferent neurones (Bynke *et al.*, 1983; Ueda *et al.*, 1984; Håkanson *et al.*, 1987).

In an earlier investigation (Griesbacher & Lembeck, 1987), in which the BK antagonist B4310 was used, concentrations of 50–500 nM had been necessary to block the BK-induced nociceptor stimulation in the rabbit ear *in vivo*. Moreover, this effect was only present during the infusion of the antagonist. In contrast, in the present investigation, the BK effect was completely inhibited for at least 1 h after a 5 min infusion of compound I in a concentration of 15 nM (Figure 6). Compound III was less potent (by a factor of about 3). Thus, this test provided evidence for the high potency and long duration of action of the new antagonists on BK-induced nociceptor stimulation.

The test on the rabbit iris sphincter muscle allowed an evaluation of the antagonism by regression analysis. It confirmed the extremely high antagonistic potency of compound I which, in a concentration as low as 0.5 nM, shifted the concentration-response curve of BK to the right by a factor of 7. Compounds II and III were slightly less potent. In contrast, compound IV was approximately 1000 times less potent than compound I (Table 4).

In summary, the present experiments have demonstrated, both *in vitro* and *in vivo*, that compound I is an extremely potent antagonist of BK in a number of different preparations. Compounds II and III were slightly less active. The antagonistic effect was specific for BK, as responses to acetylcholine, noradrenaline, and histamine were unaffected. It was not intended, however, to determine whether the new antagonists were specific for any BK receptor subtype. To investigate receptor subtype specificity quite different methods would be needed. An agonistic effect was observed only in the plasma protein extravasation test with very high concentrations of compound I, and with compound II in only one of the organs tested. The long duration of the antagonists is especially important. In some tests the inhibition of the BK response was almost irreversible. BK is metabolized by a variety of enzymes (Regoli & Barabé, 1980). A role of peptidases in the inactivation of the new BK antagonists is possible but remains to be investigated. At present it is most likely that the antagonists have a high affinity for BK receptors and that this is the major reason for their long duration of action.

Finally, the high potency and the long duration of action of the new antagonists will be of great value in the investigation of the pathophysiological role of BK. Furthermore, the lack of agonist effects should offer the possibility of therapeutic applications.

The authors wish to thank Mr W. Schluet for expert technical assistance and for preparing the drawings. The investigation was supported by the Pain Research Commission of the Austrian Academy of Sciences, the Austrian National Bank (grant No. 2811), and the Austrian Funds for Scientific Research (grant No. P5616).

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(Received July 3, 1990
Revised September 6, 1990
Accepted September 19, 1990)

Br. J. Pharmacol. (1991), **102**, 305–310 © Macmillan Press Ltd, 1991

Evidence that the unilateral activation of 5-HT_{1D} receptors in the substantia nigra of the guinea-pig elicits contralateral rotation

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1 The effects of various 5-hydroxytryptamine (5-HT) receptor agonists were examined following unilateral infusion into the substantia nigra (SN) of the guinea-pig.

2 The 5-HT_1 receptor agonists, 5-carboxamidotryptamine (5-CT) (2–25 μg), sumatriptan (10–25 μg) and RU24969 (25 μg) all induced a marked contralateral rotation. In contrast, the selective 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH DPAT, 10–25 μg) produced only a very small response, whilst the selective 5-HT_{1C}/5-HT_2 receptor agonist (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride ((±)-DOI) (25 μg) and the 5-HT_3 receptor agonist, 2-methyl 5-HT (2-Me5-HT, 25 μg) were without effect.

3 The contralateral rotation induced by 5-CT (10 μg) was attenuated following pretreatment with the non-selective 5-HT_1/5-HT_2 receptor antagonists methiothepin (1 mg kg^{-1}, s.c.) and metergoline (5–10 mg kg^{-1}, s.c.) but not the 5-HT_{1C}/5-HT_2 antagonist ritanserin (1 mg kg^{-1}, s.c.) or the 5-HT_3 antagonist, ondansetron (0.5 mg kg^{-1}, s.c.). An involvement of dopaminergic systems in the rotational response to 5-CT was implied by the antagonism of 5-CT-induced rotation by haloperidol (0.3 mg kg^{-1}, s.c.).

4 At doses lower than those required to produce contralateral rotation, 5-CT (0.08–0.4 μg) and sumatriptan (2 μg) induced a small, but nonetheless consistent, ipsilateral rotation.

5 The data with agonists and antagonists taken together suggest that 5-CT-induced contralateral rotation may be mediated by 5-HT_{1D} receptor activation but definitive classification of the receptor will not be possible until selective 5-HT_{1D}-antagonists become available. This may therefore represent the first model to study this receptor subtype *in vivo*.

Introduction

Central 5-hydroxytryptamine (5-HT) receptor subtypes have been classified into three main categories, namely 5-HT_1-like, 5-HT_2 and 5-HT_3 (Bradley *et al.*, 1986). Furthermore, the 5-HT_1-like receptor appears to consist of a heterogeneous pool of which at least four subtypes, termed 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D}, have been described (see Peroutka, 1988 for review). The 5-HT_{1D} receptor has recently attracted wide interest, in particular for its demonstration in human brain (Waeber *et al.*, 1988) and possible involvement in movement disorders such as Huntington's chorea (Waeber & Palacios, 1989). In addition, sumatriptan (GR43175) (Humphrey *et al.*, 1988), which has been shown to be effective in the treatment of migraine, (Doenicke *et al.*, 1988) also shows some selectivity for the 5-HT_{1D} binding site (Schoeffter & Hoyer, 1989).

In addition to man, central 5-HT_{1D} receptors have been found in calf, pig, dog, pigeon, monkey and guinea-pig (Heuring & Peroutka, 1987; Waeber *et al.*, 1989a,b). In each species studied to date, the distribution of this receptor subtype is similar with the highest density in regions associated with the dopamine nigrostriatal system e.g. the substantia nigra (SN), globus pallidus and striatum (Waeber *et al.*, 1989a). Functionally, 5-HT_{1D} receptors have been shown to be negatively coupled to adenylate cyclase (Schoeffter *et al.*, 1988) and they may serve as the terminal 5-HT inhibitory autoreceptor (Schlicker *et al.*, 1989). Indeed, with respect to central distribution and function, the 5-HT_{1D} receptor is very similar to the 5-HT_{1B} subtype, which appears confined to the rat, mouse and opossum (see Hoyer & Middlemiss, 1989). Thus, the 5-HT_{1D} receptor may represent an evolutionary development of the 5-HT_{1B} subtype.

In rats, both electrophysiological and behavioural studies suggest that 5-HT has an inhibitory effect on nigrostriatal dopamine function (Dray *et al.*, 1976; Pycock, 1980). Consistent with these findings, James & Starr (1980) reported that unilateral injection of 5-HT into the SN of the rat elicited ipsilateral rotation with a corresponding decrease in striatal dopamine metabolism on the injected side. Paradoxically, however, Oberlander *et al.* (1981) observed contralateral rotation to this same treatment and the intra-nigral injection of the 5-HT_1 receptor agonist, RU24969, produced behaviours consistent with an activation of the nigrostriatal dopamine pathway (Oberlander, 1983). Based on our current understanding of central 5-HT function, an involvement of 5-HT_{1B} receptors in at least some of these responses seems likely, since nigral 5-HT receptors in the rat appear to be primarily of this subtype (Pazos *et al.*, 1985;Verge *et al.*, 1986). In the search for a model in which to examine central 5-HT_{1D} receptor function *in vivo*, we therefore decided to study the effects of various 5-HT agonists following unilateral injection into the SN of the guinea-pig. In a preliminary account (Higgins *et al.*, 1990) we described how the selective 5-HT_1-like receptor agonists 5-carboxamidotryptamine (5-CT) and sumatriptan, but not 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH DPAT), induced a marked contralateral rotation when injected into the substantia nigra. Furthermore, this response may be antagonized by the non-selective 5-HT_1/5-HT_2 receptor antagonist metergoline. We now describe a more extensive analysis of this behavioural response. Based upon our current understanding of 5-HT receptor subtypes, this model may thus represent the first demonstration of central 5-HT_{1D} receptor function *in vivo*.

Methods

Animals and housing

Male, Dunkin-Hartley guinea-pigs (300–500 g, Interfauna) were used throughout. The animals were housed in groups of 5 at a temperature of 20 ± 1°C on a 12 h light/dark cycle (lights on 6 h 00 min–18 h 00 min). Animals were allowed free

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access to food (SDS FD1 guinea-pig diet) and water except during behavioural testing.

Stereotaxic surgery

Guinea-pigs were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and ketamine (40 mg kg^{-1}, i.p.) and placed in a Kopf stereotaxic frame. Guide cannulae consisting of a stainless steel tube (23 gauge) in a perspex holder, were implanted unilaterally, 8 mm above the left substantia nigra (SN) (Anterior +6.0 mm (interaural line), Lateral +2.2 mm (midline), Ventral −4.0 mm (skull surface)) with the mouthpiece set 3.2 mm below zero (Luparello, 1967). Stainless steel stylets (30 gauge) were used to keep the guide cannula patent during the 7 day recovery period. These extended 1 mm beyond the cannula tip.

Central microinjections

Seven days after surgery, the guinea-pigs were lightly restrained, the stylets removed and a microinjection needle (30 gauge) was inserted into the guide cannula such that it terminated 8 mm below the cannula tip. Drug solutions or saline vehicle were slowly infused over a 120 s period in a dose volume of 2 μl. A further 30 s was allowed for diffusion away from the cannula tip. The injection units were then removed and the stylets replaced. Unless otherwise stated, the guinea-pigs were used in one experiment only.

Testing for rotational behaviour

Immediately after the intra-nigral injection of test compound or vehicle, the guinea-pigs were placed individually in hemispherical bowls (33 cm diameter; 30 cm high) typically for a 3 h period unless otherwise stated. Rotational behaviour was automatically measured connecting the animals via a harness to a transducer which, in turn, was linked to an on-line computer (Olivetti M28) for the automatic recording of both ipsilateral (I) and contralateral (C) rotations.

For antagonist studies, the test compound or vehicle was administered subcutaneously in the nape of the neck, 30 min before the nigral injection of 5-CT (10 μg).

Testing for locomotor activity

To determine whether antagonism of 5-CT-induced rotation was due to sedation rather than pharmacological antagonism, each effective compound was assessed for overt signs of behavioural depression and locomotor activity at doses which antagonized 5-CT. All treatments were administered subcutaneously, 30 min before placing (otherwise untreated) guinea-pigs singly in activity monitors for 3 h. The transparent locomotor activity cages (length 40 cm × width 25 cm × height 30 cm) had eight infra-red photocells on each of three levels; five along the length and three across the width of the cage with a 7.5 cm space between each. The vertical distance from the floor of the cage was adjustable for the 3 levels of photocells but was set so that both normal ambulation and rearing behaviour could be monitored. Beam crossings were counted by a microcomputer in 15 min epochs over the 3 h test period.

Histology

Upon completion of testing, the guinea-pigs were killed by cervical dislocation and then injected with 2 μl of India ink into the left SN. The brains were then removed and sectioned on a freezing microtome for histological placement of injection sites. Animals in which India ink was sited outside the SN were excluded from the study.

Drugs

5-Carboxamidotryptamine (5-CT), 5-methoxy-3(1,2,3,6-tetrahydropyridin-4-yl)1H indole (RU24969), 2-methyl 5-HT, ondansetron (1,2,3,9-tetrahydro-9-methyl-3-[2-methyl-1H-imidazol-1-yl)-methyl]-4H carbazol-4-one) and sumatriptan (3-(2-dimethylamino)ethyl-N-methyl-1H-indole-5 methane sulphonamide) were all synthesized in the Neurochemistry Department, Glaxo Group Research Ltd., Ware. 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH DPAT), (±)1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride (DOI) and haloperidol were obtained from Research Biochemicals Inc. The suppliers of the remaining drugs were as follows: methiothepin maleate (Roche), metergoline (Farmitalia) and ritanserin (Janssen).

All drugs, unless otherwise stated, were dissolved or suspended in 0.9% sodium chloride solution (Steriflex). Methiothepin maleate was dissolved in 1 ml ethanol containing a few drops of 1 M HCl and made up to final volume in saline. Both ritanserin and haloperidol were dissolved in 0.1 M tartaric acid and metergoline in 1 M ascorbate before addition of saline. For each treatment, the control group received identical vehicle alone. All drug concentrations are expressed as free base.

Drugs for subcutaneous or intraperitoneal injection were administered in a dose volume of 2 ml kg^{-1}, except chloral hydrate which was injected in a volume of 10 ml kg^{-1}. The pH of all solutions was in the range 4–7. For intra-nigral injections, a dose volume of 2 μl was used.

Results

Effect of 5-carboxamidotryptamine following unilateral injection into the substantia nigra in the guinea-pig

Microinfusion of 5-CT (2–25 μg) into the left SN of the guinea-pig induced a strong contralateral (C) rotation (Figure 1). Although this response was almost immediate in onset, at higher doses (10–25 μg) the most intense period of activity was 1–4 h post injection (Figure 2). This apparent delay may be due to the induction of ataxia which impaired locomotor performance. During this time period, the animals showed a clear asymmetrical posture with the head directed away from the injected side.

At lower doses of 5-CT (0.08–2 μg), a small but nonetheless reproducible, ipsilateral (I) rotation was observed (Figure 1). The magnitude of this effect was much smaller than the contralateral response (e.g. 5-CT 0.4 μg I = 39 ± 16 rotations 3 h^{-1}; 5-CT 25 μg C = 436 ± 109 rotations 3 h^{-1}).

In order to determine the possibility of re-testing guinea-pigs, the effect of repeated nigral injections of 5-CT (10 μg) was examined. All drug injections were made into the left SN and the results are shown in Table 1. When a recovery period of 7 days was allowed between consecutive microinfusions of 5-CT (10 μg), the contralateral rotation induced by the second dose

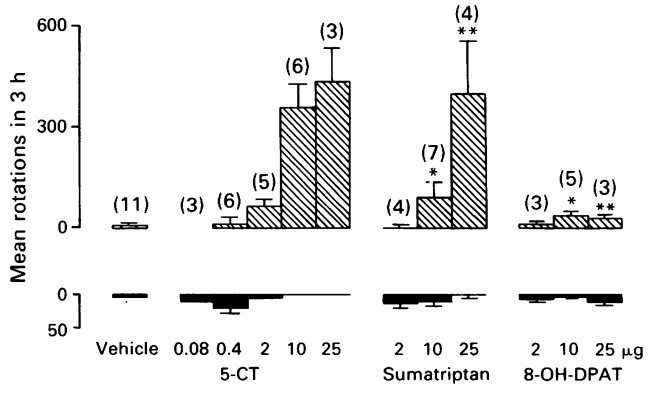


Figure 1 Rotational behaviour elicited by 5-HT_1 receptor agonists following unilateral intra-nigral injection in the guinea-pig. Contralateral (hatched columns) and ipsilateral (solid columns) rotations were recorded for 3 h. Significant differences (*$P < 0.05$; **$P < 0.01$) compared to vehicle were calculated by the paired t test. Figures in parentheses above each column refer to the number of animals used in each treatment group; vertical bars show s.e.

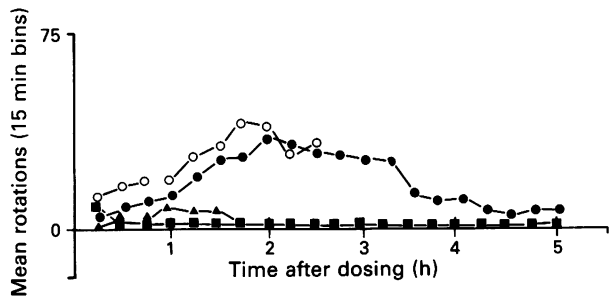


Figure 2 The duration of action of the contralateral rotation induced by unilateral intra-nigral injection of 5-carboxamido-tryptamine (5-CT) in guinea-pigs. Contralateral rotations were recorded every 15 min for up to 5 h following unilateral injection of 5-CT. The following doses refer to the total dose given to each guinea-pig: (■) 0.4 μg, (▲) 2 μg, (●) 10 μg and (○) 25 μg.

was significantly lower ($P < 0.05$, paired t test) than the response to the initial infusion. If the recovery period was extended to 14 or 21 days, the second response was still reduced, although not significantly. Because of this apparent desensitisation, all other experiments were carried out using naive animals.

The relationship between the rotational response to a 10 μg dose of 5-CT and the histological location of the injection site is shown by Figure 3. These data show that microinfusions made directly into the SN were associated with high (>150 rotations 3 h^{-1}) contralateral rotation scores to 5-CT. Animals which displayed little or no rotation to 5-CT tended to have infusion sites on the borders of, or outside, the SN.

Effect of other 5-hydroxytryptamine agonists on turning behaviour in the guinea-pig following unilateral injection into the substantia nigra

Significant contralateral rotation was induced by the infusion of sumatriptan (10–25 μg), RU24969 (25 μg) or 8-OH DPAT (10–25 μg) into the SN, although the response to 8-OH DPAT was much smaller than that elicited by the other compounds (Figure 1). In common with 5-CT, sumatriptan at a dose (2 μg) subthreshold for inducing contralateral rotation, produced a small ipsilateral response as did 8-OH DPAT (25 μg).

In contrast to the effects of the 5-HT$_1$ receptor agonists, the 5-HT$_3$ receptor agonist, 2-Me 5-HT, failed to induce any significant rotational behaviour following intra-nigral infusion (25 μg = I = 7 ± 3 rotations 3 h^{-1}; C = 11 ± 1 rotations 3 h^{-1}, $n = 4$). The selective 5-HT$_{1C/2}$ receptor agonist (±)-DOI (25 μg) was relatively ineffective although some rotational behaviour was observed (I = 58 ± 28 rotations 3 h^{-1}; C = 32 ± 30 rotations 3 h^{-1}, $n = 4$). However, there was no obvious preference for either direction and these scores may therefore reflect the behavioural hyperactivity which became apparent approximately 60 min after DOI infusion.

Table 1 Desensitization of 5-carboxamidotryptamine (5-CT)-induced contralateral rotation following intra-nigral injection in the guinea-pig

Interval between doses of 5-CT	*Contralateral rotations per 3 h induced by 5-CT*	
	First Dose	*Second Dose*
7 days	350 ± 56	212 ± 74*
14 days	374 ± 107	156 ± 36
21 days	308 ± 101	194 ± 62

Five guinea-pigs were used for each treatment group. Animals were dosed with 5-CT (10 μg) intra-nigrally on day 1 and then again on day 7, 14 or 21. Values are mean ± s.e.mean.
*Indicates a significant difference ($P < 0.05$) between response to first and second dose (paired t test).

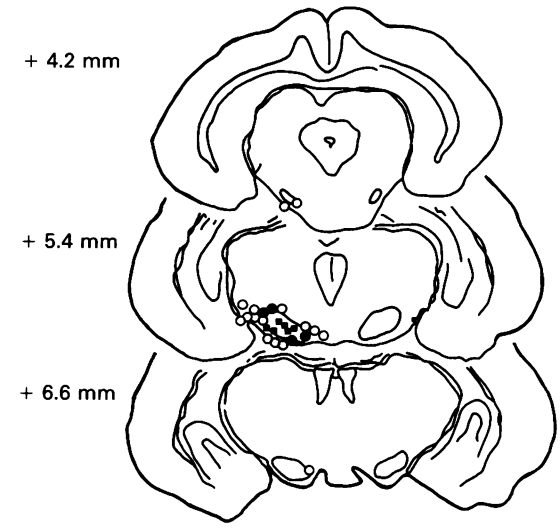


Figure 3 Correlation of the contralateral rotational behaviour induced by intra-nigral injection of 5-carboxamidotryptamine (5-CT, 10 μg) with site of injection in guinea-pig brain. Figure modified from the stereotaxic atlas of Luparello (1967). Symbols denote site of injection placement. Injections into the centre of the substantia nigra (SN) induced >150 rotations in 3 h (■). Injections on the periphery of the SN induced between 100 and 150 rotations in 3 h (●) whereas placements outside the SN caused less than 100 rotations (○). Anterior co-ordinates measured from the interaural line.

Effect of various antagonists on the contralateral rotation response produced by 5-carboxamidotryptamine (10 μg)

The contralateral rotation induced by intra-nigral infusion of 5-CT (10 μg) was dose-dependently blocked after pretreatment with methiothepin (1 mg kg^{-1}, s.c.) or metergoline (5–10 mg kg^{-1}, s.c.) (Figure 4). Following saline pretreatment, the

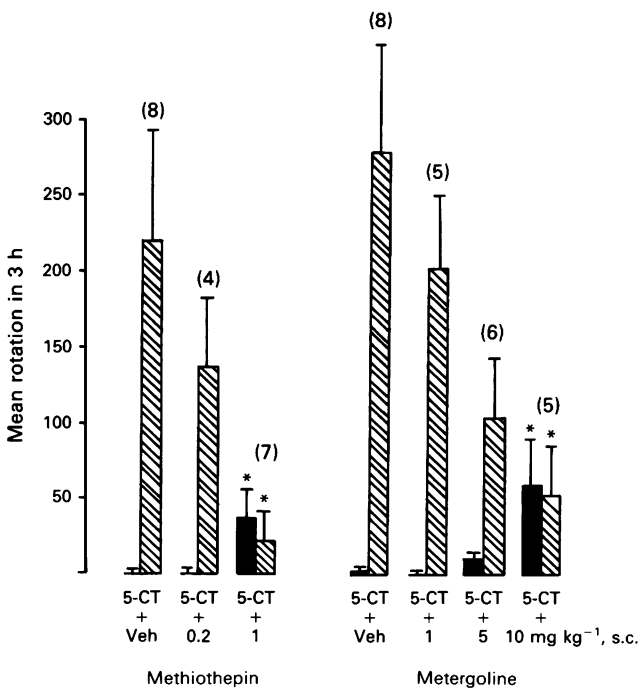


Figure 4 Antagonism of 5-carboxamidotryptamine (5-CT)-induced rotation by methiothepin and metergoline in guinea-pigs. Antagonists were given s.c. 30 min before unilateral intra-nigral injection of 5-CT (10 μg). Contralateral (hatched columns) and ipsilateral (solid columns) rotation were recorded for 3 h post 5-CT injection. Significant differences (*$P < 0.05$) compared to 5-CT/vehicle (Veh) were calculated by the unpaired t test. Figures in parentheses above each column refer to the number of animals used in each treatment group; vertical bars show s.e.

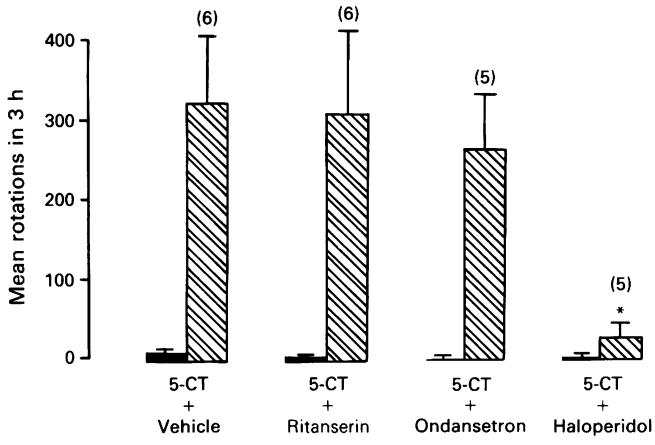


Figure 5 Effect of ritanserin, ondansetron and haloperidol on 5-carboxamidotryptamine (5-CT)-induced rotation following intra-nigral injection in guinea-pig. Ritanserin (1 mg kg^{-1}), ondansetron (0.5 mg kg^{-1}), haloperidol (0.3 mg kg^{-1}) or vehicle were given s.c. 30 min before unilateral intra-nigral injection of 5-CT (10 μg). Contralateral (hatched columns) and ipsilateral (solid columns) rotations were recorded for 3 h. Significant differences (*$P < 0.05$) compared to 5-CT/vehicle were calculated by unpaired t test. Figures in parentheses above each column refer to the number of animals used in each treatment group; vertical bars show s.e.

rotational response to 5-CT was exclusively contralateral. However, at doses of methiothepin or metergoline that significantly blocked the contralateral response, some ipsilateral rotation was observed (Figure 5).

Peak rotational responses to intra-nigral 5-CT occurred at 90–120 min post administration. The effect of the antagonists, given alone, on locomotor activity was monitored for 120 min. At doses that significantly antagonized 5-CT-induced contralateral rotation, methiothepin (1 mg kg^{-1}, s.c.), metergoline (10 mg kg^{-1}, s.c.) and haloperidol (0.3 mg kg^{-1}, s.c.) did not significantly reduce spontaneous activity in the guinea-pig (Table 2). Furthermore, no overt signs of sedation were seen in any of these groups except in the metergoline- and methiothepin-pretreated groups where 2/7 of the animals displayed hind limb abduction with a lowered body position.

Discussion

The present studies demonstrate that the unilateral microinfusion of the non-selective 5-HT$_1$ receptor agonist 5-CT, into the SN of the guinea-pig elicits a marked contralateral rotation. Various lines of evidence support an involvement of 5-HT$_{1D}$ receptors in this response. Firstly, the rank order of potency for a range of agonists to induce this behaviour following intra-nigral injection i.e. 5-CT > sumatriptan > RU24969 > 8OH-DPAT, is in good agreement with their relative potencies at inhibiting adenylate cyclase activity in calf SN, a functional 5-HT$_{1D}$ model (Schoeffter *et al.*, 1988; Schoeffter & Hoyer, 1989). This rank order is also similar to their relative affinities for 5-HT$_{1D}$ binding sites (Waeber *et al.*, 1988; Schoeffter & Hoyer, 1989). Furthermore, the selective 5-HT$_{1C}$/5-HT$_2$ receptor agonist (±)-DOI (Glennon *et al.*, 1984; Hoyer, 1988) and the 5-HT$_3$ receptor agonist 2-Me 5-HT (Richardson *et al.*, 1985) were ineffective at a dose of 25 μg, suggesting that these 5-HT receptor subtypes are not involved.

Table 2 Effects of metergoline, methiothepin and haloperidol on locomotor activity in the guinea-pig

Treatment	(mg kg^{-1}, s.c.)	*Locomotor activity counts in* 2 h	P *value*
Vehicle	—	235 ± 66	—
Metergoline	10.0	210 ± 47	0.76
Methiothepin	1.0	341 ± 26	0.14
Haloperidol	0.3	202 ± 51	0.70

*Unpaired Students t test.
Six to eight animals were used in each treatment group.

Secondly, the rotations induced by 5-CT were blocked by the non-selective 5-HT$_1$/5-HT$_2$ receptor antagonists methiothepin and metergoline at doses which failed to produce any marked behavioural effects. Pretreatment with the 5-HT$_{1C}$/5-HT$_2$ receptor antagonist, ritanserin, at 1 mg kg^{-1} failed to block the response to 5-CT. Previous studies have shown that this dose of ritanserin antagonizes (±)-DOI-induced wet dog shakes and hyperactivity in the guinea-pig, a putative central 5-HT$_2$ model in this species (Cole *et al.*, 1990). The 5-HT$_3$ receptor antagonist, ondansetron, was similarly ineffective against 5-CT-induced contralateral rotation at a pharmacologically relevant dose in the guinea-pig (Costall *et al.*, 1987). Thus, the antagonist studies are consistent with an involvement of a 5-HT$_1$ receptor subtype in the rotational response to 5-CT.

Finally, all drug microinfusions were made into the SN of the guinea-pig, a tissue containing 5-HT receptors belonging primarily, if not exclusively, to the 5-HT$_{1D}$ subtype (Waeber *et al.*, 1989b). Microinfusions of 5-CT just outside this structure failed to induce significant rotation. This strongly suggests an involvement of the SN in the behavioural effects observed.

In a wide range of animal models, dopamine has been implicated in playing a primary role in the mediation of circling behaviour (reviewed in Pycock, 1980). For instance, unilateral electrical stimulation of the SN elicits contralateral rotation in rats (which may be blocked by dopamine antagonists e.g. haloperidol), which is associated with increased striatal dopamine metabolism on the stimulated side only (Arbuthnott & Crow, 1971; Arbuthnott & Ungerstedt, 1975). The present studies also suggest that 5-CT-induced contralateral rotation in the guinea-pig is similarly dependent upon activation of ascending nigrostriatal dopamine fibres. Thus, haloperidol antagonized this rotational response at a dose that failed to affect behaviour in naive animals. This would imply that the antagonism was not simply due to sedation. One interpretation of these data is that an agonist action at nigral 5-HT$_{1D}$ receptors results in stimulation of ascending dopaminergic fibres to elicit rotation. This is the first demonstration of a functional interaction between 5-HT$_{1D}$ receptors and dopaminergic systems. At present the nature of this interaction is unclear, although a recent study (Waeber *et al.*, 1989a) may suggest that nigral 5-HT$_{1D}$ receptors are not localized on dopamine cell bodies. The histological verification of injection sites in this study (Figure 3) is consistent with this hypothesis. Thus, the greatest circling response occurred following injection into the nigra reticulata, a region containing little dopamine (dopamine cell bodies are in the nigra compacta). Based on the effects of lesions in striatum, Waeber *et al.* (1990) have suggested that 5-HT$_{1D}$ receptors appear to be presynaptically located on terminals of the pathway descending from the striatum to nigra reticulata.

Because of the time-consuming nature of the surgical operations necessary for this type of experiment, we studied the rotational responses to repeated injections of 5-CT in order to determine whether the animals could be re-used. There was a clear desensitization to a second challenge dose of 5-CT given 7 days after the first. Increasing the interval between dosing to 21 days failed to restore the second response to the initial level. Whether this is a pharmacological or behavioural desensitization, or simply due to tissue necrosis following the first injection, remains to be established but in view of this observation all the studies reported were carried out in experimentally naive animals.

An interesting finding from these studies was the ipsilateral rotation observed at doses of 5-CT and sumatriptan lower than those required to induce contralateral rotation. The

small magnitude of this response made pharmacological characterization using antagonists virtually impossible. However, following pretreatment with metergoline and methiothepin, a significant ipsilateral rotation was unmasked in animals treated with a dose of 5-CT that in control animals produced exclusively contralateral rotation. This may indicate that the ipsilateral rotation produced by 5-CT is not mediated via 5-HT$_1$/5-HT$_2$ receptors. Since both methiothepin and metergoline have affinity for the dopamine D$_2$-binding site (Leysen *et al.*, 1981) it could be argued that they prevent 5-CT-induced rotation indirectly by blocking D$_2$-binding sites (cf. haloperidol). However, unlike methiothepin and metergoline, haloperidol attenuates 5-CT-induced contralateral rotation but does not reveal the ipsilateral rotation response. This would suggest that haloperidol is acting at a different site(s) from metergoline and methiothepin. It would be interesting to examine the effects of central 5,7-dihydroxytryptamine (5,7-DHT) lesions on both the ipsi- and contralateral rotation response to 5-CT. Such studies may provide further insight into the mechanisms underlying these rotational responses. Following intra-nigral infusion in the rat, 5-HT-induced contralateral rotation is potentiated by 5,7-DHT lesions (Oberlander *et al.*, 1981) suggesting the involvement of post-synaptic 5-HT receptors within the substantia nigra. Based on both the functional and anatomical similarity between 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors (see Introduction) this equivalent response in the rat may represent an *in vivo* 5-HT$_{1B}$ receptor model.

In conclusion, 5-CT-induced contralateral rotation in the guinea-pig may represent the first animal model for the study of 5-HT$_{1D}$ receptors *in vivo*. Although the results for the agonists strongly implicate an action at the 5-HT$_{1D}$ site, definitive classification is not possible without selective 5-HT$_{1D}$ antagonists. A functional involvement of 5-HT$_{1D}$ receptors with dopaminergic systems seems evident, a finding supported by the anatomical distribution of this receptor subtype (Waeber *et al.*, 1989a,b). This may suggest a role for selective 5-HT$_{1D}$ receptor compounds in the treatment of movement disorders (see Waeber & Palacios, 1989).

We would like to thank Mrs S. Parsons, Mrs L. Elliott and Miss N. Cole for their excellent technical assistance.

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(Received July 4, 1990
Revised September 12, 1990
Accepted September 25, 1990)

Br. J. Pharmacol. (1991), **102**, 311–316 © Macmillan Press Ltd, 1991

Tissue selectivity and spasmogen selectivity of relaxant drugs in airway and pulmonary vascular smooth muscle contracted by $PGF_{2\alpha}$ or endothelin

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1 The spasmolytic effects of smooth muscle relaxant drugs with different mechanisms of action have been examined on isolated preparations of guinea-pig trachea and rat pulmonary artery. The preparations were contracted with concentrations of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or endothelin selected to give approximately 80% of the agonist maximum response on each tissue. These concentrations also caused similar levels of tone (% of tissue maximum contraction) on each tissue.

2 With endothelin as the spasmogen, the potassium channel opening drug, pinacidil, was more potent on trachea ($-\log IC_{50}$ 5.49) than on pulmonary artery (4.39), i.e. was airway-vascular selective, whereas with $PGF_{2\alpha}$ as the spasmogen it was more potent on pulmonary artery (6.01) than on trachea (5.27), i.e. was vascular-airway selective.

3 With endothelin as the spasmogen, fenoterol was also airway-vascular selective (8.35 on trachea; little effect on pulmonary artery), nitroprusside was vascular-airway selective (7.50 on pulmonary artery; 5.99 on trachea) and forskolin was non-selective (6.69 on trachea; 6.70 on pulmonary artery). Thus, the airway-vascular selectivity of the relaxant drugs varied with the drug.

4 On pulmonary artery, pinacidil, nitroprusside and forskolin were all more potent against $PGF_{2\alpha}$ than against endothelin, i.e. 42, 4 and 7 fold respectively. On trachea, these drugs were equipotent against $PGF_{2\alpha}$ and endothelin.

5 The results suggest that, in pulmonary artery, but not in trachea, the relative contribution of protein kinase C activation and calcium influx to the maintenance of tonic contractions to endothelin and $PGF_{2\alpha}$ may be different. If protein kinase C activation should be the predominant mechanism for endothelin in pulmonary artery, then it may be more difficult to reverse this with relaxant drugs that lower intracellular calcium.

6 The study indicates that the airway-vascular selectivity of relaxant drugs can be spasmogen-dependent as well as dependent on the mechanism of action of the relaxant drug. Thus, relaxant drugs, whether of interest for their airway or vascular effects, should be tested against a full range of spasmogens of likely pathophysiological importance.

Introduction

Drugs that relax airway smooth muscle are potentially useful as bronchodilators in asthma and drugs that relax pulmonary vascular smooth muscle might be useful as vasodilators in the treatment of pulmonary hypertension. From a therapeutic viewpoint it is important that smooth muscle relaxant drugs are effective against those spasmogens that may be involved pathophysiologically in the disease state. Furthermore, a bronchodilator drug should show selectivity in the lung for airway, as compared with pulmonary vascular, smooth muscle if side effects are to be minimized.

In a previous study we showed airway-vascular selectivity in the lung for the relaxant drug pinacidil, a potassium channel opening drug (Southerton *et al.*, 1988). The study involved the reversal of endothelin-induced contractions and pinacidil was more potent, as a spasmolytic agent, on tracheal smooth muscle (guinea-pig or rat) than on rat pulmonary artery (O'Donnell *et al.*, 1990). To explain this observation we suggested that it might be related to differences between airway and pulmonary arterial smooth muscle in either (a) the level of tone induced by endothelin, (b) the receptor reserve for endothelin and/or (c) the contractile mechanism(s) for endothelin.

Since each of the above postulates relates to the spasmogen used in that study, i.e. endothelin, one aim of this study was to establish whether the same selectivity was seen for pinacidil if the tissues were contracted with an alternative spasmogen. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was selected since it is a potent spasmogen on both tissues. In addition, we have examined the airway-vascular selectivity in the lung of drugs with mechanisms that differ from that of pinacidil (with endothelin as the spasmogen). The drugs selected were fenoterol (β-adrenoceptor agonist), forskolin (a direct activator of adenylate cyclase), nitroprusside (an activator of soluble guanylate cyclase) and felodipine (a calcium entry blocking drug). To extend our understanding of these relaxant drugs on airway and vascular smooth muscle in the lung, we have also examined whether their potency on either of these tissues is affected by the spasmogen selected to contract the tissues, i.e. endothelin or $PGF_{2\alpha}$.

A preliminary account of some of these data was presented to the 23rd meeting of the Australasian Society of Clinical and Experimental Pharmacologists, Sydney, December 1989 (Kay *et al.*, 1990; Zeng & O'Donnell, 1990).

Methods

Female, English shorthair, guinea-pigs (250–400 g) and male Wistar rats (300–400 g) were used in this study.

Isolated tracheal preparations (guinea-pig)

Tracheal rings, with the cartilage cut, were set up in Krebs solution at 37°C and responses were mainly recorded isometrically (10 mN resting force). In some experiments with pinacidil, isotonic recording (5 mN constant force) was also used. Since the data obtained by these two recording methods

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were indistinguishable they were combined. Indomethacin (3 μM) was present throughout the experiment to prevent the generation of tone and to maintain a steady baseline. Four preparations from each trachea were set up and, after two or three submaximal contractions to carbachol, to equilibrate the preparations, a reference contractile response to 10 μM carbachol (maximum) was obtained on each preparation.

Isolated pulmonary artery preparations (rat)

Single ring preparations of pulmonary artery were set up at a resting force corresponding to a transmural pressure of approximately 10 mmHg (7 mN for 2 mm and 10 mN for 3 mm ring preparations) in physiological salt solution (PSS) at 37°C as described by Wanstall & O'Donnell (1988). The endothelium was removed by gentle rubbing of the intimal surface. Developed force (mN) in the circular muscle was recorded isometrically. After a 1 h equilibration period, the preparations were contracted with noradrenaline (0.1 μM). When the contraction was stable, acetylcholine (1 μM) was added and the lack of a relaxant response indicated successful removal of the endothelium. After washing the tissues with PSS, a reference contractile response to K^+-depolarizing PSS (in which 80 mM NaCl was replaced with 80 mM KCl) was obtained.

Experimental protocol and data analyses

Preparations were contracted with the selected concentration of $PGF_{2\alpha}$ or endothelin (see Results). Once the response had stabilized, a cumulative concentration-response curve to pinacidil, forskolin, nitroprusside, fenoterol or felodipine was obtained. Increments in concentrations were made once each response had equilibrated until no further relaxation occurred. Contractions to the spasmogens could be maintained for the time required to complete the concentration-relaxation curves for the spasmolytic drug (a maximum of 90 min).

Responses to the relaxant drugs (spasmolytic effect) were expressed as percentage reversal of the spasmogen contraction, i.e. 100% reversal represents a return of tone in the preparation to the starting baseline. IC_{50} values (molar concentrations producing 50% reversal of spasmogen-induced contraction) were interpolated from these curves and $-\log IC_{50}$ values were used as a measure of spasmolytic potency of the relaxant drugs.

Drugs and solutions

The drugs used were: acetylcholine chloride (Sigma), carbamylcholine (carbachol, Sigma), endothelin (porcine, endothelin-1, Peptide Institute), felodipine (gift from AB Hässle), fenoterol (gift from Boehringer-Ingelheim), forskolin (Calbiochem-Behring), indomethacin (Sigma), nitroprusside (Sigma), (−)-isoprenaline acid tartrate (Sigma), (−)-noradrenaline acid tartrate (Sigma), pinacidil (racemic, gift from Leo Pharmaceuticals), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Sigma).

Stock solutions were prepared as follows: acetylcholine, carbachol, nitroprusside and $PGF_{2\alpha}$ as 10 mM, and endothelin as 10 μM, solutions in deionized water: isoprenaline, noradrenaline and pinacidil (10 mM) and fenoterol (100 mM) in 0.01 M HCl; felodipine (1 mM) and forskolin and indomethacin (10 mM) in absolute ethanol. Dilutions were made on the day of the experiment with the appropriate physiological solution or, for forskolin, with ethanol, and discarded after the experiment. The total amount of ethanol added to the organ bath in the forskolin experiments did not exceed 0.2% v/v.

The composition of the Krebs solution was (mM): NaCl 114, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.7. The composition of the PSS was (mM): NaCl 118, KCl 5.9, $CaCl_2$ 1.5, $MgSO_4$ 0.72, $NaHCO_3$ 25, glucose 11.7 and ascorbic acid 1.14. In the K^+-depolarizing PSS, 80 mM KCl replaced 80 mM NaCl giving a final KCl concentration of 85.9 mM. All the solutions were gassed with 95% O_2: 5% CO_2.

Statistical analyses

Mean values are quoted with their standard errors (s.e.). The significance of differences between mean values of $-\log IC_{50}$ values was assessed by Student's t test.

Results

Concentrations of spasmogens selected

The concentrations of $PGF_{2\alpha}$ and endothelin used to contract the preparations were selected because they gave approximately 80% of the spasmogen maximum response. The concentrations selected, viz. on trachea 0.03 μM endothelin and 1 μM $PGF_{2\alpha}$ and on pulmonary artery 0.01 μM endothelin and 10 μM $PGF_{2\alpha}$, reflect the different potencies of these spasmogens on the two tissues (Figure 1).

Retrospective analyses of the contractions to the above concentrations on all the preparations used in the experiments with the relaxant drugs revealed that the selected concentrations caused approximately the same level of tone viz. the contractions (expressed as a percentage of the maximum response to 10 μM carbachol on trachea or to 80 mM K^+ on pulmonary artery) were: on trachea $PGF_{2\alpha}$ 68 ± 3.4% (25); endothelin 63 ± 3.8% (24); on pulmonary artery $PGF_{2\alpha}$ 76 ± 3.5% (20); endothelin 83 ± 5.4% (20).

Selectivity of relaxant drugs between tissues

The spasmolytic effects of pinacidil on trachea are compared with those on pulmonary artery in Figure 2. Although pinacidil was airway-vascular selective if endothelin was the spasmogen, i.e. was more potent on trachea than on pulmonary artery, it was not airway-vascular selective if $PGF_{2\alpha}$ was the spasmogen. On the contrary, it was vascular-airway selective, i.e. significantly more potent on the pulmonary artery than on the trachea (Figure 2, Table 1). A similar finding for $PGF_{2\alpha}$ in a previous study failed to reach statistical significance (O'Donnell et al., 1990).

When endothelin was used as the spasmogen, airway-vascular selectivity was not universally seen for the other relaxant drugs tested (Figure 3). Nitroprusside was vascular-

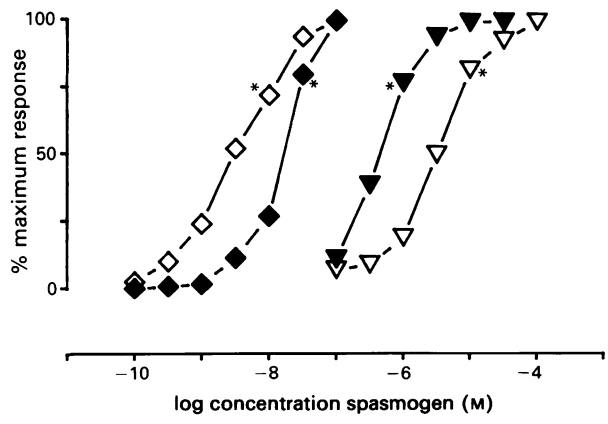


Figure 1 Potency of spasmogens. Mean concentration-response (contraction) curves to endothelin on pulmonary artery (◇) and trachea (◆) and to prostaglandin $F_{2\alpha}$ on pulmonary artery (▽) and trachea (▼). The responses are expressed as a percentage of the maximum contraction to spasmogen on each preparation. Points are mean values, from 8, 13, 10 and 25 experiments respectively; s.e. mean are all less than the size of the symbols. The asterisks indicate the concentrations of spasmogens selected for the spasmolytic experiments.

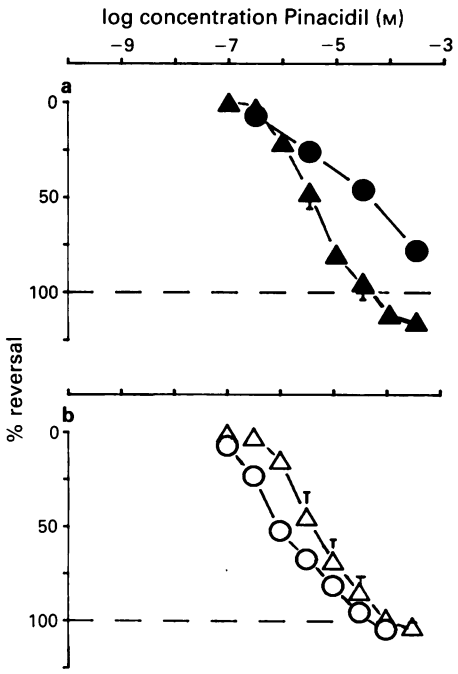


Figure 2 Spasmolytic effects of pinacidil. Mean concentration-response (relaxation) curves to pinacidil on trachea (▲ △) and pulmonary artery (● ○) contracted with endothelin (a, closed symbols) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (b, open symbols). The concentrations of endothelin and $PGF_{2\alpha}$ used on each tissue are indicated in Figure 1. Relaxation responses are expressed as percentage reversal of the induced contraction. Points are mean values and vertical bars are s.e. mean, shown only where these are greater than the size of the symbol. For the number of experiments refer to Table 1.

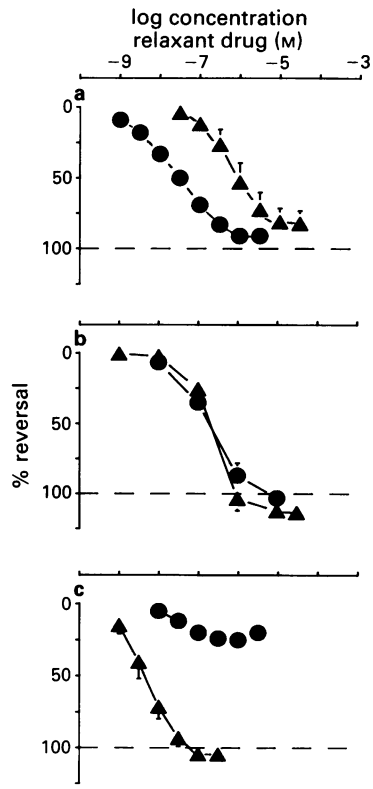


Figure 3 Spasmolytic effects of nitroprusside (a), forskolin (b) and fenoterol (c). Mean concentration-response (relaxation) curves on preparations of trachea (▲) or pulmonary artery (●) contracted with endothelin (for endothelin concentrations see Figure 1). Relaxation responses are expressed as percentage reversal of the induced contraction. Points are mean values and vertical bars are s.e.mean, shown only where these are greater than the size of the symbol. For the number of experiments refer to Table 1.

airway selective, forskolin was equipotent on both tissues and fenoterol was airway-vascular selective in that it was potent and gave full reversal of contractions on trachea but had little effect on pulmonary artery. The differences between tissues for these three relaxant drugs were qualitatively similar if $PGF_{2\alpha}$ was the spasmogen (Table 1).

Selectivity of relaxant drugs between spasmogens on trachea or pulmonary artery

On trachea, the spasmolytic potencies of pinacidil, nitroprusside, forskolin and fenoterol were independent of whether endothelin or $PGF_{2\alpha}$ was the spasmogen (Table 1). In contrast, on pulmonary artery, all these drugs were more potent against $PGF_{2\alpha}$ than endothelin (Table 1).

On pulmonary artery, the reversal of both spasmogens by fenoterol was small (maximum reversal <50%). However, in another series of experiments, a different β-adrenoceptor agonist isoprenaline, was examined. The relaxant responses to isoprenaline were more pronounced than those obtained with

Table 1 Spasmolytic potencies (− log IC_{50} values), on guinea-pig trachea and rat pulmonary artery preparations, of relaxant drugs against prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or endothelin

Relaxant drug	*$PGF_{2\alpha}$*		*Endothelin*	
	Trachea	*Artery*	*Trachea*	*Artery*
Pinacidil	5.27 ± 0.20# (9)	6.01 ± 0.01*** (4)	5.49 ± 0.13† (8)	4.39 ± 0.19 (4)
Nitroprusside	6.19 ± 0.12### (4)	8.10 ± 0.12* (4)	5.99 ± 0.27# (4)	7.50 ± 0.12 (4)
Forskolin	6.98 ± 0.32 (4)	7.52 ± 0.06** (4)	6.69 ± 0.10 (4)	6.70 ± 0.15 (4)
Fenoterol	8.59 ± 0.15 (4)		8.38 ± 0.15 (4)	

Values are mean − log IC_{50} ± s.e.mean. Number of preparations from different animals are shown in parentheses. See Results for concentrations of spasmogens used to contract the preparations. No values are given for fenoterol on artery because the maximum relaxation was <50%.

Significantly lower on trachea than on artery #$0.05 > P > 0.01$ ###$P < 0.001$.

† Significantly higher on trachea than artery †$0.05 > P > 0.01$.

* Significantly higher than value against endothelin on artery *$0.05 > P > 0.01$ **$0.01 > P > 0.001$ ***$P < 0.001$.

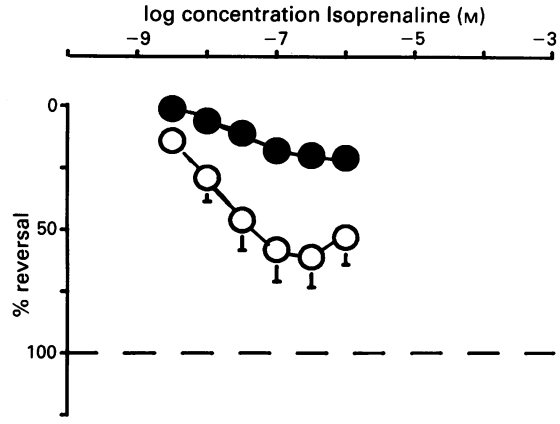


Figure 4 Spasmolytic effect of isoprenaline on rat pulmonary artery. Mean concentration-response (relaxation) curves on preparations contracted with prostaglandin $F_{2\alpha}$ (10 μM, ○) or endothelin (0.01 μM, ●). Relaxation responses are expressed as percentage reversal of the induced contraction. Points are mean values from 4 experiments and vertical bars are s.e.mean, shown only where these are greater than the size of the symbol.

fenoterol, and isoprenaline was clearly more effective against $PGF_{2\alpha}$ than against endothelin (Figure 4).

On pulmonary artery the spasmolytic potencies of the drugs with noradrenaline (0.1 μM, approximately 80% maximum) as the spasmogen were also determined. All the drugs were significantly ($P < 0.05$) more potent against noradrenaline than against endothelin ($-\log IC_{50}$ values against noradrenaline: pinacidil 5.13 ± 0.11 (4); nitroprusside 8.21 ± 0.03 (4); forskolin 7.49 ± 0.05 (4); for values against endothelin see Table 1).

Although a series of experiments was carried out on both tissues with felodipine, data are not included. This is because concentration-response curves to felodipine could not be obtained reliably against either spasmogen on trachea and, on pulmonary artery, felodipine (100 nM) caused only 11–30% reversal of $PGF_{2\alpha}$ and no reversal of endothelin. On those tracheal preparations where felodipine (100 nM) did have some effect, the reversal of either spasmogen was very slow to develop and varied greatly in magnitude from preparation to preparation. Some tracheal preparations contracted in response to 3 μM felodipine, a response not mimicked by the ethanol solvent.

Discussion

The relative potencies (spasmolytic) of a number of different smooth muscle relaxant drugs on two isolated tissues from the lungs, i.e. trachea (guinea-pig) and pulmonary artery (rat), were studied. The preparations were contracted with $PGF_{2\alpha}$ or endothelin. The results suggest that differences might exist between these representative airway and vascular smooth muscle preparations with respect to (a) the mechanisms of action of these two spasmogens and (b) the relative importance of the different mechanisms that initiate relaxation in the smooth muscle cells.

Selectivity between tissues

The observation that the potassium channel opening drug, pinacidil, was apparently airway-vascular selective as a relaxant drug in the lung, i.e. was more potent in reversing contractions to endothelin on trachea than on pulmonary artery (O'Donnell *et al.*, 1990; this study), was interesting since this is a desirable property in a bronchodilator drug. However, if the drug is to be of value as a bronchodilator then ideally it should also show airway-vascular selectivity against other spasmogens as well. Unfortunately, in the present study this was not seen when $PGF_{2\alpha}$ was used to contract the tissues and against this spasmogen pinacidil was vascular-airway selective. This agrees with data obtained on guinea-pig pulmonary artery and trachea by Mellemkjaer *et al.* (1989).

This observation initially supported the view (see the Introduction) that the airway-vascular selectivity of pinacidil might be a direct consequence of the use of endothelin as the spasmogen and thus we expected to see the same selectivity with other relaxant drugs when examined against endothelin. However, the tissue selectivity of smooth muscle relaxant drugs which have mechanisms of action that differ from that of pinacidil, varied between drugs. Fenoterol also showed airway-vascular selectivity but nitroprusside was vascular-airway selective and forskolin showed no selectivity. The selectivity of felodipine could not be determined because its effects could not be quantified on either tissue. Thus, contrary to our original view (see above), it is unlikely that the airway-vascular selectivity of pinacidil can be solely attributed to the use of endothelin as the spasmogen, although this may still be a contributing factor (see below). The tissue selectivity of different relaxant drugs must also take into account the mechanisms whereby the drug produces relaxation in each of the tissues.

Pinacidil acts by opening potassium channels thereby causing hyperpolarization (Southerton *et al.*, 1988). Although several types of potassium channel are present in airway smooth muscle (Kotlikoff, 1989), whether or not these differ from those present in pulmonary artery either in type(s) or in their sensitivity to potassium channel opening drugs has not yet been elucidated. Also, it has been proposed that higher concentrations of pinacidil cause relaxation of smooth muscle by an additional, as yet undefined, mechanism (Hermsmeyer, 1988; Nielsen-Kudsk *et al.*, 1988; Wanstall *et al.*, 1989; O'Donnell *et al.*, 1990) and this may complicate the interpretation of its effects. Thus, it is still not possible to offer an explanation for the airway-vascular selectivity of pinacidil against endothelin.

Fenoterol was also airway-vascular selective. The adenosine 3′:5′-cyclic monophosphate (cyclic AMP) system, which is implicated in the actions of β-adrenoceptor agonists, is operative in both airway and vascular smooth muscle (Gold, 1980; Lincoln & Fisher-Simpson, 1983; Hardman, 1984; Torphy, 1987). From the data in this study there is no reason to believe that this system differs qualitatively or quantitatively between these tissues in that forskolin was equipotent in relaxing trachea and pulmonary artery whether contracted with $PGF_{2\alpha}$ or endothelin. Forskolin increases the intracellular concentration of cyclic AMP via a direct activation of adenylate cyclase (Seamon & Daly, 1983) whereas β-adrenoceptor agonists, such as fenoterol, do so indirectly via activation of β-adrenoceptors (Hardman, 1984). Thus, the airway-vascular selectivity seen for fenoterol in the present study (against $PGF_{2\alpha}$ or endothelin) could represent a greater population of β-adrenoceptors in guinea-pig trachea than in rat pulmonary artery. There is already evidence to suggest that the β-adrenoceptor population of rat pulmonary artery may be small in that complete (100%) relaxation of these preparations by β-adrenoceptor agonists is obtained only if using concentrations of spasmogen which cause low levels of contractions. For example, in a previous study we used a concentration of KCl (15 mM) that gave only about 20% of the maximum contraction (O'Donnell & Wanstall, 1981).

Nitroprusside was vascular-airway selective. This drug relaxes smooth muscle by increasing the intracellular concentration of cyclic GMP via activation of soluble guanylate cyclase (Rapoport & Murad, 1983) and airway and vascular smooth muscle both possess the guanylate cyclase-cyclic GMP system (Fiscus *et al.*, 1984; Lincoln & Johnson, 1984; Murad *et al.*, 1985). However, there may be quantitative differ-

ences in this system between tissues. If it is more important in vascular smooth muscle than in airways then this could account for the observation that pulmonary artery was more responsive than trachea to nitroprusside in the present study. Other agents that act through cyclic GMP, e.g. atriopeptin (Labat *et al.*, 1988) and nitric oxide (Shikano *et al.*, 1987; Buga *et al.*, 1989), have also been reported to be vascular-airway selective.

Selectivity between spasmogens

The present study has provided some indirect evidence that, at least in rat pulmonary artery, there may be some difference(s) in the mechanisms involved in the contractions to $PGF_{2\alpha}$ and endothelin. On pulmonary artery, pinacidil, nitroprusside, and forskolin (fenoterol and felodipine data could not be obtained) were all more potent in reversing contractions to $PGF_{2\alpha}$ than endothelin, i.e. they showed spasmogen selectivity. This selectivity was not seen in matching experiments on guinea-pig trachea. Since this selectivity (a) involves only the pulmonary artery preparations and (b) occurred with a range of relaxant drugs which did not have a common mechanism of action (see above), it is likely that the explanation for it could relate to differences in the contractile mechanisms for $PGF_{2\alpha}$ and endothelin on rat pulmonary artery. It is unlikely to be due to differences in tone in that the selected concentrations of spasmogen gave comparable levels of tone on this tissue.

In the present study relaxant drugs were used to reverse established contractions to spasmogen and thus it is the mechanism(s) involved in tonic (sustained) contractions rather than in phasic (initial) contractions that is relevant. The latter is considered to involve largely the release of intracellular calcium via inositol trisphosphate but the mechanism(s) underlying tonic contractions may be more complex. In vascular smooth muscle, the two major mechanisms thought to be responsible for tonic contractions to spasmogens are (1) the influx of extracellular calcium through either voltage-operated calcium channels or non-specific cation channels, and (2) activation of protein kinase C as a result of an increase in the affinity of this enzyme for calcium. The first of these mechanisms produces an increase in intracellular free calcium concentration whereas the second can operate at resting levels of intracellular calcium (Sybertz *et al.*, 1986; Jiang & Morgan, 1987).

In vascular smooth muscle, the only specific information on the mechanisms of the tonic contractions to either $PGF_{2\alpha}$ or endothelin has been obtained on blood vessels other than rat pulmonary artery. In rat aorta, $PGF_{2\alpha}$ has been reported to act via protein kinase C although it is still not clear whether this results from the production of the protein kinase C activator, diacyl glycerol, as a result of phosphoinositol hydrolysis (Suba & Roth, 1987; Heaslip & Sickels, 1989). Nevertheless. it is reasonable to assume that protein kinase C activation also occurs in rat pulmonary artery. In addition, there is probably also some influx of calcium through voltage-operated channels since, in the present study, responses to $PGF_{2\alpha}$ could be reversed to a small extent by felodipine. Also, calcium influx has been shown to be important for $PGF_{2\alpha}$-induced vasoconstriction in perfused lungs from rats (Santoian *et al.*, 1989). Tonic contractions to endothelin in various blood vessels have also been reported to involve protein kinase C activation, probably via diacyl glycerol production (Griendling *et al.*, 1989; Sugiura *et al.*, 1989; Steffan & Russell, 1990). There is also some evidence for influx of extracellular calcium (Van Renterghem *et al.*, 1988; Steffan & Russell, 1990) but, if this should occur in rat pulmonary artery, it does not seem to be through dihydropyridine-sensitive voltage dependent channels since felodipine could not reverse endothelin contractions in the present study. Thus, both of the mechanisms referred to above could potentially play a role in the tonic contractions of rat pulmonary artery to both $PGF_{2\alpha}$ and endothelin. However, the relative contributions of the two mechanisms may differ for $PGF_{2\alpha}$ and endothelin.

Each of the relaxant drugs used in the present study, whatever the initial mechanism of action, finally prevents, or reverses, an increase in the intracellular free calcium concentration. Thus, they would all be expected to be more effective against contractions involving calcium influx than against those involving an increase in the affinity of protein kinase C for calcium. Support for this concept has recently been obtained in a study on rat aorta (Obianime & Dale, 1989) in which several relaxant drugs, including those used in the present study, were more effective against the α-adrenoceptor agonist methoxamine (which, like other α-adrenoceptor agonists, probably causes both calcium influx and protein kinase C activation) than against the phorbol ester, 4β-phorbol dibutyrate (which acts only by directly activating protein kinase C). It is possible that, in the present study, the relaxant drugs were all more effective against $PGF_{2\alpha}$ than endothelin because of similar spasmogen differences. In rat pulmonary artery, $PGF_{2\alpha}$ (like α-adrenoceptor agonists) acts via calcium influx as well as protein kinase C, whereas the main mechanism responsible for the tonic contractions to endothelin may be the activation of protein kinase C, in which case, like for the phorbol esters, contractions to endothelin could still be maintained in the face of low intracellular free calcium concentrations. This would cause the relaxant drugs to be less effective.

On guinea-pig tracheal smooth muscle, none of the relaxant drugs showed selectivity between spasmogens. Thus, the present data provide no evidence for a difference in the contractile mechanisms for $PGF_{2\alpha}$ and endothelin on this tissue. However, if the contractile mechanism for $PGF_{2\alpha}$ and endothelin differ from one another on pulmonary artery (see above) but not on trachea, then the contractile mechanism for at least one of the spasmogens must, inter alia, also differ between pulmonary artery and trachea. Our data do not indicate which of the two spasmogens might differ and, since we used pulmonary artery preparations from rats and tracheal preparations from guinea-pigs, we cannot entirely rule out the contribution of a species difference to our results. However, in a previous study, pinacidil did not differ in its effects on preparations of trachea from guinea-pigs or rats contracted with endothelin (O'Donnell *et al.*, 1990) and thus a between-species difference for trachea is unlikely. We have not compared rat with guinea-pig pulmonary artery preparations.

In general, the mechanisms responsible for tonic contractions of airway smooth muscle (Rodger, 1988) resemble those cited above for vascular smooth muscle. However, to our knowledge, there are no reports specifically on the mechanisms for $PGF_{2\alpha}$ or endothelin in guinea-pig trachea, or on any airway preparation. Data obtained in the present study for the calcium entry blocking drug felodipine were very variable and difficult to quantify and did not help to clarify the possible role of the influx of extracellular calcium in the tonic responses to these two spasmogens on this tissue. It is clear that more specific information is needed on the mechanisms whereby contractions to $PGF_{2\alpha}$ and endothelin are maintained in guinea-pig trachea before meaningful comparisons between airway and vascular smooth muscle in the lung can be made.

Nevertheless, if our speculation is correct, i.e. that at least one of the spasmogens differs in its mechanism between airway and vascular smooth muscle, and, if differences in spasmogen mechanisms can give rise to selectivity as discussed above for pulmonary artery, then it is possible that other relaxant drugs will be found which, like pinacidil, show airway-vascular selectivity against some of the more recently discovered, and less well studied, spasmogens such as endothelin.

Conclusion

In conclusion, we suggest that our data indicate that the mechanism of the tonic contraction to endothelin on pulmonary artery may be different from that of $PGF_{2\alpha}$. We have

other data that suggest that endothelin may also differ from other spasmogens (O'Donnell & Wanstall, 1990). Irrespective of whether this speculation proves to be correct, the study has confirmed that information on smooth muscle relaxant drugs obtained on airway tissue cannot necessarily be extrapolated to pulmonary vascular tissue, and *vice-versa*, even if using the same spasmogen. Furthermore, data obtained with one spasmogen may not reflect what happens if the spasmogen is changed. The provoking spasmogen(s) is still not known for conditions such as asthma and pulmonary hypertension, conditions for which the tissues used in this study are frequently used in the screening of new relaxant drugs. Thus, it is important to include a full range of potential spasmogens, including recently discovered potential endogenous mediators, when comparing relaxant drugs with different mechanisms if a full profile of activity of the drugs, and hence of their future clinical potential, is to be obtained

This research was supported by the National Health & Medical Research Council of Australia. J.C.W. is an NH&MRC Research Scientist and X.P.Z. an NH&MRC Visiting Overseas Scholar. We thank Agatha Gambino for excellent technical assistance and Dr Ian Ahnfelt-Rønne, Leo Pharmaceuticals, for the gift of pinacidil and AB Hässle for the gift of felodipine.

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(Received June 22, 1990
Revised September 18, 1990
Accepted September 20, 1990)

Br. J. Pharmacol. (1991), **102**, 317–322 © Macmillan Press Ltd, 1991

Pressor effects following microinjection of 5-HT_{1A} receptor agonists into the raphe obscurus of the anaesthetized rat

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1 The effects of electrical stimulation and microinjections (90 nl) of the 5-HT_{1A} receptor agonists, flesinoxan and 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), and glutamate into the raphe obscurus on blood pressure, heart rate and phrenic nerve activity (central inspiratory drive) were investigated in rats anaesthetized with α-chloralose.

2 Electrical stimulation of the raphe obscurus caused a rise in blood pressure which was associated with bradycardia, while glutamate (2.7 nmol) caused only a rise in blood pressure.

3 Flesinoxan (1.3 nmol) and 8-OH-DPAT (0.7 nmol) increased blood pressure by 9 ± 1 and 14 ± 2 mmHg, respectively and did not affect heart rate. For both agonists the effect on blood pressure was shown to be dose-dependent; again no effect on the heart rate was observed over the dose-ranges chosen.

4 Microinjections of the non-selective 5-HT_{1A} receptor antagonists, (±)-pindolol (2.7 nmol) or methiothepin (5.2 nmol), into the raphe obscurus prevented the increase in blood pressure caused by microinjection of flesinoxan. However, (±)-pindolol caused a sustained rise in blood pressure of 15 ± 1 mmHg while methiothepin caused a transient rise in blood pressure. Neither drugs affected heart rate. The ability of methiothepin to attenuate the pressor effect of flesinoxan was found to be partially reversed after 30 min.

5 It is suggested that activation of 5-HT_{1A} receptors within the raphe obscurus can cause sympathoexcitation.

Introduction

The 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor agonists, flesinoxan and 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), have been shown to lower arterial blood pressure in rats (Gradin *et al.*, 1985; Martin & Lis, 1985; Fozard *et al.*, 1987; Dreteler *et al.*, 1990), cats (McCall *et al.*, 1987; Ramage & Fozard, 1987; Ramage *et al.*, 1988; Wouters *et al.*, 1988b) and dogs (Laubie *et al.*, 1989) by a central action. In the rat, brain areas that contain 5-HT_{1A} binding sites are the hypothalamus, midbrain raphe nuclei, the nucleus tractus solitarius and medullary raphe nuclei (Pazos & Palacios, 1985; Vergé *et al.*, 1986; Thor *et al.*, 1990). All these areas are known to be involved in the central regulation of blood pressure (Loewy & Neil, 1981; Spyer, 1981). 5-Hydroxytryptaminergic neurones from the nucleus raphe pallidus (B_1) and raphe obscurus (B_2) project directly to the intermediolateral cell column (Dahlström & Fuxe, 1965; Loewy, 1981; Loewy *et al.*, 1981), the main site of origin of the sympathetic preganglionic neurones, which are the final site at which integration of cardiovascular control can occur within the central nervous system (Coote, 1988).

The present study was carried out to determine if the raphe obscurus is involved in the central hypotensive action of flesinoxan and 8-OH-DPAT by observing the effects on blood pressure and heart rate following microinjection of these 5-HT_{1A} receptor agonists into the raphe obscurus. Furthermore, as phrenic motoneurones in the cat have been shown to be surrounded by 5-HT immunoreactive fibres (Holtman *et al.*, 1984) and chemical and electrical stimulation of the raphe obscurus in the cat causes excitation of phrenic motoneurones i.e. increases central inspiratory drive (Holtman *et al.*, 1986), the effect of microinjection into the raphe obscurus of flesinoxan and 8-OH-DPAT was also examined on phrenic nerve activity in the rat.

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A preliminary account of some of these observations has been given (Dreteler, 1990).

Methods

Male Sprague-Dawley rats (250–375 g; Royal Free Hospital School of Medicine, London) were anaesthetized initially with halothane to allow the implantation of cannulae (polythene) into the right femoral artery and vein. Subsequently, α-chloralose (70 mg kg^{-1}) was given intravenously (i.v.), tracheotomy was performed and the left femoral artery and vein were cannulated so that arterial blood gases could be measured and an i.v. infusion could be given. The animals were then placed in a stereotaxic head frame. The dorsal aspect of the brainstem was exposed by retraction of the skin and muscles at the base of the skull followed by removal of the occipito-alantoid membrane and portions of the occipital bone. The animals were artificially ventilated (frequency 80 cycles min^{-1}, tidal volume 3–4 ml) with room air enriched with oxygen following neuromuscular blockade with decamethonium iodide (1 mg per animal, i.v.). Arterial blood gases and pH were monitored throughout the experiment and maintained between 90–130 mmHg for Po_2, 40–50 mmHg for Pco_2 and 7.3–7.4 for pH. Slow i.v. injections of sodium bicarbonate (1.0 M) or adjustments in respiration were made as necessary to maintain pH and blood gases within this range. The Pco_2 was kept high in order to produce activity in the phrenic nerve. During the experiment an i.v. infusion (6 ml kg^{-1} h^{-1}) of a solution of 50 ml gelofusine plasma substitute, 50 ml distilled water, 0.2 g glucose, 0.84 g sodium bicarbonate and 150 mg decamethonium iodide was given, to counteract the development of non-respiratory acidosis and to maintain blood volume and neuromuscular blockade. Rectal temperature was monitored and maintained between 37 and 38°C by means of a homeothermic blanket system. Blood pressure

was recorded from the right femoral artery by means of a pressure transducer (Gould Statham P23XL) and heart rate was electronically derived from the blood pressure signal. Mean arterial blood pressure was calculated by adding one third of the pulse pressure to the diastolic pressure.

Phrenic nerve activity recordings

In animals in which phrenic nerve activity was recorded the nerve was exposed by deflecting the left scapula forward and dissecting the nerve free from the surrounding connective tissue. The nerve was cut and the central end was placed on bipolar silver hook electrodes for the recording of nerve activity. The number of events above the noise level was counted over a 5 s period using a spike processor (Digitimer D 130).

Microinjections into the raphe obscurus

Microinjections were made into the raphe obscurus with 5 barrel glass electrodes having a tip size of 25–35 μm. One barrel was filled with a 1:1 mixture of Wood's metal and Indium for electrical stimulation. Electrical stimuli were produced by a Digitimer DS2 stimulator (40 Hz, 100 μA, 0.5 ms, 5 s). The other four barrels were filled with the experimental drugs and saline. The pH of the saline solution was always adjusted to the pH of the test solutions. The electrode was positioned 1.0–1.5 mm rostral to the obex along the midline and advanced 1.50–1.75 mm down from the brain surface according to Paxinos & Watson (1982). Once in position, 90 nl volumes of test solutions were applied by means of positive pressure injection. In all animals the site of injection was marked by application of Pontamine sky blue (2% in saline) via one of the barrels of the electrode. At the end of each experiment the animals were perfused with fixative (10% formalin; 1% glutaraldehyde solution) and the brain was removed and stored in the fixative until it was sectioned. Brains were sectioned on a freeze microtome (100 μm). The brain sections were mounted and stained with neutral red for histological verification of the injection site.

Experimental protocols

Electrical and chemical stimulation and microinjection of single doses of flesinoxan or 8-OH-DPAT were performed in separate experiments. In experiments used to construct dose-response curves for the blood pressure effects of microinjection of flesinoxan or 8-OH-DPAT into the raphe obscurus, the time between drug injections was 15–30 min, depending on the time needed for blood pressure to return to its basal value. In the antagonist studies, a submaximal dose of flesinoxan (1.3 nmol) was microinjected initially and once the blood pressure had returned to its basal level either (±)-pindolol (2.7 nmol) or methiothepin (5.2 nmol) was microinjected, followed 5 min later by another microinjection of flesinoxan. Injections of flesinoxan were repeated at 10–15 min intervals. To investigate if the submaximal pressor response was reproducible, control experiments were performed. In these experiments only flesinoxan (1.3 nmol) was microinjected at time intervals as described for the antagonist studies.

Drugs

α-Chloralose (Sigma); L-glutamate, (Sigma); flesinoxan ((+)-(R)-N-[2-[4-(2,3-dihydro-2-hydroxymethyl-1,4-benzodioxin-5-yl)- 1-piperazinyl]ethyl]-4- fluorobenzamide · HCl, Duphar B.V.); 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino) tetralin · HBr, Research Biochemicals Inc.) and methiothepin monomethanesulphonate (Jilek *et al.*, 1980; Pharmaceutical Biochem. Research Inst., Prague, Czechoslovakia) were dissolved in saline. (±)-Pindolol (Sandoz) was dissolved in 0.01 N HCl and saline and buffered to pH 7.4.

Statistical analysis

Data are expressed as mean ± s.e.mean. Paired Student's t test was used to estimate the significance between mean values; $P < 0.05$ was considered statistically significant.

Results

Effect of electrical and glutamate stimulation of the raphe obscurus

The baseline values for blood pressure and heart rate as well as the effects of the different treatments on blood pressure, heart rate and phrenic nerve activity are presented in Table 1.

Electrical stimulation of the raphe obscurus caused an immediate rise in mean arterial blood pressure (Figure 1a). This rise in blood pressure (30 ± 6 mmHg) was accompanied by a decrease in heart rate (37 ± 9 beats per minute (b.p.m.)). In two experiments there was a small increase in phrenic nerve activity (< 15%), but in the other experiments there was no effect. To examine the effects of stimulating only the cell bodies in the raphe obscurus and avoiding stimulation of fibres of passage, the excitatory amino acid L-glutamate was microinjected into this nucleus. Injection of L-glutamate (2.7 nmol) into the raphe obscurus (Figure 1b) caused an increase in blood pressure of 22 ± 6 mmHg, had hardly any effect on heart rate and caused an increase in phrenic nerve activity (53 ± 10%). The increase in phrenic nerve activity consisted of an increase in the number of bursts (Figure 1b) and not of an increase in the number of events in each burst. The glutamate effects on blood pressure lasted 1–2 min. Electrical stimulation or chemical stimulation with glutamate outside the raphe obscurus (Figure 1a and b) had no, or slight depressor effects. Injection of 90 nl of saline into the raphe obscurus never affected any of the variables measured.

Effects of microinjection of flesinoxan or 8-OH-DPAT into the raphe obscurus

The baseline values for blood pressure and heart rate and the effects of microinjection of flesinoxan and 8-OH-DPAT into

Table 1 Effects of electrical stimulation (ES) and microinjection of glutamate, flesinoxan, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) or saline into the raphe obscurus of the anaesthetized rat on mean arterial blood pressure (MAP), heart rate (HR) and phrenic nerve activity (PNA)

Treatment	*Dose* (nmol)	n	*Baseline MAP* (mmHg)	*MAP* (mmHg)	*Baseline HR* (b.p.m.)	*HR* b.p.m.	% PNA
ES		7	129 ± 5	30 ± 6*	404 ± 14	−37 ± 9*	NE
Glutamate	2.7	7	98 ± 6	22 ± 6*	410 ± 14	NE	53 ± 10*
Flesinoxan	1.3	9	101 ± 6	9 ± 1*	400 ± 14	NE	NE
8-OH-DPAT	0.7	7	98 ± 7	14 ± 2*	407 ± 19	NE	NE
Saline		6	104 ± 4	NE	407 ± 18	NE	NE

Data are expressed as mean ± s.e.mean. * $P < 0.05$ versus baseline values. NE: no effect.

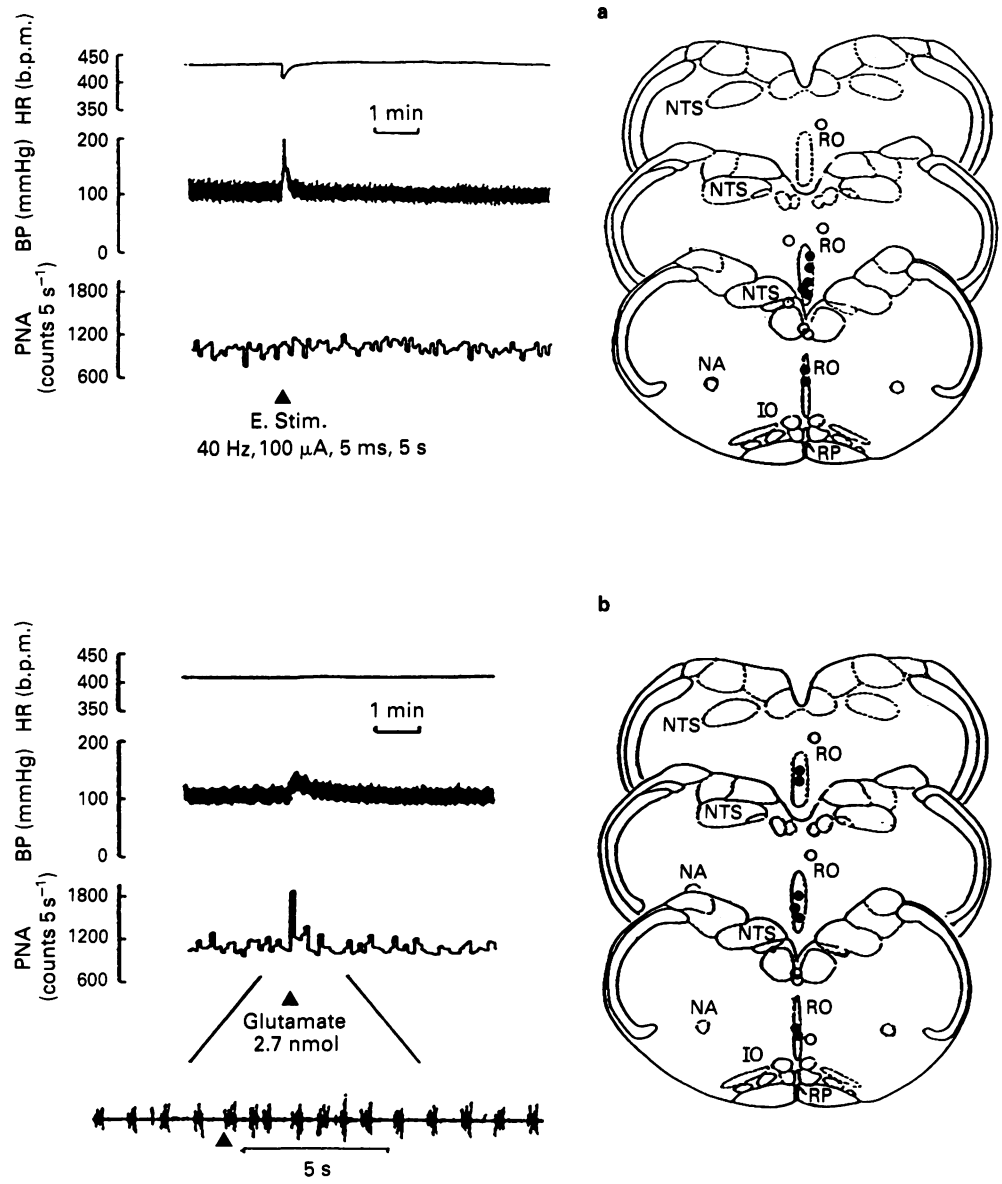


Figure 1 Left: Effect of electrical stimulation (a) and chemical stimulation with glutamate (b) of the raphe obscurus on heart rate (HR), blood pressure (BP) and phrenic nerve activity (PNA). Right: Injection sites in rat brainstem at the level 1.0–2.0 mm rostral to the obex. RO, nucleus raphe obscurus; NA, nucleus ambiguus; NTS, nucleus tractus solitarius; RP, nucleus raphe pallidus; IO, inferior olive; (●) injection sites where positive effects were found; (○) injection sites where no or different effects were found.

the raphe obscurus on blood pressure, heart rate and phrenic nerve activity are shown in Table 1.

Microinjection of flesinoxan (1.3 nmol; Figure 2a) or 8-OH-DPAT (0.7 nmol; Figure 2b) into the raphe obscurus caused an instantaneous significant increase in blood pressure of 9 ± 1 mmHg and 14 ± 2 mmHg, respectively which lasted between 5 and 10 min. However, the rise in blood pressure caused by these drugs was not associated with any changes in heart rate or phrenic nerve activity. Microinjection of either flesinoxan or 8-OH-DPAT outside the raphe obscurus nucleus (Figure 2a and b) had no effects on blood pressure, heart rate or phrenic nerve activity.

In separate experiments dose-response curves were constructed (Figure 3) for the effects of flesinoxan and 8-OH-DPAT on blood pressure. Successive microinjections of increasing doses of flesinoxan (0.22–2.6 nmol) caused dose-dependent increases in blood pressure (baseline value 93 ± 4 mmHg) which at the highest dose used reached 27 ± 8 mmHg. Administration of 8-OH-DPAT (0.09–1.4 nmol) into the raphe obscurus also produced a dose-dependent rise in blood pressure (baseline value 91 ± 8 mmHg) reaching a maximum of 21 ± 6 mmHg at the highest dose given. Again, even at the highest doses administered, both flesinoxan and 8-OH-DPAT failed to affect heart rate (baseline values 437 ± 12 and 425 ± 19 b.p.m., respectively) or phrenic nerve activity.

Effect of pretreatment with (±)-pindolol or methiothepin on repeated microinjections of submaximal pressor doses of flesinoxan into the raphe obscurus

In control experiments the pressor responses caused by microinjection of successive doses of 1.3 nmol flesinoxan, were reproducible. Microinjection of (±)-pindolol (2.7 nmol; $n = 4$) caused a maintained rise in blood pressure of 15 ± 1 mmHg with no change in heart rate (baseline values 101 ± 3 mmHg and 394 ± 14 b.p.m., respectively). In these experiments the pressor response to flesinoxan was reduced from 10 ± 2 mmHg before to 2 ± 1 mmHg 5 min after pretreatment with (±)-pindolol. Microinjection of methiothepin (5.2 nmol; $n = 5$) caused an increase in blood pressure of 7 ± 2 mmHg (baseline value 104 ± 4 mmHg) with no change in heart rate (baseline value 439 ± 4 b.p.m.). Blood pressure returned to

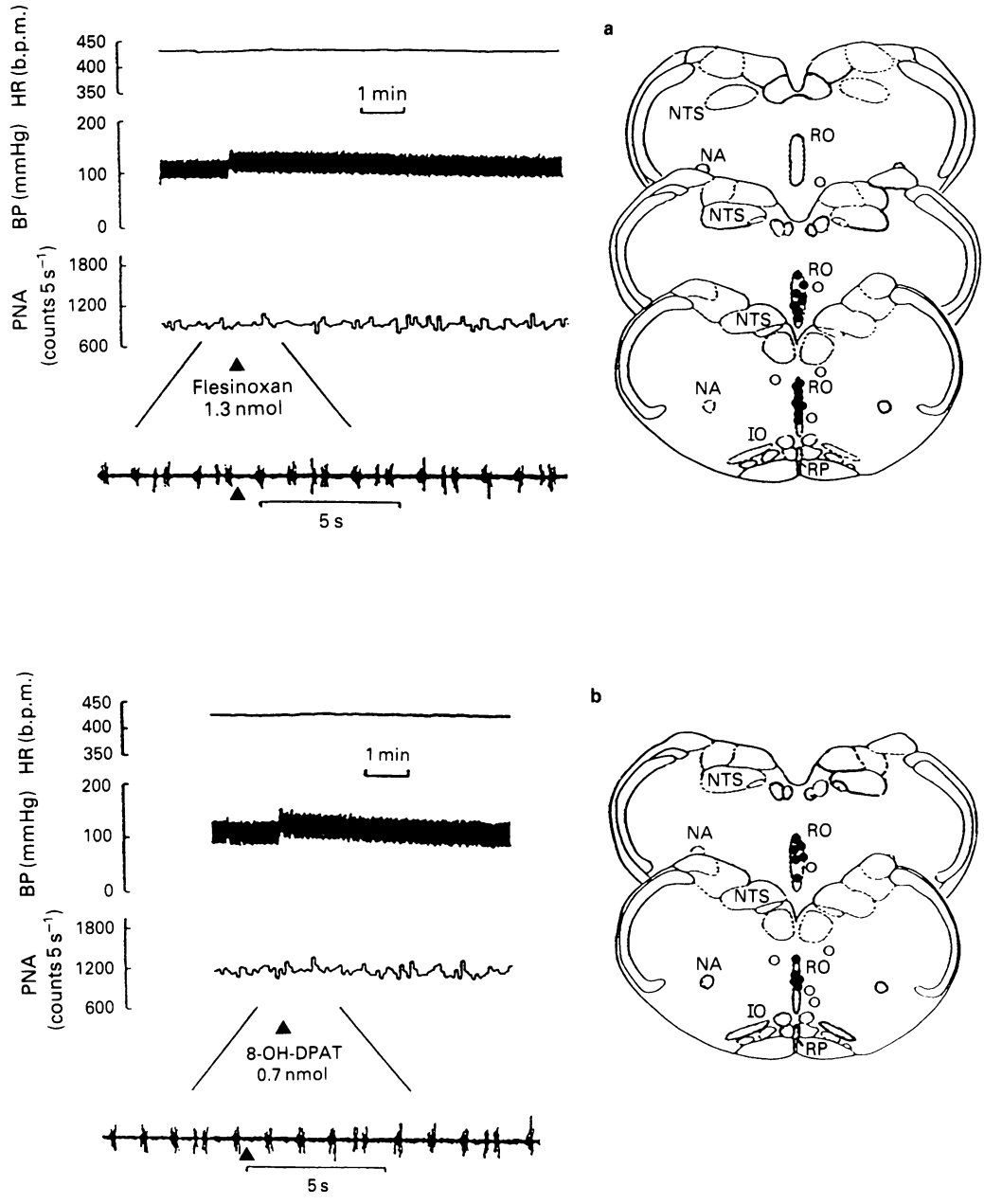


Figure 2 Left: effect of microinjection of flesinoxan (a) and 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (b) into the raphe obscurus on heart rate, blood pressure and phrenic nerve activity. Right: injection sites in rat brainstem at the level 1.0–2.0 mm rostral to the obex. RO, nucleus raphe obscurus; NA, nucleus ambiguus; NTS, nucleus tractus solitarius; RP, nucleus raphe pallidus; IO, inferior olive; (●) injection sites where positive effects were found; (○) injection sites where no effects were found.

near the baseline value (108 ± 9 mmHg) between 3–4 min after the injection of methiothepin. The increase in blood pressure produced by flesinoxan (13 ± 2 mmHg) was abolished 5 min after the microinjection of methiothepin. However, in 3 experiments 30 min after the microinjection of methiothepin the pressor response to flesinoxan had returned to 6 ± 1 mmHg.

Discussion

In the present study both electrical stimulation and chemical stimulation with glutamate and the selective 5-HT_{1A} receptor agonists, flesinoxan (Wouters *et al.*, 1988a,b) and 8-OH-DPAT (Middlemiss & Fozard, 1983), consistently caused a pressor response when stimuli were applied within, but not outside, the brain region known as the raphe obscurus. In the case of electrical stimulation the pressor effect was accompanied by a decrease in heart rate whereas the pressor effect caused by chemical stimulation failed to cause any change in heart rate. As stimulation with glutamate does not produce any changes in heart rate, the bradycardia caused by electrical stimulation may be due to stimulation of axons of passage. However, the rise in blood pressure caused by electrical stimulation of the raphe obscurus may have initiated a baroreceptor-mediated reflex bradycardia while the pressor response after chemical stimulation with glutamate and also the 5-HT_{1A} receptor agonists may have caused a change in baroreceptor reflex preventing the expected bradycardia. The reason for the failure to observe reflex bradycardia to the pressor effect of chemical stimulation with glutamate or the 5-HT_{1A} receptor agonists of the raphe obscurus remains to be determined.

The effects of electrical stimulation on phrenic nerve activity varied from a small increase to no change in activity, whereas stimulation with glutamate caused an increase in phrenic nerve activity. However, microinjections of flesinoxan or 8-OH-DPAT had no effect on phrenic nerve activity. This difference between glutamate and flesinoxan and 8-OH-DPAT presumably reflects activation of different receptors. Flesinox-

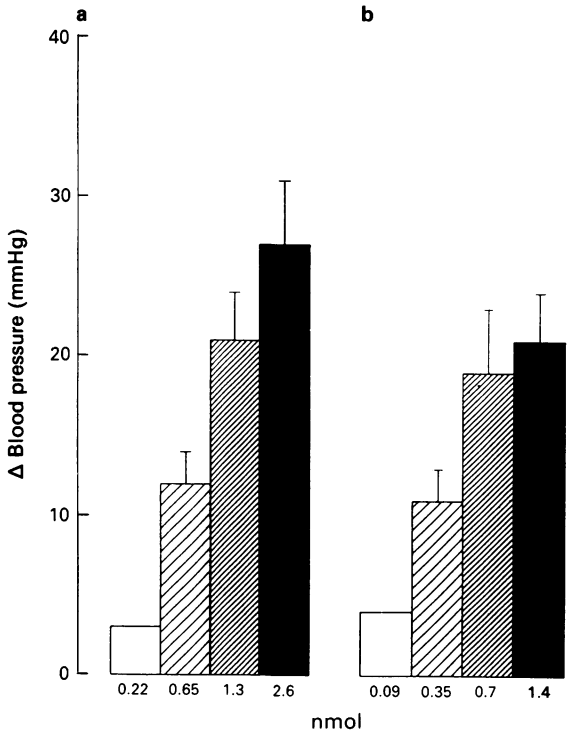


Figure 3 Effect of microinjection of consecutive doses of (a) flesinoxan (0.22, 0.65, 1.3 and 2.6 nmol) or (b) 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 0.09, 0.35, 0.7 and 1.4 nmol) into the raphe obscurus of the anaesthetized rat on mean arterial blood pressure. Data are expressed as mean (columns) with s.e.mean shown by vertical bars ($n = 5$).

an and 8-OH-DPAT, although structurally unrelated, are selective agonists with a comparable high affinity for the 5-HT$_{1A}$ receptor (Schoeffter & Hoyer, 1988; Hoyer, 1988; van Wijngaarden *et al.*, 1990). Both drugs proved to be equipotent in eliciting a dose-dependent pressor response after microinjection into the raphe obscurus supporting the view that the 5-HT$_{1A}$ receptor might mediate these blood pressure effects. Indeed, the non-selective 5-HT$_1$ receptor antagonist, methiothepin (Schoeffter & Hoyer, 1988; Hoyer, 1988), which inhibits the cardiovascular effects of 8-OH-DPAT and flesinoxan in the anaesthetized rat (Fozard *et al.*, 1987; Dreteler *et al.*, 1990), appeared to block reversibly the action of flesinoxan microinjected into the raphe obscurus. Moreover, (±)-pindolol, which also displays an antagonist action at 5-HT$_{1A}$ receptors (Schoeffter & Hoyer, 1988) and is known to block the cardiovascular effects of flesinoxan in the cat (Wouters *et al.*, 1988b) and in the rat (although in this species some involvement of functional antagonism could not be excluded; Dreteler *et al.*, 1990), also attenuated the pressor effect of flesinoxan in the present study. Again, functional antagonism could not be excluded as (±)-pindolol caused a sustained rise in blood pressure similar to that observed for flesinoxan and 8-OH-DPAT. This increase in blood pressure observed with (±)-pindolol may reflect an agonist action at 5-HT$_{1A}$ receptors (Hjorth & Carlsson, 1986). Methiothepin also caused a transient rise in pressure. However, an explanation for this action of methiothepin remains to be determined. Taken together, these observations suggest that the pressor effect elicited by flesinoxan and 8-OH-DPAT is mediated via 5-HT$_{1A}$ receptor activation in the raphe obscurus. 5-HT$_{1A}$ receptors do not seem to be involved in the increase in phrenic nerve activity caused by glutamate activation of the raphe obscurus. An increase in phrenic nerve activity has also been observed for injection of glutamate into the raphe obscurus in the cat (Holtman *et al.*, 1986) but the present results differ in that glutamate caused an increase in the number of bursts indicating an increase in frequency of respiration and not, as seen in the cat, an increase in the number of events in each burst indicating a greater depth of inspiration.

In the rat, stimulation of the midline region of the rostral medulla with glutamate has been reported to cause a small decrease in blood pressure (Minson *et al.*, 1986). In the present study, stimulation of neurones in the posterior part of the raphe obscurus by glutamate elicited a pressor response. This disparity in effects observed in the two studies may be due to the stimulation of different anatomical areas within the raphe obscurus. In this respect, Yusoff & Coote (1988) have shown that electrical stimulation of different areas of the raphe obscurus in the rat, though increasing skeletal muscle and skin sympathetic nerve activity, either increased or decreased renal sympathetic nerve activity. In the rat, microinjections of 5-HT$_{1A}$ receptor agonists into other raphe nuclei e.g. raphe magnus and pallidus (Valenta & Singer, 1990) or the dorsal raphe nucleus (Connor & Higgins, 1990), in doses equivalent to the doses used in the present study, caused a decrease in blood pressure and heart rate, while microinjections into the median raphe had no effect (Connor & Higgins, 1990). The falls in blood pressure and heart rate observed after injection of the 5-HT$_{1A}$ receptor agonists into the dorsal raphe were blocked by N-methylatropine, suggesting that these cardiovascular effects were vagally mediated (Connor & Higgins, 1990). These results, together with the results from the present study, suggest that the various raphe nuclei have complex effects on autonomic outflow. In this respect, electrical and chemical stimulation of the raphe obscurus in the rat has been reported to enhance gastric motility (McCann *et al.*, 1989).

It is now well established that 5-HT$_{1A}$ receptor agonists lower blood pressure via a reduction in sympathetic output (Ramage & Fozard, 1987; Ramage *et al.*, 1988; Saxena & Villalon, 1990). Activation of central 5-HT$_{1A}$ receptors causes inhibition (Colino & Halliwell, 1987; Sprouse & Aghajanian, 1987) of neuronal activity, which in the dorsal raphe is thought to be mediated via a somatodendritic autoreceptor (Vergé *et al.*, 1985; Sprouse & Aghajanian, 1987). In contrast, the present results indicate that in the raphe obscurus, activation of 5-HT$_{1A}$ receptors, as well as neuronal excitation by glutamate or electrical stimulation, elicits sympathoexcitation. However, it is not known whether the 5-HT$_{1A}$ receptors in the raphe obscurus are located postsynaptically causing direct excitation of the neurones or presynaptically causing inhibition of an inhibitory input to these cells.

In conclusion, microinjection of the 5-HT$_{1A}$ receptor agonists flesinoxan and 8-OH-DPAT into the raphe obscurus of the anaesthetized rat dose-dependently increases blood pressure without any change in heart rate. Since these drugs lower blood pressure following systemic administration, the consequences of the activation of raphe obscurus activation are apparently overruled by the sympathoinhibition resulting from the activation of the other sites, such as the dorsal raphe nucleus and raphe magnus and pallidus. However, the pathways that underlie the blood pressure effects caused by microinjection of the 5-HT$_{1A}$ agonists into the raphe obscurus and the way in which this nucleus might be involved in central cardiovascular regulation need further investigation.

We wish to thank Mr R.G. Bogle and Dr S.L. Shepheard for their help and advice and Mr S. Wilkinson for technical assistance. These experiments were carried out while G.H.D. was on sabbatical leave from Duphar B.V.

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(Received July 24, 1990
Revised October 5, 1990
Accepted October 11, 1990)

Br. J. Pharmacol. (1991), **102**, 323–330 © Macmillan Press Ltd, 1991

Role of 5-HT_1-like receptors in the reduction of porcine cranial arteriovenous anastomotic shunting by sumatriptan

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1 The new tryptamine derivative sumatriptan (GR43175) is effective in the treatment of migraine. Since several antimigraine agents reduce cranial arteriovenous anastomotic blood flow in the anaesthetized pig, we have investigated the carotid haemodynamic effects of sumatriptan.

2 Sumatriptan (10, 30, 100 and 300 $\mu g\,kg^{-1}$, i.v.) reduced total common carotid blood flow, exclusively by affecting its arteriovenous anastomotic fraction; the capillary fraction even increased with the highest doses.

3 These reductions in the carotid arteriovenous anastomotic ('shunt') blood flow were mediated by a 5-HT_1-like receptor, as methiothepin, but not ketanserin, antagonized the responses to sumatriptan.

4 Sumatriptan increased the difference in oxygen saturation between arterial and jugular venous blood, which is likely to be a consequence of the reduction of the carotid shunt blood flow.

5 The selective reduction in arteriovenous anastomotic blood flow produced by sumatriptan may reflect its antimigraine action, thought to involve vasoconstriction of those cranial vessels, be they 'shunt' vessels or not, which are distended and inflamed during a migraine attack.

Introduction

Sumatriptan, a new tryptamine derivative, has a high affinity for 5-HT_{1D} (K_i = 17 nM) and 5-HT_{1B} (K_i = 27 nM) binding sites, an appreciable affinity for 5-HT_{1A} (K_i = 100 nM) binding sites and minimal affinity for other 5-hydroxytryptamine (5-HT), adrenaline, dopamine, muscarine or benzodiazepine binding sites (Peroutka & McCarthy, 1989). Furthermore, sumatriptan seems to discern between two subtypes of 5-HT_{1D} binding sites (Sumner & Humphrey, 1989). Functionally, sumatriptan causes contraction of different isolated blood vessels (Humphrey *et al.*, 1989a; 1990; Saxena & Ferrari, 1989), like the dog saphenous vein (Humphrey *et al.*, 1988) and middle cerebral artery (Humphrey *et al.*, 1989a) and the dog, monkey and human basilar artery (Connor *et al.*, 1989; Parsons *et al.*, 1989), by acting on a 5-HT_1-like receptor. In the anaesthetized dog and cat, sumatriptan increases the carotid arterial resistance (Feniuk *et al.*, 1989; Perren *et al.*, 1989; MacLennan & Martin, 1990). In the dog this increase is mediated by a 5-HT_1-like receptor (Feniuk *et al.*, 1989). Regional blood flow measurements with radioactive microspheres in the cat have shown that the increase in the carotid resistance is confined to its arteriovenous anastomotic fraction (Perren *et al.*, 1989; MacLennan & Martin, 1990); the receptor mechanism of this effect is not known.

Sumatriptan is effective in the treatment of the acute migraine attack and can abolish both the headache and the associated symptoms of migraine (Doenicke *et al.*, 1988; Perrin *et al.*, 1989). The selective reduction of cranial arteriovenous anastomotic blood flow in several anaesthetized species, amongst these the domestic pig, is a property shared with many antimigraine agents, including ergotamine (Johnston & Saxena, 1978; Spierings & Saxena, 1980a; Saxena *et al.*, 1983; Bom *et al.*, 1989a), dihydroergotamine (Spierings & Saxena, 1980a), methysergide (Saxena & Verdouw, 1984) and isometheptene (Spierings & Saxena, 1980b). In addition, 5-HT (Saxena & Verdouw, 1982; Saxena *et al.*, 1986) and other drugs with affinity for 5-HT receptors – 5-carboxamidotryptamine (Saxena & Verdouw, 1985), BEA 1654 (Verdouw *et al.*, 1985), 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, Bom *et al.*, 1989b), RU 24969 (Bom *et al.*, 1989c) and indorenate (Villalón *et al.*, 1990) – also constrict carotid arteriovenous anastomoses by acting on 5-HT_1-like receptors.

Since sumatriptan possesses both antimigraine properties and 5-HT_1-like agonist activity, we set out to study its effect on porcine carotid blood flow and its distribution, with special emphasis on the arteriovenous anastomotic fraction, with the radioactive microsphere method (Saxena *et al.*, 1980). By using the 5-HT receptor antagonists methiothepin (5-HT_1 and 5-HT_2) and ketanserin (5-HT_2), we also tried to establish whether indeed sumatriptan acted via a 5-HT_1-like receptor. A part of this study has been presented to the British Pharmacological Society (Den Boer *et al.*, 1990).

Methods

General

After an overnight fast 33 domestic pigs (Yorkshire x Landrace; 16–22 kg) were anaesthetized with 120 mg azaperone i.m. and 150 mg metomidate i.v. (both from Janssen Pharmaceutica, Beerse, Belgium), intubated and connected to a respirator (Bear 2E, BeMeds AG, Baar, Switzerland) for intermittent positive pressure ventilation with a mixture of room air and oxygen. Respiratory rate, tidal volume and oxygen supply were adjusted to keep arterial blood gas values within physiological limits (pH: 7.35–7.48; P_{CO_2}: 35–48 mmHg; P_{O_2}: 100–120 mmHg). Anaesthesia was maintained with a continuous i.v. infusion of pentobarbitone sodium (Sanofi, Paris, France) at 20 $mg\,kg^{-1}\,h^{-1}$ for the first hour and thereafter at 12 $mg\,kg^{-1}\,h^{-1}$.

Catheters were placed in the inferior vena cava via a femoral vein for the administration of drugs and in the aortic arch via a femoral artery, connected to a Statham pressure transducer (P23 Dc, Hato Rey, Puerto Rico) for the measurement of arterial blood pressure and the withdrawal of arterial blood for determining blood gases (ABL-2, Radiometer, Copenhagen, Denmark). Mean arterial blood pressure (MAP) was calculated from the systolic (SAP) and diastolic (DAP) arterial pressures: MAP = (SAP + 2 × DAP)/3. The common carotid arteries were dissected free and the cervical vagosympathetic trunks were cut. Blood flow was measured in one of

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the common carotid arteries with a flow probe (internal diameter: 2.5 or 3 mm) connected to a sine-wave electromagnetic flow meter (Transflow 600-system, Skalar, Delft, The Netherlands). Heart rate was measured with a tachograph triggered from the blood pressure or the flow signal, depending on their shape. A 0.5 mm (external diameter) needle, connected to a polyethylene tubing was inserted into the common carotid artery against the direction of the blood flow for the administration of radioactive microspheres. At the same side the jugular vein was cannulated in order to obtain venous blood samples for determining blood gases.

During the experiment body temperature was kept at about 37°C and the animal was continuously infused with 100 ml h^{-1} saline to compensate for fluid losses.

Distribution of common carotid blood flow

The distribution of common carotid blood flow was determined with 15 ± 1 (s.d.) μm diameter microspheres 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 about 200,000 microspheres, labelled with one of the isotopes, was mixed and injected into the carotid artery against the direction of the blood flow to ensure uniform mixing. At the end of the experiment the animals were killed and the heart, kidneys, lungs and the different cranial tissues were dissected out, weighed and put in vials. The radioactivity in these vials was counted for 5–10 min in a gamma-scintillation counter (Packard, Minaxi Autogamma 5000) using suitable windows for discriminating the different isotopes.

The ratio between the radioactivity in a particular tissue and the total radioactivity was calculated with a set of specially developed computer programmes (Saxena *et al.*, 1980). By multiplying this ratio with the total carotid blood flow value at the time of the injection, blood flow to the tissues (nutrient blood flow) was determined. No radioactivity could be detected in the heart or the kidneys, so all microspheres reaching the venous side by arteriovenous anastomoses were trapped in the lungs. Therefore, the amount of radioactivity in the lungs was used as an index for the arteriovenous anastomotic part of the common carotid blood flow (see Johnston & Saxena, 1978; Saxena & Verdouw, 1982). The respective conductances were determined by dividing blood flow by mean arterial blood pressure.

Experimental protocol

After a stabilization period of 1 h the animals were divided into five groups. The first group ($n = 6$) received 10 ml boluses of saline at the times the other animals received the respective doses of sumatriptan; in this group the stability of the preparation was evaluated. The four other groups received sumatriptan in cumulative doses of 10, 30, 100 and 300 μg kg^{-1}, administered every 15 min, after pretreatment with either saline ($n = 8$), ketanserin (0.5 mg kg^{-1}, $n = 6$), methiothepin (1 mg kg^{-1}, $n = 7$) or methiothepin (3 mg kg^{-1}, $n = 6$). All pretreatments were given i.v. over 30 min. After the pretreatment and after each dose of sumatriptan, measurements of heart rate, mean blood pressure, carotid blood flow and its distribution and arterial jugular venous blood gases were made.

Data presentation and statistical analysis

All data have been expressed as means ± s.e.mean. The significance of the differences between the variables within one group was evaluated with 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). Between groups the respective changes at the same dose of sumatriptan were evaluated with Student's t test. Statistical significance was accepted at $P < 0.05$ (two-tailed).

Drugs

Apart from the anaesthetics the drugs used in this study were: sumatriptan (GR43175; Glaxo Research Laboratories, Ware), ketanserin tartrate (gift: Dr J.M. Van Nueten, Janssen Pharmaceutica, Beerse, Belgium), methiothepin maleate (gift: Hoffman La Roche B.V., Mijdrecht, The Netherlands) and heparin sodium (Thromboliquine, Organon Teknika B.V., Boxtel, The Netherlands) to prevent clotting of the catheters. Sumatriptan was dissolved in physiological saline. Ketanserin tartrate and methiothepin maleate were dissolved in 5 ml propylene glycol 20% in distilled water and subsequently diluted with 45 ml physiological saline. All doses refer to the respective salts.

Results

Stability of the preparation during saline treatment

The effects of four consecutive bolus injections of saline after saline pretreatment are presented in Table 1. No significant changes occurred during the experiment in total common carotid blood flow and its distribution in arteriovenous anastomotic and nutrient blood flow. Heart rate also remained constant, but there was a small decrease in mean arterial blood pressure (−5 ± 2%).

Changes in the systemic haemodynamics by sumatriptan

The effects of i.v. infusions of sumatriptan on heart rate and blood pressure after pretreatment with either physiological saline, ketanserin (0.5 mg kg^{-1}) or methiothepin (1 or 3 mg kg^{-1}) are summarized in Table 2. None of the pretreatments resulted in a significant difference in mean arterial blood pressure, compared to the saline pretreated group, though after ketanserin blood pressure was slightly decreased. In the group pretreated with the higher dose of methiothepin, the initial heart rate was significantly lower than in the saline pretreated group.

Sumatriptan induced a small dose-dependent decrease in heart rate, which was not present after ketanserin. Also after methiothepin 3 mg kg^{-1} the bradycardia after the highest dose of sumatriptan was blunted, although it must be noted that the baseline (pre-sumatriptan) heart rate in this group was lower.

There was a small (in most cases statistically not significant) decrease in mean blood pressure during the experiments in all groups except in the ketanserin pretreated group, in which the baseline blood pressure was already lower.

Table 1 Stability of the preparation during the experimental period where 5 i.v. bolus injections of physiological saline were given ($n = 6$)

	Time of injection after the start of the experiment (min)				
	0	15	30	45	60
HR	87 ± 6	86 ± 6	86 ± 6	86 ± 6	84 ± 6
MAP	78 ± 7	77 ± 8	77 ± 8	76 ± 8	75 ± 8*
Car F	198 ± 18	190 ± 19	194 ± 17	187 ± 14	186 ± 13
AVA F	151 ± 22	147 ± 22	153 ± 23	148 ± 20	140 ± 17
Nut F	46 ± 7	43 ± 7	40 ± 7	38 ± 7*	46 ± 7

HR, heart rate (beats min^{-1}); MAP, mean arterial pressure (mmHg); Car, total carotid; AVA, arteriovenous anastomotic; Nut, nutrient; F, flow (ml min^{-1}). All values have been presented as means ± s.e.mean. * $P < 0.05$ vs. baseline.

Table 2 Effects of intravenous bolus injections of sumatriptan (10, 30, 100 and 300 $\mu g\,kg^{-1}$) on systemic haemodynamic variables after pretreatment with saline, ketanserin (0.5 $mg\,kg^{-1}$), methiothepin (1 $mg\,kg^{-1}$) or methiothepin (3 $mg\,kg^{-1}$)

		Sumatriptan ($\mu g\,kg^{-1}$)			
Pretreatment	*Baseline*	10	30	100	300
Heart rate (beats min^{-1})					
Saline	93 ± 2	90 ± 2*	88 ± 2*	85 ± 3*	82 ± 3*
Ketanserin	88 ± 7	86 ± 6	83 ± 5	82 ± 4	84 ± 5
Methiothepin 1	91 ± 4	90 ± 5	87 ± 5	82 ± 4*	78 ± 4*
Methiothepin 3	84 ± 2	83 ± 2	82 ± 2*	80 ± 3*	78 ± 3†*
Mean arterial blood pressure (mmHg)					
Saline	85 ± 4	86 ± 4	85 ± 4	83 ± 6	76 ± 8
Ketanserin	75 ± 7	75 ± 6	76 ± 5	74 ± 4	74 ± 3
Methiothepin 1	89 ± 7	86 ± 5	82 ± 5	82 ± 6	83 ± 6
Methiothepin 3	89 ± 5	88 ± 7	86 ± 6	85 ± 6	83 ± 6*

All values have been presented as means ± s.e.mean.
* $P < 0.05$ vs. baseline.
† $P < 0.05$ vs. the corresponding dose in saline pretreated animals.

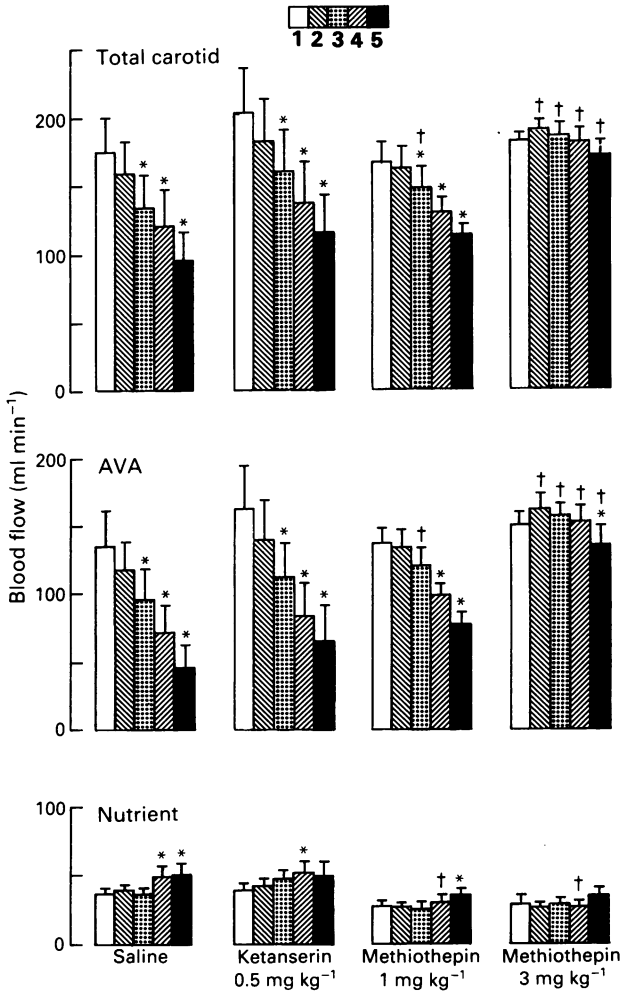


Figure 1 Effect of sumatriptan i.v. on the total carotid blood flow and its arteriovenous anastomotic (AVA) and nutrient (tissue) fractions (ml min^{-1}) in pigs, pretreated i.v. with saline ($n = 8$), ketanserin 0.5 $mg\,kg^{-1}$ ($n = 6$), methiothepin 1 $mg\,kg^{-1}$ ($n = 7$) or methiothepin 3 $mg\,kg^{-1}$ ($n = 6$). (1) Before sumatriptan (after pretreatment); (2) sumatriptan 10 $\mu g\,kg^{-1}$; (3) sumatriptan 30 $\mu g\,kg^{-1}$; (4) sumatriptan 100 $\mu g\,kg^{-1}$ and (5) sumatriptan 300 $\mu g\,kg^{-1}$. All values have been presented as means with s.e.mean shown by vertical bars. * $P < 0.05$ vs baseline; † $P < 0.05$ vs the corresponding dose of sumatriptan in the saline pretreated animals.

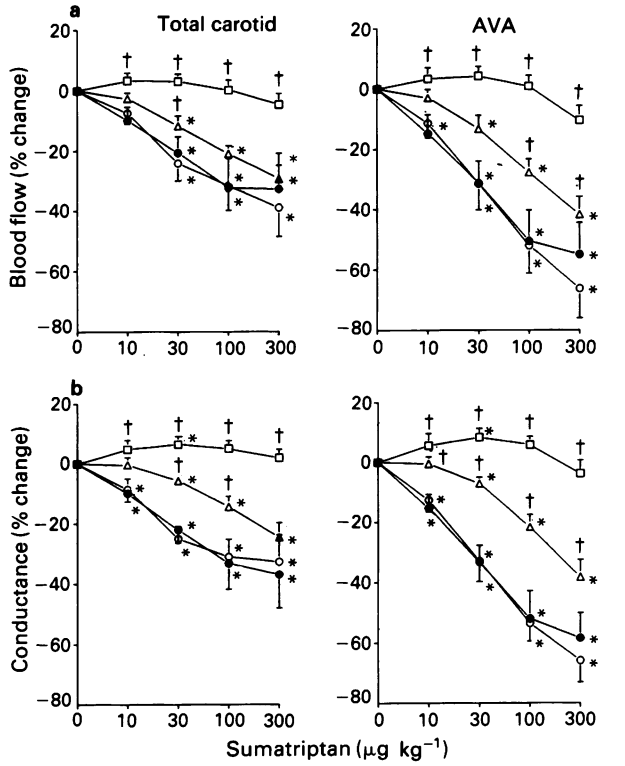


Figure 2 Effect (% change from baseline) of sumatriptan i.v. on the total carotid and arteriovenous anastomotic (AVA) blood flow (a) and conductance (b) in pigs, pretreated i.v. with either saline (○, $n = 8$), ketanserin 0.5 $mg\,kg^{-1}$ (●, $n = 6$), methiothepin 1 $mg\,kg^{-1}$ (△, $n = 7$) or methiothepin 3 $mg\,kg^{-}$ (□, $n = 6$). All values have been presented as means with s.e.mean shown by vertical bars. * $P < 0.05$ vs baseline. † $P < 0.05$ vs the corresponding dose of sumatriptan in the saline pretreated animals.

Changes in the carotid haemodynamics

The effects of i.v. injections of sumatriptan on the total carotid blood flow and its distribution into arteriovenous anastomotic (non-nutrient) and arteriolar (nutrient) blood flow are shown in Figure 1 (absolute values) and Figure 2a (% changes from baseline). In all groups about 80% of the carotid blood flow was initially diverted through arteriovenous anastomoses. In the saline-pretreated animals sumatriptan caused a dose-dependent decrease in arteriovenous anastomotic blood flow. At the highest dose of sumatriptan (300 $\mu g\,kg^{-1}$ or 440 $\mu g\,kg^{-1}$ cumulative dose) arteriovenous anastomotic blood flow had decreased by 66 ± 10%. Total carotid blood flow had decreased by only 39 ± 9%, because nutrient (tissue) blood flow had increased by 36 ± 18%. In the animals pretreated with ketanserin (0.5 $mg\,kg^{-1}$) none of these responses was modified, either on the arteriovenous anastomoses or on the arteriolar fraction. However, pretreatment with methiothepin at 1 $mg\,kg^{-1}$ attenuated and at 3 $mg\,kg^{-1}$ almost completely blocked the effects of sumatriptan on the arteriovenous anastomoses. Methiothepin also attenuated the effect of sumatriptan on nutrient blood flow, especially after 100 $\mu g\,kg^{-1}$ of sumatriptan.

Since arterial blood pressure was not much changed during the experiments, the pattern of changes in the vascular conductances by sumatriptan was similar to that in the blood flows. As depicted in Figure 2b, ketanserin did not modify, but methiothepin antagonized the sumatriptan-induced increases in both total carotid and arteriovenous anastomotic conductance.

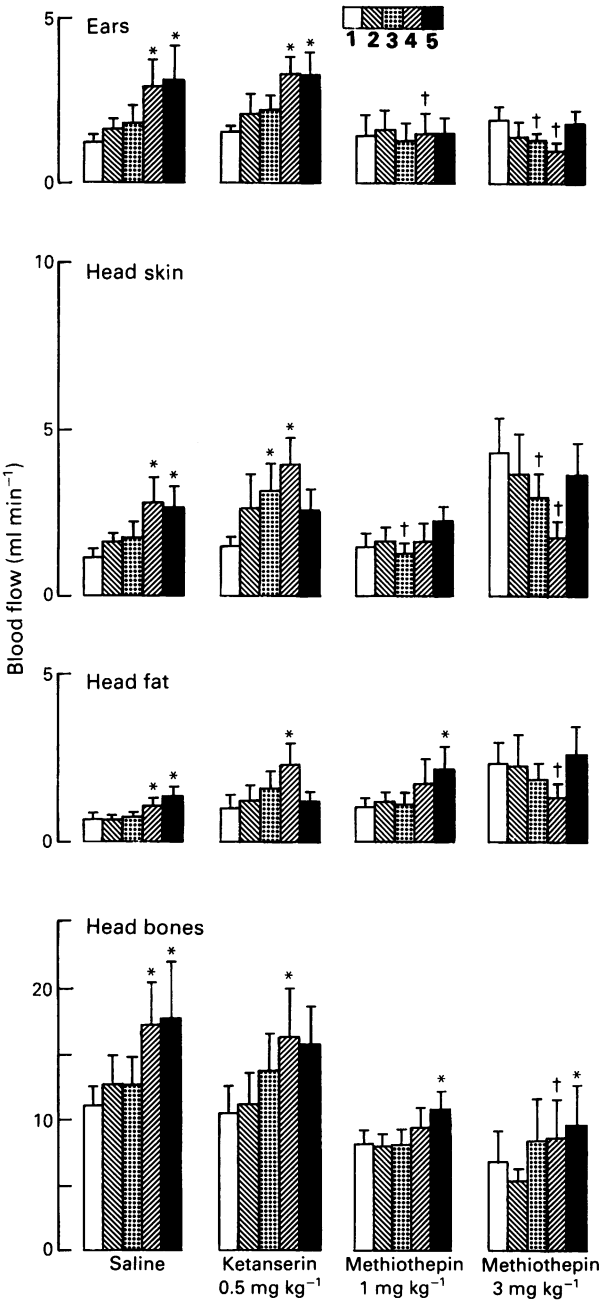


Figure 3 Effect of sumatriptan i.v. on the regional carotid blood flow distributed to the ears, skin, fat and bones (ml min^{-1}) in pigs, pretreated i.v. with either saline ($n = 8$), ketanserin 0.5 mg kg^{-1} ($n = 6$), methiothepin 1 mg kg^{-1} ($n = 7$) or methiothepin 3 mg kg^{-1} ($n = 6$). (1) Before sumatriptan (after pretreatment); (2) sumatriptan 10 μg kg^{-1}; (3) sumatriptan 30 μg kg^{-1}; (4) sumatriptan 100 μg kg^{-1} and (5) sumatriptan 300 μg kg^{-1}. All values have been presented as means with s.e.mean shown by vertical bars. * $P < 0.05$ vs baseline; † $P < 0.05$ vs the corresponding dose of sumatriptan in the saline pretreated animals.

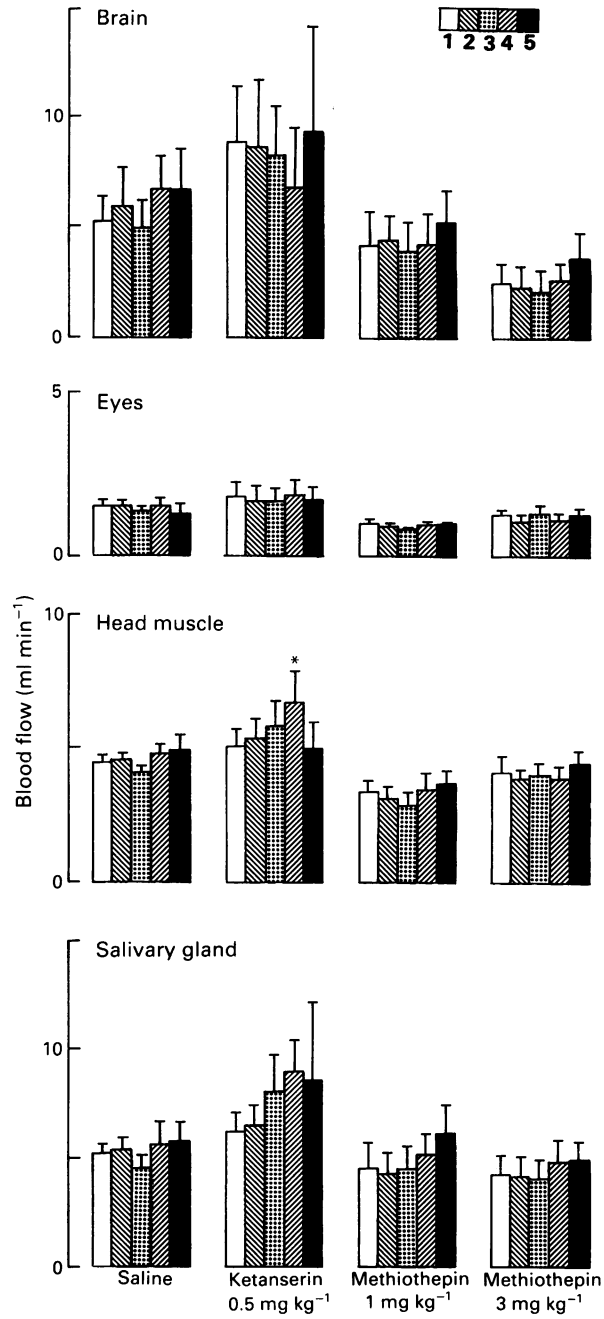


Figure 4 Effect of sumatriptan i.v. on the regional carotid blood flow distributed to the brain, eyes, head muscle and salivary gland (ml min^{-1}) in pigs, pretreated i.v. with either saline ($n = 8$), ketanserin 0.5 mg kg^{-1} ($n = 6$), methiothepin 1 mg kg^{-1} ($n = 7$) or methiothepin 3 mg kg^{-1} ($n = 6$). (1) Before sumatriptan (after pretreatment), (2) sumatriptan 10 mg kg^{-1}; (3) sumatriptan 30 μg kg^{-1}; (4) sumatriptan 100 μg kg^{-1} and (5) sumatriptan 300 μg kg^{-1}. All values have been presented as means with s.e.mean shown by vertical bars. * $P < 0.05$ vs baseline. No significant differences between the corresponding doses of saline and (2) the drug were found.

The effects of sumatriptan on the distribution of arteriolar, nutrient blood flow to the different tissues are shown in Figures 3 and 4. Sumatriptan increased the arteriolar blood flow to the ears (up to 188 ± 78%), head skin (up to 148 ± 47%), head bones (up to 47 ± 27%) and head fat (up to 148 ± 74%), whereas the arteriolar blood flow to the muscles, eyes, brain and salivary glands remained unchanged. The sumatriptan-induced increase in arteriolar blood flow to the above mentioned tissues was unchanged by ketanserin. Methiothepin, however, attenuated this response to 30 and 100 μg kg^{-1} of sumatriptan.

Changes in blood gases

Sumatriptan caused an increase in the difference in oxygen saturation between arterial and jugular venous blood (Table 3). There was a clear tendency towards attenuation of this effect in the methiothepin pretreated groups. Though the oxygen saturation changes by sumatriptan in the methiothepin- and saline-treated groups did not differ significantly ($P > 0.05$), the values after the two highest doses of sumatriptan in animals treated with methiothepin (3 mg kg^{-1}), in contrast to those in the saline-treated animals, were not significantly different from the baseline values (Table 3).

Table 3 Effects of intravenous bolus injections of sumatriptan (10, 30, 100 and 300 $\mu g\,kg^{-1}$) on the arteriovenous difference in oxygen saturation (%) after pretreatment with saline, ketanserin (0.5 $mg\,kg^{-1}$), methiothepin (1 $mg\,kg^{-1}$) or methiothepin (3 $mg\,kg^{-1}$)

		Sumatriptan ($\mu g\,kg^{-1}$)			
Pretreatment	*Baseline*	10	30	100	300
Saline	9.4 ± 4.2	9.9 ± 4.1	13.2 ± 6.3	13.7 ± 5.6*	15.3 ± 5.2*
Ketanserin	7.6 ± 2.5	7.7 ± 2.4	8.4 ± 2.6	9.8 ± 2.5*	11.0 ± 3.6*
Methiothepin 1	5.6 ± 1.4	6.4 ± 1.1	6.6 ± 1.2	7.8 ± 1.5*	7.6 ± 1.0*
Methiothepin 3	5.3 ± 1.2	4.3 ± 1.2	6.0 ± 2.1	7.1 ± 2.8	7.3 ± 2.1

All values have been presented as means ± s.e.mean. * $P < 0.05$ vs baseline.
No significant differences between the corresponding doses of saline and the drug were found.

Discussion

Systemic haemodynamic changes after sumatriptan

After each dose of sumatriptan a small decrease in heart rate was observed. This is likely to be an effect of the drug, as in the saline-treated group no significant changes in heart rate were observed. Furthermore, in anaesthetized dogs almost identical falls in heart rate occurred after sumatriptan administration (Feniuk *et al.*, 1989). The mechanism of this decrease in heart rate is unknown. After ketanserin pretreatment the sumatriptan-induced decreases in heart rate were slightly blunted. This is unlikely to have been caused by the 5-HT_2 or α_1-adrenoceptor antagonist action of ketanserin, since sumatriptan has minimal affinity for these receptors (Peroutka & McCarthy, 1989). Likewise, after 3 $mg\,kg^{-1}$ of methiothepin the highest dose of sumatriptan caused significantly less heart rate reduction. In this case the baseline heart rate differed from the other groups. However, it should be noted that these changes were rather small, so that their significance is doubtful. In clinical studies with sumatriptan no changes in heart rate have been reported (Perrin *et al.*, 1989).

Unlike the antimigraine drug ergotamine which usually increases blood pressure by increasing total peripheral resistance (see Saxena & De Vlaam Schluter, 1974; Johnston & Saxena, 1978), sumatriptan did not increase arterial blood pressure. This lack of a hypertensive effect of sumatriptan can be explained by its more selective vasoconstrictor action on cranial blood vessels. In anaesthetized dogs sumatriptan had no influence on total peripheral resistance at doses which induced a marked increase in carotid resistance (Feniuk *et al.*, 1989).

Carotid haemodynamics

Sumatriptan elicited a dose-dependent reduction in common carotid blood flow, which was directly related to a decrease in its arteriovenous anastomotic fraction, as found in the cat (Perren *et al.*, 1989; MacLennan & Martin, 1990). The decrease in the arteriovenous anastomotic blood flow was also reflected in the decrease in the oxygen saturation in the jugular venous blood. Pretreatment with ketanserin (0.5 $mg\,kg^{-1}$) did not change the effects of sumatriptan. This dose of ketanserin is adequate to block 5-HT_2 receptor-mediated responses (Saxena & Lawang, 1985; Schalekamp & Wenting, 1990). In contrast to ketanserin, methiothepin antagonized the sumatriptan-induced reductions in both total carotid and arteriovenous anastomotic blood flows. Therefore, sumatriptan appears to reduce arteriovenous anastomotic blood flow via stimulation of a 5-HT_1-like receptor. In this sense sumatriptan behaves in a similar way to 8-OH-DPAT (Bom *et al.*, 1989b), RU 24969 (Bom *et al.*, 1989c) and indorenate (Villalón *et al.*, 1990). Whereas 1 $mg\,kg^{-1}$ of methiothepin markedly antagonized the reduction in arteriovenous anastomotic blood flow by the above-mentioned agonists, in the present experiments the antagonist potency of this dose of methiothepin was less marked; for a full inhibition of the response of sumatriptan a dose of 3 $mg\,kg^{-1}$ of methiothepin was needed. A possible reason for the difference may be the greater selectivity of sumatriptan which does not appear to affect the carotid circulation via a 5-HT_2-receptor mechanism, since ketanserin left the responses unchanged. In contrast, with 5-HT, RU 24969 and indorenate a small ketanserin-sensitive 5-HT_2 receptor component is also noticed in the reduction of arteriovenous anastomotic blood flow (see Saxena & Verdouw, 1984; Verdouw *et al.*, 1984; Bom *et al.*, 1989c; Villalón *et al.*, 1990). RU 24969 is also known to activate 5-HT_2 receptors (Feniuk & Humphrey, 1989). It may be that the slightly greater antagonist activity of methiothepin against these agonists reflects this additional 5-HT_2 receptor component; indeed, methiothepin is a more potent 5-HT_2 receptor than a 5-HT_1-like receptor antagonist (Bradley *et al.*, 1986).

Sumatriptan caused a modest increase in the arteriolar fraction of the carotid blood flow to the ears, head skin, head bones and fat. In view of vagosymapthectomy carried out in the present experiments, this vasodilatation is unlikely to be due to inhibition of noradrenaline release from sympathetic neurones by sumatriptan (Humphrey *et al.*, 1988; 1990). It is possibly a consequence of the decrease in arteriovenous anastomotic flow with resultant diversion of blood flow to the tissues. However, a small effect of sumatriptan via the vascular 5-HT_1-like receptors mediating vasodilatation cannot be ruled out (Bradley *et al.*, 1986; Saxena & Villalón, 1990). Previous experiments had shown that the reduction of arteriovenous anastomotic blood flow, as for example in case of indorenate, is not necessarily associated with an increase in the nutrient fraction of the carotid blood flow (Villalón *et al.*, 1990) and sumatriptan can elicit endothelium-dependent relaxation in the pig coronary artery (Schoeffter & Hoyer, 1989a). In any case, if such a dilator action of sumatriptan is present in the cephalic vascular beds, it is very weak compared to 5-HT (Saxena & Verdouw, 1982) or 5-carboxamidotryptamine (Saxena & Verdouw, 1985), both of which induce massive vasodilatation and redness in the skin. The attenuation of the vasodilator effect by methiothepin is compatible with both a direct and an indirect effect of sumatriptan.

Nature of the 5-HT_1-like receptors involved in the reduction of cephalic arteriovenous anastomotic blood flow

In view of the heterogeneity of 5-HT_1-like receptors (Bradley *et al.*, 1986; Humphrey & Feniuk, 1988; Saxena & Villalón, 1990) one may ask which of the several subtypes of the 5-HT_1-like receptors mediates the reduction in arteriovenous anastomotic blood flow by sumatriptan in the pig? Sumatriptan has a high affinity for 5-HT_{1D} and 5-HT_{1B} binding sites and a reasonably high affinity for 5-HT_{1A} sites (Peroutka & McCarthy, 1989; Schoeffter & Hoyer, 1989b). Because the other putative 5-HT_{1A} receptor agonists BEA 1654 (Verdouw *et al.*, 1985), 8-OH-DPAT (Bom *et al.*, 1989b) and indorenate (Villalón *et al.*, 1990) also reduce arteriovenous shunting, it is tempting to suggest that 5-HT_{1A} receptors are involved. However, the selective 5-HT_{1A} receptor agonist ipsapirone (Peroutka, 1986) was inactive in the pig carotid circulation

(Bom et al., 1988) and the reduction in arteriovenous shunting by RU 24969, a 5-HT_{1A} and 5-HT_{1B} receptor agonist, is not amenable to blockade by (±)-pindolol (Bom et al., 1989c), an antagonist at both these receptors (Hoyer, 1988). Therefore, neither 5-HT_{1A} nor 5-HT_{1B} receptors seem to be involved. The involvement of 5-HT_{1C} receptors is also unlikely because of the very high potency of 5-carboxamidotryptamine in the present experimental model (Saxena & Verdouw, 1985).

Compared to the above 5-HT_1-like receptor subtypes, the 5-HT_{1D} receptor would seem a more likely candidate, since sumatriptan, ergotamine, dihydroergotamine, methysergide, RU 24969, 8-OH-DPAT and indorenate all bind with reasonable affinity to the 5-HT_{1D} receptor (see Hoyer, 1989). On the other hand, the sumatriptan-induced contraction of the dog saphenous vein (Humphrey et al., 1988, 1990) and the indorenate-induced reduction in the porcine carotid arteriovenous anastomotic blood flow (Villalón et al., 1990) are both resistant to metergoline, which has a high affinity for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} receptors (Hoyer, 1988) and has been shown to antagonize a putative 5-HT_{1D} receptor-mediated effect, the endothelium-dependent relaxation of the pig coronary artery by 5-HT (Schoeffter & Hoyer, 1990). Though in another putative functional model for 5-HT_{1D} receptors, inhibition of adenylate cyclase production in calf substantia nigra, metergoline behaved almost as a full agonist (Schoeffter et al., 1988), no agonist effect of metergoline was observed in the cranial arteriovenous anastomoses (Villalón et al., 1990). Taken together, the evidence to-date indicates that the 5-HT_1-like receptor mediating the reduction of arteriovenous anastomotic blood flow in the pig does not fully correspond to any of the brain subtypes characterized so far. Rather the receptor would seem to be the same as that characterized in the dog saphenous vein and intracerebral vessels in a variety of species (Perren et al., 1991).

Do sumatriptan and ergotamine constrict porcine arteriovenous anastomoses by acting on different receptors?

We have earlier reported that the constriction of arteriovenous anastomoses by ergotamine, being resistant to blockade by phentolamine (0.5 mg kg^{-1}), pizotifen (0.5 mg kg^{-1}), ketanserin (0.5 mg kg^{-1}) and methiothepin (1.0 mg kg^{-1}), is not mediated by either α-adrenoceptors or 5-HT_2 and 5-HT_1-like receptors (Saxena et al., 1983; Bom et al., 1989a). However, 1 mg kg^{-1} of methiothepin proved little more effective against sumatriptan in the present study than it did against ergotamine earlier (Bom et al., 1989a). In view of these new data with sumatriptan, the question of involvement of the 5-HT_1-like receptor in the ergotamine-induced constriction of the shunt vessels remains open until the use of 3 mg kg^{-1} dose of methiothepin against ergotamine in this model.

Is the constriction of arteriovenous anastomoses likely to contribute to the antimigraine activity of sumatriptan?

In a study of 7 migraine patients during an attack, Heyck (1969) found the jugular venous oxygen saturation to be elevated on the affected side; treatment with dihydroergotamine normalized the oxygen saturation on the affected side. Heyck suggested that one explanation for this phenomenon could be an abnormal dilatation of arteriovenous anastomoses during the migraine attack, increasing the amount of shunted blood from which no oxygen can be extracted (see also Saxena & Ferrari, 1989; Saxena, 1990). Arteriovenous anastomoses do exist in human cranial non-cerebral tissues such as the dura mater and skin (Rowbotham & Little, 1965; Kerber & Newton, 1973), but it is fair to point out that they may not convey the same volume of blood as in anaesthetized domestic animals and that there is no direct information on their behaviour during a migraine attack. Nevertheless, in view of the possible involvement of arteriovenous anastomoses in migraine and the fact that the reduction of arteriovenous anastomotic blood flow in our animal experimental model is a feature sumatriptan shares with several other antimigraine agents like ergotamine (Johnston & Saxena, 1978; Spierings & Saxena, 1980a; Saxena et al., 1983; Bom et al., 1989a), dihydroergotamine (Spierings & Saxena, 1980a), methysergide (Saxena & Verdouw, 1984) and isometheptene (Spierings & Saxena, 1980b), it seems reasonable to suggest that the constriction of arteriovenous anastomoses may play a part in the mechanism of therapeutic action of sumatriptan and other antimigraine drugs.

It has recently been shown that ergotamine, dihydroergotamine, methysergide and sumatriptan inhibit the extravasation of plasma proteins from dural blood vessels following stimulation of the trigeminal ganglion and that this action could be responsible for their antimigraine action (Markowitz et al., 1988; Saito et al., 1988; Buzzi & Moskowitz, 1990). It should, however, be noted that, except for sumatriptan, the doses of these antimigraine drugs needed to inhibit protein extravasation were higher than those employed in patients against migraine attacks or those required to cause a substantial decrease in arteriovenous anastomotic blood flow. Furthermore, it is possible that the reduction in plasma extravasation by these drugs is secondary to the constriction of the leaking vessels within the carotid vascular bed. The lack of inhibition of neurogenic and capsaicin-induced plasma extravasation by angiotensin II and phenylephrine (see Markowitz et al., 1988; Saito et al., 1988; Buzzi & Moskowitz, 1990) does not necessarily signify independence from the vasoconstrictor effect of the antimigraine drugs, since these results were not backed up by simultaneous measurement of dural (or carotid) blood flow or even arterial blood pressure.

In conclusion this study shows that sumatriptan reduces porcine carotid blood flow by selectively affecting its arteriovenous anastomotic fraction and that these effects are mediated by a 5-HT_1-like receptor. The reduction in arteriovenous anastomotic blood flow may play a role in its antimigraine efficacy. Alternatively, since the cerebral anatomy in man is somewhat different from that in domestic animals, this action may reflect a similar selective cranial vasoconstrictor action in man on the particular vessels, yet to be identified, thought to be distended and inflamed during a migraine attack. These vessels may be the meningeal vessels and evidence has already been provided that sumatriptan will constrict the human dural vasculature (Humphrey et al., 1989b), where arteriovenous anastomoses have been demonstrated (Rowbotham & Little, 1965; Kerber & Newton, 1973).

This work was partially supported by Glaxo Group Research, Ware, Herts. C.M.V. was supported by a Science and Technology Grant from the E.E.C.

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(Received July 3, 1990
Revised October 5, 1990
Accepted October 11, 1990)

Br. J. Pharmacol. (1991), 102, 331–336 © Macmillan Press Ltd, 1991

Characterization and actions of human umbilical endothelium derived relaxing factor

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1 A bioassay cascade superfusion technique was utilized to study the properties of endothelium derived relaxing factor (EDRF) from human umbilical vein (HUV) and compare its actions on umbilical, chorionic plate and bovine pulmonary arterial strips.

2 Histamine (1 μM), bradykinin (1 μM) and A-23187 (0.3 μM, 1 μM) but not acetylcholine (1 μM) released EDRF.

3 The non-innervated human foetoplacental vessels, i.e., umbilical and chorionic plate arteries, do relax to EDRF by a guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-mediated mechanism.

4 The sensitivity of the human umbilical arterial strips to EDRF was less than that of the chorionic plate arterial strips. Bovine pulmonary arterial strips were the most sensitive to the relaxant actions of human umbilical EDRF.

Introduction

The umbilical and placental vessels lack innervation (Reilly & Russell, 1977; Spivack, 1980). Regulation of vascular tone in the foetal extracorporeal circulation must therefore depend on the release of vasoactive substances which are locally produced or conveyed through the blood stream. The endothelial cells of human umbilical vessels produce the eicosanoids prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) (Tuvemo *et al.*, 1976; Bjoro *et al.*, 1986) and these influence the tone of placental and umbilical vessels (Tuvemo *et al.*, 1976; Glance *et al.*, 1985; Bjoro & Stray-Pedersen, 1986).

More recently, we (Buga *et al.*, 1989) and others (Van de Voorde *et al.*, 1987) demonstrated the release of EDRF from human umbilical vessels. The aim of the present study was to investigate the pharmacological properties of human umbilical venous EDRF, using as a detector the bovine pulmonary artery (Ignarro *et al.*, 1987), and compare the responses to those obtained on human umbilical and chorionic plate arteries.

Methods

Preparation and superfusion of the arterial strips

Human umbilical cords and chorionic plate arteries were obtained from normal deliveries at UCLA. Freshly obtained cords were placed and maintained in cold oxygenated Krebs solution. The chorionic plate arteries were dissected from the foetal side of the freshly delivered placenta and were transported in cold oxygenated Krebs solution. The vessels were used within 2 h of delivery. The bovine pulmonary vessels were obtained from lungs that were rapidly excised from cows that had been slaughtered at a nearby abbatoir and immediately submerged in ice cold salt solution contained in a plastic bag. The bag was sealed, placed in a cooler packed with ice and brought to the laboratory.

For bovine pulmonary vascular arterial strips (BPA), the 2nd arterial branch extending into the larger lobe was used (Ignarro *et al.*, 1987). The umbilical artery (UA) was isolated from the middle segment of the umbilical cord. The main chorionic plate arteries (CPA) were dissected from the foetal side (chorionic plate) of the placenta. The vessels were used to prepare helically cut strips (2 cm long and 4 mm wide) that were used for superfusion. Endothelial cells were removed from the arterial strips by gently rubbing the internal surface with a moistened filter paper for 30–40 s. The vascular endothelium denuded strips were then mounted for recording changes in length as has been previously described (Ignarro *et al.*, 1987; Palmer *et al.*, 1987) by means of cotton ties that were fixed to either end of the tissues. These vascular strips were mounted in series to constitute the cascade.

Bioassay

The bioassay was carried out by the cascade superfusion technique as originally described by Vane (1964). Isolated segments of the umbilical cord (10 cm) were fitted at either end of the umbilical veins with polyethylene tubing and submerged in chambers containing oxygenated Krebs solution at 37°C. The umbilical vein was perfused (3.5 ml min^{-1}) with oxygenated Krebs solution at 37°C by use of a constant perfusion pump and the perfusate was allowed to superfuse the cascade of endothelium denuded vascular smooth muscle preparations. Preparations were equilibrated for at least 90 min under tension (2 g for UA and CPA and 4 g for BPA) in oxygenated Krebs solution delivered by superfusion (3.5 ml min^{-1}) by a separate line (in addition to the perfusate) over the cascade of preparations at 37°C. Just prior to superfusing strips with vessel perfusates, the strips were contracted submaximally by 10 μM phenylephrine or a mixture of 10 μM phenylephrine and 0.01 μM U46619, delivered by superfusion. The vascular segments used for perfusion as well as all strips were pretreated with indomethacin (10 μM) in order to prevent the formation of prostaglandins especially prostacyclin (Gryglewski *et al.*, 1986). The treatment with indomethacin was started 30 min prior to bioassay and continued for the duration of the experiment.

Pharmacological properties of human umbilical EDRF

In the initial experiments to study the pharmacological properties of human umbilical venous (HUV) EDRF, endothelium denuded bovine pulmonary strips were used as detector tissues. The time delay between each strip was 2.5 s as was the delay between the perfused vessel and the first vascular strip. In these studies, at the beginning and end of each experiment, glyceryl trinitrate (GTN) was superfused (0.1 μM) over the strips. The strips that were insensitive to glyceryl trinitrate were replaced with fresh strips. The responses to the strips

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were electronically adjusted so that a given dose of GTN (0.1 μM) produced equal relaxation. This was done to see the pattern of response when an unstable substance was superfused over the tissues.

In experiments designed to evaluate the actions of HUV EDRF on UA and CPA, the sequence of tissues in the cascade varied. In the first set of experiments, the first two tissues were UA strips with the CPA being the third or bottom tissue in the cascade. In the second set of experiments, the CPA strips were the top two tissues in the cascade with the BPA strip being the third or bottom tissue in the cascade. In both sets of experiments, the time delay between each strip was 0.5 s as was the delay between the perfused vessel and the first vascular strip. This was achieved by adjusting the distance between the cascade of tissues while the rate of flow of the perfusate and superfusate were kept constant and was similar to that in previous experiments. This short time interval was necessary to compare the potency of EDRF in causing vascular relaxation in these before its inactivation. The percentage of relaxation produced by EDRF for each vessel was calculated. S-nitroso acetyl penicillamine (SNAP) (1 μM) was also used to test the responsiveness of these vessels in addition to glyceryl trinitrate (GTN) at the start of the experiments.

Determination of cyclic GMP levels

Cyclic GMP (guanosine 3′:5′-cyclic monophosphate) determinations were made in the arterial smooth muscle preparations that had been equilibrated under tension and precontracted as described above. The cascade bioassay technique was employed and the top or first tissue was the UA strip. The second strip down the cascade was the CPA. In a separate series of experiments, SNAP (1 μM) which releases nitric oxide (NO) was superfused over the cascade of strips or 1 μM A-23187 was perfused through a segment of human umbilical vein and the perfusate allowed to superfuse the strips described above. The strips were quick-frozen at 90 s after the start of arterial relaxation. Tone was monitored until the time of quick freezing. Tissues were homogenized, extracted and analyzed for cyclic GMP by radioimmunoassay as described previously (Ignarro *et al.*, 1987).

None of the test agents added to the strips interfered directly with antigen-antibody binding in the radioimmunoassay procedure. Recoveries of standard amounts of added cyclic GMP were determined periodically and values ranged from 95% to 102%. Therefore, no corrections of sample recoveries were made. All samples were run in a single assay to minimize problems with inter-assay variation.

Statistical analysis of the results was performed using one-way analysis of variance with Duncan's t range or Student's t test for unpaired values. The level of statistically significant difference was $P < 0.05$.

Chemicals

Phenylephrine hydrochloride, bradykinin, histamine dihydrochloride, indomethacin, A23187 (calcium ionophore), methylene blue, pyrogallol, haemoglobin, and superoxide dismutase (bovine liver) were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Glyceryl trinitrate (nitroglycerin, 10% w/w triturated mixture in lactose) was a gift from ICI Americas, Inc. (Wilmington, Delaware, U.S.A.). U46619 ([15S-hydroxy-11α,9α[epoxymethano]prosta-5Z, 13E-denoic acid) was provided by The Upjohn Co. (Kalamazoo, Michigan, U.S.A.), and was dissolved in absolute ethanol (5 mg ml^{-1}). Dilutions were prepared in distilled water. Deoxyhaemoglobin was prepared from haemoglobin by reduction with dithionite in deoxygenated Krebs-bicarbonate solution. Oxyhaemoglobin solutions were prepared by slowly bubbling oxygen into the deoxyhaemoprotein solutions for 20 min at 4°C (Ignarro *et al.*, 1986). S-nitroso-N-acetyl-penicillamine (SNAP) was synthesized and handled as described previously (Ignarro *et al.*, 1981). All concentrations indicated in the text are expressed as final bath concentrations. Krebs-bicarbonate solution consisted of (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.5, $NaHCO_3$ 25, $MgSO_4$ 1.1, KH_2PO_4 1.2 and glucose 5.6. Oxygenated Krebs solution signifies Krebs-bicarbonate solution bubbled and equilibrated with a mixture of 95% O_2 plus 5% CO_2.

Results

Release of EDRF from human umbilical vein and its detection on endothelium-denuded bovine pulmonary artery strips

Direct superfusion of acetylcholine (ACh), bradykinin (BK), histamine or A-23187 (all 1 μM) over the endothelium-denuded arterial strips elicited no observable responses, confirming the absence of endothelium on the detector tissues. Superfusion of GTN over the arterial strip cascade caused relaxant responses that were of comparable magnitude in all three strips thus reflecting the chemically stable nature of the drug in comparison to EDRF. The perfusate from the intact HUV in the absence of added vasodilators caused a small relaxation of the first two endothelium-denuded arterial strips (Figure 1). Perfusion of HUV with ACh (1 μM) failed to relax further any of the arterial strips. Addition of BK (1 μM) (data not shown), histamine (1 μM), or A-23187 (0.3 μM, 1 μM) to the perfusate caused further relaxation of all three arterial strips, and the magnitude of relaxation was concentration-dependent. (Figure 1). The decrement in the magnitude of the relaxation in the three strips is attributed to the short half life of the EDRF released from the perfused vein.

Properties of EDRF

Based on the 2.5 s time delay between each of the three superfused vascular strips, an approximation of the biological half life of EDRF was made directly from the recorded tracings and was found to be 3–5 s for human umbilical vein EDRF under the standardized experimental conditions employed. The addition of superoxide dismutase (SOD) (100 u ml^{-1}) to the medium perfusing the umbilical venous segment, resulted in an increased magnitude and duration of EDRF-elicited relaxant responses. Superoxide dismutase increased the stability of the EDRF released in the presence of perfused A-23187 (Figure 2). The apparent half-life of EDRF released by A-23187 in the presence of SOD was estimated to be 12–16 s.

When the cascade of vascular strips were superfused with pyrogallol (10 μM) which rapidly generates superoxide anion from oxygen containing solutions (Marklund & Marklund,

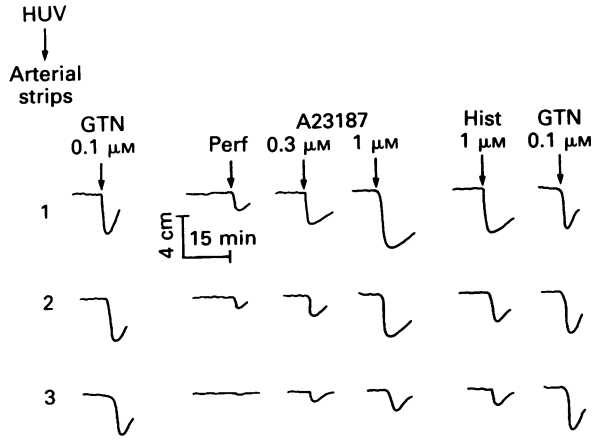


Figure 1 Relaxation by human umbilical vein (HUV) EDRF of endothelium-denuded bovine pulmonary arterial (BPA) strips arranged in cascade. The decrement in the responsiveness of the tissues down the cascade is shown demonstrating the unstable nature of the substance: GTN = glyceryl trinitrate; Hist = histamine.

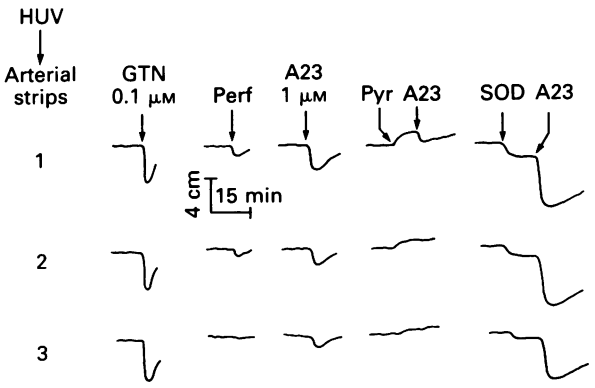


Figure 2 Pyrogallol (Pyr) shortens and superoxide dismutase (SOD) prolongs the half-life of EDRF.

1974), a slight contraction of the top tissue was seen. Pyrogallol also abolished the relaxation induced by perfusion of A-23187 (Figure 2). These observations suggest that the superoxide radical rapidly inactivated EDRF.

Superfusion of vascular strips with oxyhaemoglobin (1 μM) during perfusion of the HUV caused a small and sustained contraction (Figure 3). Oxyhaemoglobin also markedly antagonized or abolished EDRF-elicited relaxant response to perfused A-23187. In contrast, relaxation to superfused GTN was unaltered or only slightly antagonized by oxyhaemoglobin (Figure 3). Superfusion of vascular strips with methylene blue (10 μM) produced inhibitory effects that were similar to those produced by oxyhaemoglobin, but in addition, the relaxant responses to GTN were inhibited (Figure 3).

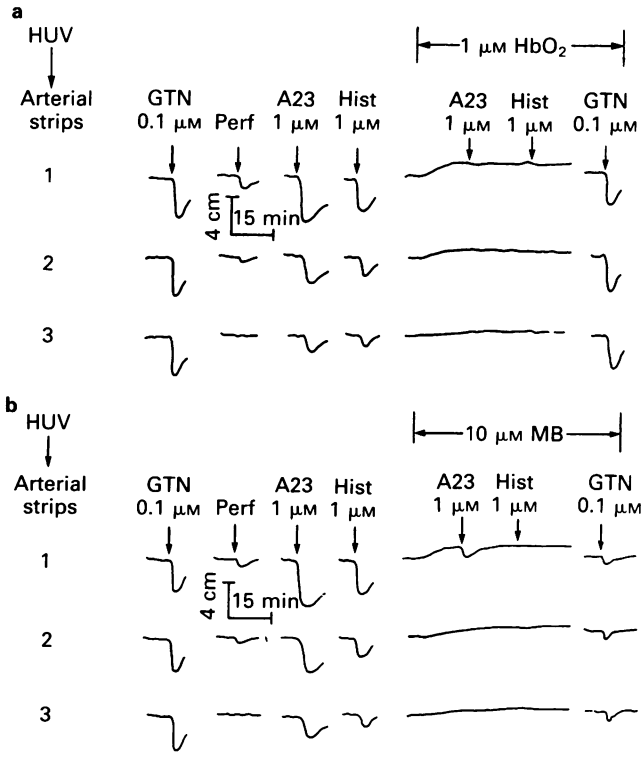


Figure 3 (a) Shows antagonism of EDRF elicited arterial smooth muscle relaxation by oxyhaemoglobin (HbO_2); in (b) antagonism of both EDRF and glyceryl trinitrate (GTN) elicited arterial smooth muscle relaxation by methylene blue (MB) is shown.

Comparison of actions of human umbilical vein EDRF on umbilical artery, chorionic plate arteries and bovine pulmonary arteries

When SNAP (1 μM) was superfused over the vascular strips, the magnitude of relaxation of the UA strips was less than that of the CPA strips (Figure 4). Perfusion of A-23187 (0.3 μM, 1 μM) through the HUV produced concentration-related relaxations of the vascular strips. The relaxations were more evident on the top UA strip and the bottom CPA strip (Figure 4). The mean percentage relaxation of the vascular strips are shown in Table 1. When SOD (100 u ml^{-1}) was superfused over the vascular strips and two different concentrations of A-23187 (0.3 μM, 1 μM) were added to the perfusate, the relaxant responses of the UA and CPA were potentiated when compared to responses obtained in the absence of SOD. In the presence of SOD and at a time when the relaxant response was at its maximum, superfusion of oxyhaemoglobin (1 μM) antagonized the relaxation (Figure 4).

In the next series of experiments in which two CPA strips and one BPA strip were used, superfusion of SNAP relaxed all

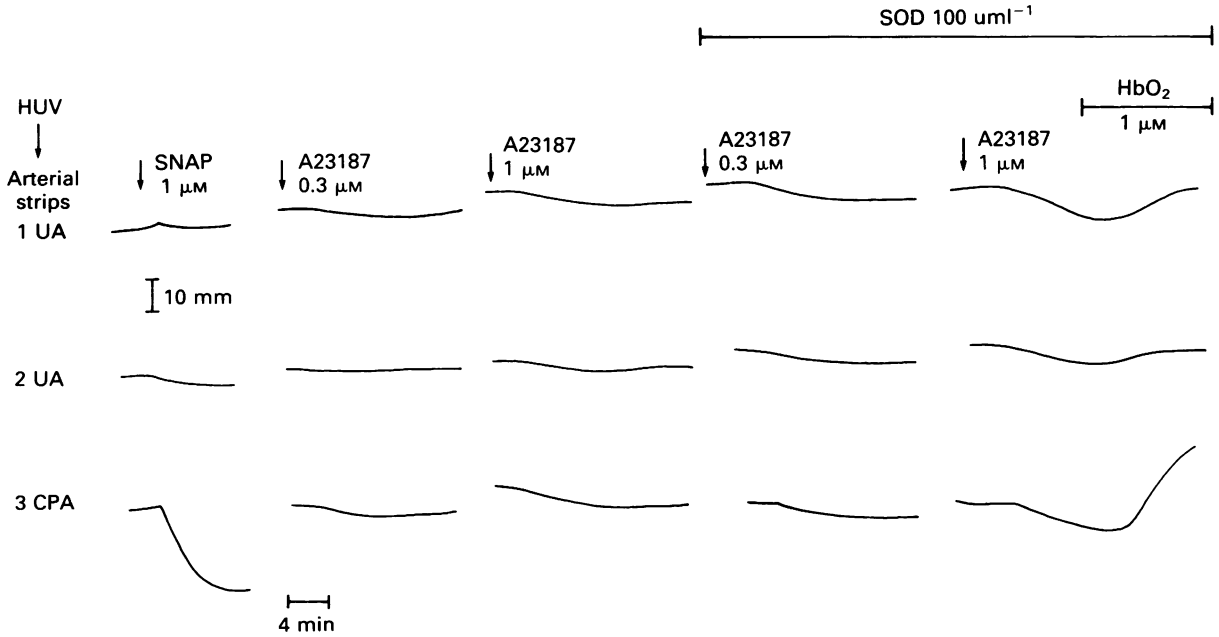


Figure 4 Relaxation by human umbilical vein (HUV) EDRF of endothelium-denuded human umbilical arterial (UA) strips and chorionic plate arterial (CPA) strip arranged in a cascade. The magnitude of relaxation produced is greater in the CPA than in the UA. SOD = superoxide dismutase; SNAP = S-nitroso-N-acetylpenicillamine

Table 1 Mean (±s.e.mean) percentage relaxation of the endothelium denuded umbilical (UA), chorionic plate (CPA), or bovine pulmonary arterial (BPA) strips to superfused S-nitroso-N-acetyl penicillamine (SNAP) or when A-23187 in two different concentrations was perfused through the human umbilical vein and the perfusate allowed to superfuse the cascade of tissues

	Arterial strips	*SNAP* (1 μM)	*A-23187* (0.3 μM)	*A-23187* (1 μM)
A	1 UA	12.6 (3.2)	8.0 (2.2)	18.3 (2.3)
	2 UA	10.6 (3.0)	4.6 (1.0)	14.6 (3.5)
	3 CPA	40.3* (8.5)	13.3* (2.6)	36.6* (5.1)
B	1 CPA	41.3 (5.0)	13.6 (3.4)	31.6 (5.1)
	2 CPA	38.1 (3.7)	6.0 (1.8)	20.6 (7.9)
	3 BPA	73.3* (8.2)	24.3* (4.7)	57.0* (8.4)

$n = 4$–5 in each set of experiments. **A** and **B** indicate the arrangement of the strips in the cascade.
* Values significantly different from those obtained from tissues just above it in the cascade.

three arterial strips. The percentage relaxation of the BPA strip was of a greater magnitude when compared to the CPA strips in spite of the BPA strip being the bottom tissue in the cascade (Figure 5). Concentration-related relaxation of these vessels was produced when A-23187 was added to the Krebs solution perfusing the HUV. The mean percentage relaxations of the vascular strips are shown in Table 1. In the presence of SOD, the responses of A-23187 were potentiated and this potentiation was greater in the BPA strip which was the third or bottom tissue in the cascade (Figure 5).

Cyclic GMP accumulation

Perfusion of umbilical vein with A-23187 (1 μM) caused relaxation and an increase in cyclic GMP levels in superfused UA and CPA strips.

When SNAP (1 μM) was superfused over the UA and CPA strips, a relaxation associated with cyclic GMP accumulation was also observed. The percentage relaxation and cyclic GMP accumulation are shown in Table 2.

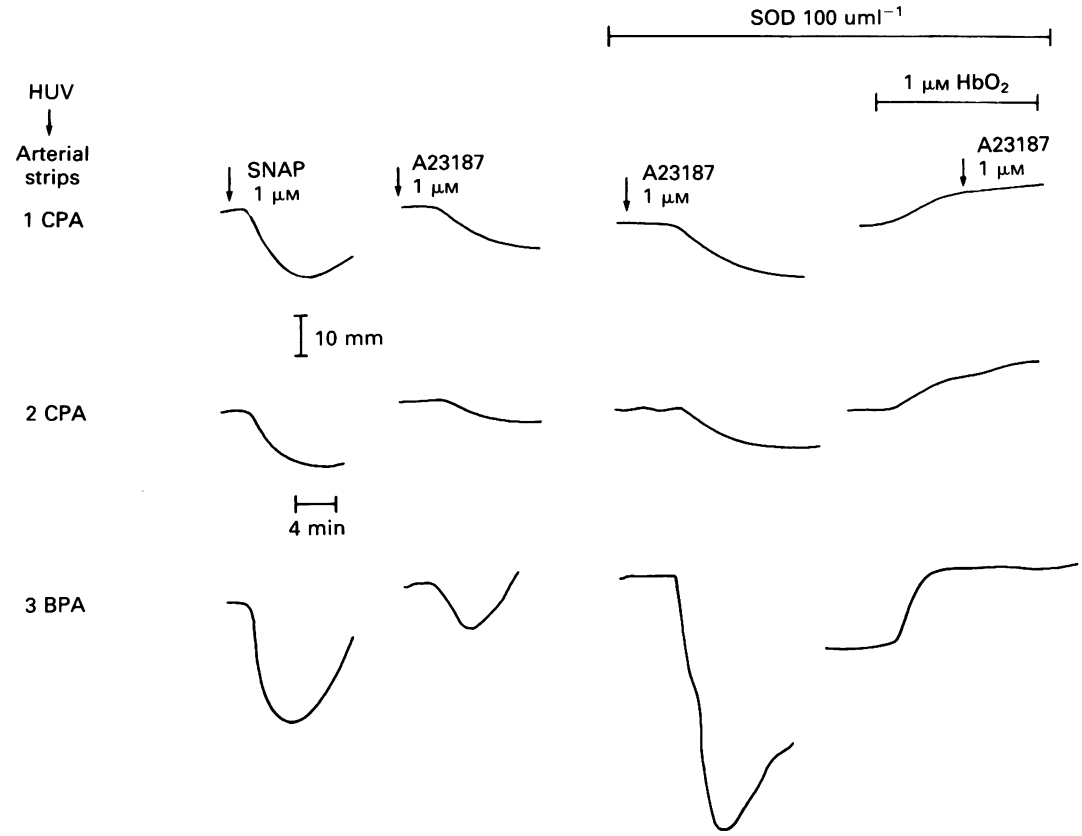


Figure 5 Relaxation by human umbilical vein (HUV) EDRF of endothelium-denuded chorionic plate arteries (CPA) and bovine pulmonary arteries (BPA) arranged in cascade. The magnitude of relaxation is greater in the BPA than in the CPA. Superoxide dismutase (SOD) when superfused over the detector tissues potentiated and oxyhaemoglobin (HbO_2), even in the presence of SOD, antagonized human umbilical EDRF-elecited vascular relaxation.

Table 2 Stimulation of cyclic GMP accumulation and relaxation in the umbilical (UA) and chorionic plate (CPA) arteries by EDRF and S-nitroso-N-acetyl penicillamine (SNAP)

		A-23187 (1 μM)		*SNAP* (1 μM)	
Cascade		*Cyclic GMP*	*% relaxation*	*Cyclic GMP*	*% relaxation*
Strip 1	UA	22 ± 3* (10)	18 ± 3	24.3 ± 3* (13)	14 ± 3
Strip 2	CPA	23 ± 3* (5)	21 ± 6	25.3 ± 5* (6.0)	27 ± 4

Strips of endothelium denuded umbilical and chorionic plate arteries were mounted in a cascade and precontracted as described in the Methods. SNAP (1 μM) was delivered over the cascade by superfusion. A-23187 (1 μM) was perfused through a segment of human umbilical vein as described in the Methods. Strips were quick frozen at 90 s after the beginning of arterial relaxation. Control strips were quick frozen after precontraction, in the absence of additions. Relaxation is expressed as the percentage decrease in agonist-elicited tone. Cyclic GMP levels are expressed as pmol g^{-1} tissue. Numbers in parentheses signify fold increase above (±s.e.mean) resting levels of cyclic GMP (1.8 ± 0.1 pmol g^{-1} tissue for umbilical artery and 4.2 ± 0.4 for chorionic plate artery). Results are expressed as means of 4–6 strips. The numbers 1 and 2 signify the cascade arrangement of the strips.
* Values significantly different from basal values.

Discussion

The primary objective of the present study was to elucidate whether the human foetoplacental vessels, which are not innervated, are capable of generating an endothelium derived relaxing factor and characterize its properties including its effects on umbilical and chorionic plate arteries.

We, therefore, elected to demonstrate the release of EDRF from HUV and to elucidate whether the properties of human umbilical EDRF were similar to that of bovine (Ignarro *et al.*, 1987) and porcine (Gryglewski *et al.*, 1986; Palmer *et al.*, 1987) EDRF. We also wished to compare the actions of HUV EDRF on UA, CPA and BPA in order to assess whether EDRF-induced cyclic GMP mediated vascular relaxation is less efficient in human foetoplacental vessels as has been suggested by other workers (Van de Voorde *et al.*, 1987). Bovine pulmonary artery was selected for comparison as this vessel relaxes to EDRF (Ignarro *et al.*, 1987) and carries deoxygenated blood similar to human umbilical and chorionic plate arteries.

The EDRF released from HUV perfused with endothelium-dependent vasodilators caused relaxation of the precontracted endothelium denuded strips of BPA arranged in series when the vessel perfusates were allowed to superfuse the strips. The decremental nature of the relaxant responses down the cascade of vascular strips revealed the highly unstable nature of the EDRF. The estimated half-life of EDRF released from human umbilical vein under the experimental conditions employed in the present study was 3 to 5 s. The potentiation of action of EDRF by SOD and its antagonism by pyrogallol is similar to that reported for bovine (Ignarro *et al.*, 1987; 1988) and porcine (Gryglewski *et al.*, 1986; Palmer *et al.*, 1987) EDRF. Acetylcholine failed to release venous EDRF presumably because of the absence of muscarinic receptors on the venous endothelial cells (Ignarro *et al.*, 1988). The relaxant responses were not due to prostacyclin as indomethacin was always present in the perfusate and superfusate. Similar release of EDRF by histamine from human umbilical artery, vein and umbilical vascular endothelial cells in culture has been reported (Van de Voorde *et al.*, 1987). However, these authors failed to observe any relaxation of umbilical vascular preparations by histamine when these preparations were mounted for isometric tension recording in the presence and absence of indomethacin and precontracted with 5-hydroxytryptamine (5-HT). They therefore suggested that the umbilical arteries may have a poor relaxant response to cyclic GMP-mediated vascular relaxation. It has also been suggested that cyclic GMP accumulation in umbilical vessels induced by acetylcholine and histamine, may be associated with vascular contraction rather than relaxation (Clyman *et al.*, 1975). Our results demonstrate that the umbilical and chorionic plate arteries do relax by cyclic GMP-mediated mechanisms although not as efficiently as other vessels under similar experimental conditions. The difference in sensitivity of UA and CPA to EDRF is not surprising as similar differences in sensitivity to other vasoactive substances such as angiotensin II, bradykinin and 5-HT have previously been reported (Tulenko, 1979). The contractile responses of UA to endothelium-dependent vasodilators as reported in earlier studies (Altura *et al.*, 1972), were probably due to endothelial damage during preparation of the vascular strips, when care to preserve the endothelium may not have been taken.

The physiological role of EDRF in the foetoplacental circulation is unknown. von Euler (1938) observed that when 5 percent haemoglobin was mixed with Locke solution to perfuse the human placenta *in vitro*, there occurred a consistent increase in peripheral resistance and perfusion pressure. The observation in this study that oxyhaemoglobin antagonized the vascular relaxant actions of EDRF may explain the haemoglobin-induced increase in perfusion pressure reported by von Euler (1938), in the placental circulation and may therefore suggest a role for flow-induced release of EDRF in maintaining vascular tone in the foetoplacental circulation. An interesting feature of the foetoplacental circulation is that the oxygen tension is very low in the foetal side and the placenta acts as a very efficient barrier to the transfer of oxygen from the maternal to the foetal circulation (Assali, 1988). The precise mechanism by which the nerve-free vessels of the foetoplacental circulation are dilated *in vivo* but spontaneously close after birth is not known. It has been suggested that increase in O_2 tension that occurs immediately after birth may be an important mechanism for spontaneous closure of the umbilical arteries (Oberhansli-Weiss *et al.*, 1972). All studies, including the present, which have reported on the release and actions of EDRF on foetoplacental vessels were undertaken under relatively high oxygen tension (i.e., 95% O_2 and 5% CO_2). It is thus possible that the decreased sensitivity *in vitro* of UA and CPA to EDRF may be due to the relatively high Po_2.

An increase in prostacyclin formation by human umbilical artery under low oxygen tension when compared to that produced under high oxygen tension has been reported (Stuart *et al.*, 1984; Bjoro *et al.*, 1987). Further studies need to be performed to evaluate whether the release and actions of EDRF in human foetoplacental vessels are modified by differences in oxygen tension.

This work was supported in part by USPH Grants HL40872, HL35014 and a grant from the Laubisch Fund for Cardiovascular Research. Appreciation is extended to Christopher Doyle for editorial assistance.

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(Received June 25, 1990
Revised September 7, 1990
Accepted September 29, 1990)

Br. J. Pharmacol. (1991), **102**, 337–344 © Macmillan Press Ltd, 1991

Differences between the effects of cromakalim and nifedipine on agonist-induced responses in rabbit aorta

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1 The effects of cromakalim on endothelium-denuded rabbit aortic strips were compared with those of the calcium (Ca^{2+}) entry blocking agent, nifedipine.

2 Pre-incubation with cromakalim or nifedipine had no significant effect on the initial phasic component of noradrenaline (NA)-induced responses.

3 Cromakalim (0.3–10 μM), but not nifedipine, inhibited the maintained tonic contractions produced by NA. The effects of cromakalim were antagonized by raising extracellular [K^+] or by glibenclamide.

4 Nifedipine inhibited contractions produced by KCl (40 mM) whereas cromakalim had no effect.

5 In Ca^{2+}-free physiological salt solution (PSS), cromakalim produced a significant inhibition of both the refilling of and the release of Ca^{2+} from NA-releasable Ca^{2+} stores, whereas nifedipine was ineffective.

6 In tissues preloaded with $^{42}K^+$ cromakalim (0.3–10 μM) produced a concentration-dependent increase in the $^{42}K^+$ efflux rate coefficient. NA (0.3 μM) also produced an increase in the rate of efflux of $^{42}K^+$, an effect which was not antagonized by nifedipine (0.3 μM).

7 When microelectrodes were used, cromakalim (1–10 μM) produced a maintained concentration-dependent membrane hyperpolarization. However, low concentrations of cromakalim (< 1 μM) which relaxed the aorta had no effect on membrane potential. NA had no significant effect on membrane potential.

9 It is concluded that the ability of cromakalim to relax NA-induced contractions in rabbit aorta is not exerted by the indirect closure of nifedipine-sensitive Ca^{2+} channels. Instead, cromakalim may exert a direct inhibitory action on Ca^{2+} uptake into and release from Ca^{2+} stores and additionally inhibit the pathway through which Ca^{2+} passes from the extracellular fluid to intracellular Ca^{2+} stores.

Introduction

The pharmacology of a new class of smooth muscle relaxant drugs, the effects of which are associated with the opening of membrane potassium (K^+) channels has recently been reviewed (Hamilton & Weston, 1989). One of these agents, cromakalim (BRL 34915) is a potent vasodilator both *in vitro* (Hamilton *et al.*, 1986) and *in vivo* (Buckingham *et al.*, 1986) and is currently undergoing Phase 3 clinical trials as an antihypertensive agent (Nguyen *et al.*, 1987; Pilgrim *et al.*, 1987; Vandenburg *et al.*, 1987; Lebel *et al.*, 1988).

Evidence in favour of a causal relationship between cromakalim-induced smooth muscle relaxation and K^+-channel opening has been derived from both electrophysiological measurements in rat and guinea-pig smooth muscle (Allen *et al.*, 1986; Hamilton *et al.*, 1986; Southerton *et al.*, 1988) and from observations of an increased rate of $^{86}Rb^+$ exchange in a variety of tissues (Kreye & Weston, 1986; Weir & Weston, 1986a,b; Coldwell & Howlett, 1987; Cook & Quast, 1987; Quast, 1987). However, several discrepancies have also been reported, suggesting that relaxation may occur independently of changes in ion flux and membrane potential (Shetty & Weiss, 1987; Hollingsworth *et al.*, 1987; 1988; Quast & Baumlin, 1988).

In blood vessels the opening of K^+-channels and the consequent closure of voltage-operated Ca^{2+}-channels effectively short circuits agonist-induced depolarization. It has therefore been suggested that the inhibitory actions of cromakalim will be exerted on those tissues in which depolarization plays a major role in agonist-induced excitation (Hamilton *et al.*, 1986). Thus, cromakalim would be expected to show a profile of action similar to that of the Ca^{2+} entry blocking drugs. However, in rabbit aorta, contractions to angiotensin II (AII) are inhibited to a greater extent by cromakalim than by the dihydropyridine, isradipine (Cook *et al.*, 1988). This suggests that indirect inhibition of Ca^{2+} entry through voltage-operated, dihydropyridine-sensitive Ca^{2+} channels cannot alone explain the inhibitory effects of cromakalim.

In the present investigation we have compared the effects of cromakalim and nifedipine on contractile responses to noradrenaline (NA) in rabbit aorta, a tissue in which responses to this agonist are electrically silent (Cauvin *et al.*, 1984). In this way it was hoped to extend our understanding of the inhibitory effects of cromakalim in blood vessels. Preliminary accounts of some of these observations have already been presented (Bray *et al.*, 1988b,c,d).

Methods

Tissue bath experiments

Preparation of tissues Male half-lop rabbits (2.5–3.5 kg) were killed by stunning and bleeding. A portion of the thoracic aorta was removed, and cut into four rings each approximately 0.5 cm in length. Each ring was cut longitudinally to form a flat sheet and the endothelium removed by gently rubbing the intimal surface with a moistened cotton bud. The resulting aortic preparations were then mounted for isometric tension recording, under a resulting tension of 2 g, in a tissue bath containing MOPS-buffered physiological salt solution (PSS) at 37°C, pH 7.4 and gassed with 100% O_2. After a 30 min equilibration period tension on the strips was readjusted to 2 g and they were left for a further 30 min.

Effects of agents on phasic and tonic contractions The antispasmogenic effects of cromakalim and nifedipine on the

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initial phasic responses to NA and potassium chloride (KCl), were examined by cumulative exposure to NA or KCl. This was followed by incubation with either cromakalim or nifedipine for 40 min prior to re-exposure to the spasmogen in the continuing presence of the relaxant drug.

The ability of cromakalim or nifedipine to relax the maintained tonic phase of contractions to NA or KCl was also examined. Tissues were exposed to NA (0.3 μM) or KCl (40 mM). When maximal, maintained tonic contractions to these concentrations of spasmogen had been obtained, cromakalim or nifedipine was added cumulatively to the tissue bath at 10 min intervals.

Antagonism by glibenclamide Tissues were pretreated for 30 min with glibenclamide (0.1–1 μM) prior to exposure to NA 0.3 μM. Cumulative concentration-response experiments with cromakalim were then performed in the continuing presence of glibenclamide as previously described. From the above experiments relaxant and spasmogen concentrations were chosen for use in the following experiments.

Antagonism by high $[K^+]$ Tissues were incubated with nifedipine (0.3 μM) for 40 min followed by exposure to KCl (40 mM) for 15 min. This concentration of nifedipine was the maximally effective relaxant concentration used in the present study, which retained selectivity for blockade of voltage-operated Ca^{2+} channels (Bean, 1985). In the presence of KCl a small contraction ensued. When the maintained phase of this contraction had been achieved, NA (0.3 μM) was added for 8 min, followed by cumulative addition of cromakalim at 10 min intervals in the continuing presence of KCl and NA. Control tissues were treated similarly but were not exposed to either KCl or cromakalim.

Ca^{2+}-free experiments

Effects of agents on Ca^{2+} store refilling Aortic strips were equilibrated in Ca^{2+}-free PSS containing EGTA (2 mM), $MgCl_2$ (10 mM) and nifedipine (0.3 μM) (EGTA-PSS; see Drugs and Solutions) for 15 min followed by exposure to NA (1 μM), which produced a phasic mechanical contraction. The tissues were washed in EGTA-PSS for 10 min and then exposed to PSS containing Ca^{2+} (1 mM) (Ca^{2+}-PSS) for 2, 5, 10, 15, or 25 min, these periods of exposure to Ca^{2+}-PSS being randomized for every experiment. After washing in EGTA-PSS for 15 min to remove extracellular Ca^{2+}, the tissues were re-exposed to NA. In some experiments cromakalim (10 μM) was added 10 min prior to the initial exposure to NA in EGTA-PSS and remained during the periods of exposure to Ca^{2+}-PSS.

Results were calculated as the response to NA after refilling of the store for 'x' min. They were expressed as a percentage of the initial contraction to NA in EGTA-PSS in the presence of cromakalim but with a full Ca^{2+} store. In this way the component of contraction attributable to inhibition of Ca^{2+} release by these agents was automatically subtracted and the result reflected only the effect of cromakalim on the refilling process.

Effects on Ca^{2+} release Tissues were exposed to EGTA PSS (see Drugs and Solutions) for 15 min, followed by an initial exposure to NA (1 μM) for 8 min. They were then bathed in normal PSS for 25 min to refill the Ca^{2+} store. After washing in EGTA-PSS for 5 min, they were exposed to EGTA-PSS + cromakalim for a further 10 min. The preparations were then challenged again with NA (1 μM) for 8 min (in the continuing presence of EGTA-PSS) and the ensuing contraction was expressed as % of the initial contraction to NA in EGTA-PSS. Time-matched control experiments were also carried out in which no cromakalim was present prior to and during the second exposure to NA (1 μM). To assess any possible effect of nifedipine (0.3 μM) which was normally present in the EGTA-PSS, on the tension changes produced by NA (1 μM), the responses to NA (1 μM) in EGTA-PSS obtained using the above experimental design were compared to those elicited by NA (1 μM) in EGTA-PSS containing no nifedipine.

$^{42}K^+$ efflux experiments

Endothelium-free strips of rabbit aorta were attached to a perspex gassing manifold and after a 15 min period in PSS, they were loaded with $^{42}K^+$ (1.57 μCi ml^{-1}) for 180 min. $^{42}K^+$ was then allowed to efflux from the tissues into 3 ml aliquots of PSS during 2 min collection periods. After 8 such periods tissues were exposed to PSS alone, PSS containing cromakalim, (0.3, 1, 3 or 10 μM), NA (0.3 μM), or NA (0.3 μM) + nifedipine (0.3 μM) for the next five collection periods. For the last four collection periods the tubes contained PSS alone. The tissues were blotted and their $^{42}K^+$ content was determined together with that in the collecting tubes by gamma counting. The efflux data were expressed in terms of the rate coefficient (fractional loss of $^{42}K^+$ from the tissue standardized for a 1-min period) expressed as a percentage.

Microelectrode experiments

A segment of endothelium-free aorta was prepared as already described. It was placed, intimal side uppermost, in a recording chamber through which PSS was flowing at a rate of 2 ml min^{-1}. The tissue was fixed in the Sylgard floor of the chamber with fine pins and allowed to equilibrate for 1 h. Glass microelectrodes were inserted into the smooth muscle layer through the internal elastic lamina. When the tissue was bathed in normal PSS or in PSS plus cromakalim, impalements of many minutes duration were possible. However, when tissues were exposed to PSS containing either NA or KCl, the electrode was always rapidly dislodged. Thus to assess membrane potential changes under these conditions, a serial penetration technique was employed. Prior to exposure to NA or KCl, five control impalements were made. After a stable recording of at least 1 min, the electrode was deliberately withdrawn and the resulting deflection taken as the indicator of resting membrane potential. Tissues were then exposed to either NA or KCl and penetrations were made during the 5th and 15th min of exposure to these agents. In tissue bath experiments, maintained, tonic contractions to NA and KCl became established during this time period. Only stable recordings which lasted for at least 1 min in the presence of NA or KCl were considered reliable enough for subsequent analysis.

Drugs and solutions/Statistical analysis

The following drugs were used (±)-cromakalim (Beecham), stock solution in 70% ethanol; (−)-noradrenaline bitartrate (Sigma), stock solution in 0.1 M HCl; nifedipine (Bayer), stock solution in absolute ethanol (protected from light); glibenclamide (Hoechst), stock solution in absolute ethanol. Dilutions were made in twice distilled water.

The compositon of the physiological salt solution (PSS) was as follows (mM): NaCl 129.7, KCl 5.9, $CaCl_2$ 2.54, $MgCl_2$ 1.19, MOPS 10 and glucose 11.1. The pH of this solution was adjusted to 7.4 while being maintained at 37°C and gassed with 100% O_2.

The EGTA-PSS had the following composition (mM): NaCl 129.7, KCl 5.9, $MgCl_2$ 10, MOPS 10, glucose 11.1, EGTA 2, and nifedipine 0.0003. EGTA was dissolved in NaOH and added to the PSS. The pH of the solution was then adjusted to 7.4 while being maintained at 37°C and gassed with 100% O_2. In some experiments, nifedipine was omitted from this solution.

When KCl was used as the spasmogen, the stated concentration excludes the KCl (5.9 mM) already present in the PSS.

Experiments with nifedipine were performed under sodium light.

Differences in tissue responses were assessed by either a two-tailed unpaired Student's t test or by two-factor Analysis of Variance (AV). Where appropriate, values are quoted as means ± s.e.mean.

Results

Effect of nifedipine and cromakalim on phasic and tonic responses to noradrenaline

In rabbit aorta, NA (0.0125–7.8 μM) produced a concentration-dependent phasic tension increase which was not significantly inhibited by pre-incubation for 40 min with either cromakalim (0.3–10 μM) or nifedipine (0.03 μM) (Figure 1) (AV; $P > 0.05$, $n = 4$–5). Pre-incubation with higher concentrations of nifedipine (0.1–0.3 μM) produced significant reduction of responses to NA at the upper end of the concentration range (Figure 1).

In contrast to its effects on the initial phase of NA-induced contraction, cromakalim (0.3–10 μM) produced a slowly developing inhibition of the established tonic phase of contraction induced by NA (0.1–0.3 μM) (Figure 2). In these experiments a 10 min contact time with each concentration of cromakalim was allowed. However, this was insufficient to allow full expression of the inhibitory effects of cromakalim. Full relaxation of a NA (0.3 μM)-induced contraction could be achieved after exposure to cromakalim (0.3 μM) after 75 ± 11 min (mean ± s.e.mean, $n = 4$). Nifedipine was without effect on the tonic phase of NA-induced contractions (Figure 2).

Antagonism by glibenclamide

The ability of cromakalim to relax the tonic phase of NA (0.3 μM)-induced contractions was antagonized by pre-incubation of the tissues with the K^+-channel blocking agent glibenclamide (0.1–1 μM) in a concentration-dependent manner (Figure 2). In the presence of glibenclamide (1 μM) the maximum relaxant effect of cromakalim was reduced by 52 ± 4% (mean ± s.e.mean, $n = 6$).

Antagonism by high $[K^+]$

Contractions to NA (0.3 μM) and inhibition of these mechanical responses by cumulative application of cromakalim (0.1–10 μM) were not significantly affected by pre-incubation with nifedipine (0.3 μM) (see Figure 3). In the presence of nifedipine, the magnitude of the NA-induced contraction was 2.0 ± 0.2 g ($n = 9$). The mechanical response to KCl (40 mM) was reduced from 2.5 ± 0.23 g to 0.36 ± 0.06 g ($P < 0.01$, $n = 5$), in the presence of nifedipine (0.3 μM), indicating effective blockade of Ca^{2+}-activated K^+-channels. In such depolarized conditions, the total contraction developed by nifedipine pre-treated tissues on exposure to NA (0.3 μM) was 2.4 ± 0.4 g, $n = 4$, a value not different from that produced by NA in the absence of KCl ($P > 0.05$). Subsequent exposure to increasing concentrations of cromakalim (0.1–10 μM) failed to produce any relaxation of an established NA contraction in the presence of nifedipine + KCl (Figure 3).

Effect of nifedipine and cromakalim on phasic and tonic responses to KCl

KCl (10–80 mM) produced a concentration-dependent contraction of rabbit aortic strips, an effect which was not inhibited by pre-incubation with cromakalim up to 10 μM (Figure 1). Although mean responses to low concentrations of KCl (10–20 mM) were reduced by cromakalim (10 μM), this effect was not significant (AV; $P > 0.05$, $n = 6$) (Figure 1). Pre-incubation with nifedpine (0.03–0.3 μM) significantly depressed KCl-induced contractions (Figure 1). Similarly, nifedipine relaxed the maintained tonic contraction produced by KCl (40 mM)

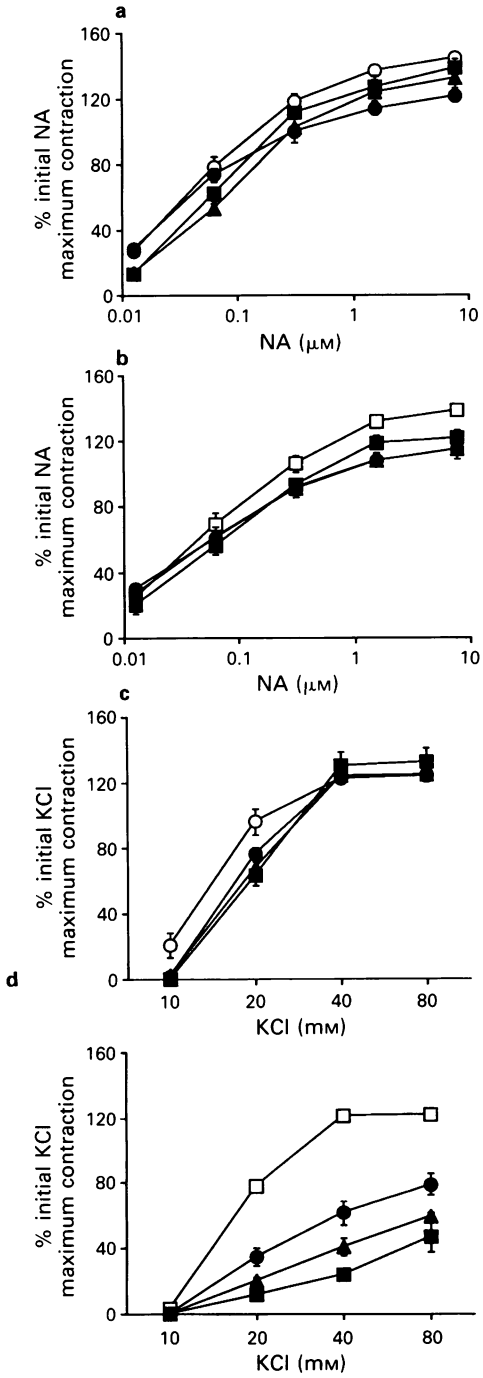


Figure 1 Effect of cromakalim (a, c) or nifedipine (b, d) on phasic contractions produced by cumulative application of noradrenaline (NA; a, b) or KCl (c, d). (a, c): Control responses (○); responses in the presence of cromakalim 0.3 μM (●), 3 μM (▲) 10 μM (■). (b, d) Control responses (□); responses in the presence of nifedipine 0.03 μM (●), 0.1 μM (▲), 0.3 μM (■). Ordinate scales; % of the initial control maximum contraction to NA or KCl. Each point is the mean derived from 4–5 experiments with s.e.mean shown by vertical bars. Some s.e. bars have been omitted for clarity. No significant changes in response to either NA or KCl were detected in concurrent, time-matched control experiments.

whereas cromakalim was without effect on these contractions ($P > 0.05$, $n = 4$, data not shown).

Effects in Ca^{2+}*-free PSS (EGTA-PSS)*

In Ca^{2+}-free PSS, NA (1 μM) induced a rapidly-developing contraction which declined to a low, maintained level. A second exposure to NA induced only a small, but maintained contraction. Subsequent addition of 1 mM Ca^{2+} to the PSS

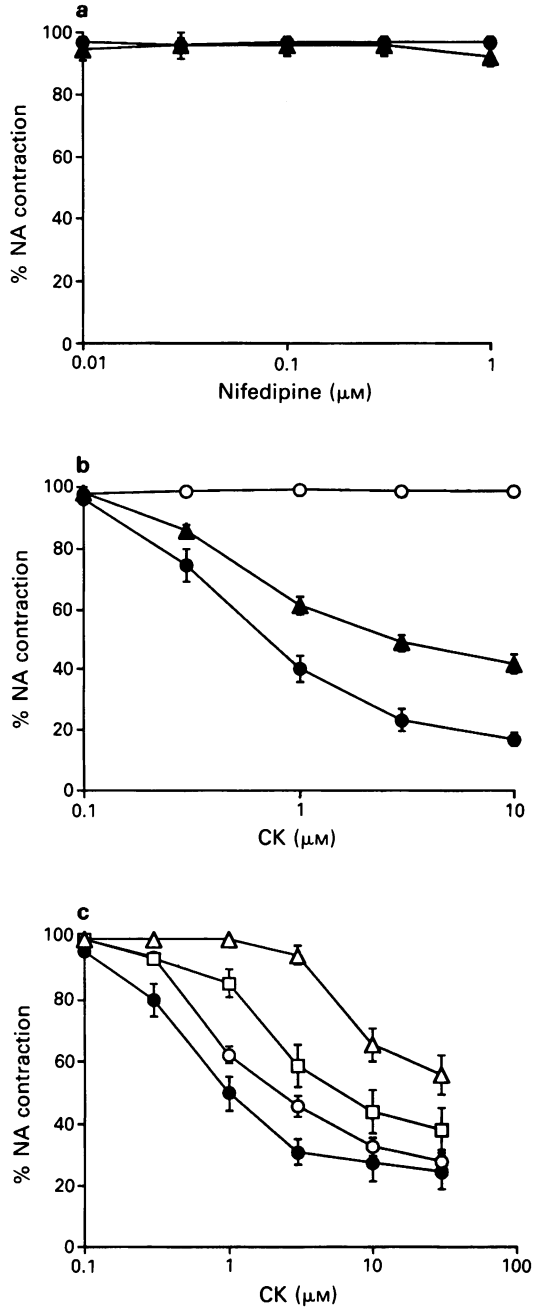


Figure 2 Effects of (a) nifedipine and (b) cromakalim (CK) on the established tonic phase of contractions produced by noradrenaline (NA) 0.1 μM (●) or 0.3 μM (▲). CK solvent control (○). (c) Relaxation of tonic contractions to NA 0.1 μM by CK alone (●) and in the presence of glibenclamide 0.1 μM (○), 0.3 μM (□), 1 μM (△). Ordinate scales: % of control NA contraction. Each point is the mean derived from 4–6 experiments with s.e.mean shown by vertical bars. No significant time-dependent relaxation of NA tonic contractions was detected in concurrent, time-matched control experiments.

produced a time-dependent return of NA contractions to control levels. This was interpreted as a measure of the time-dependent refilling of the NA-sensitive Ca^{2+} store. When cromakalim (10 μM) was included in the PSS, the rate of store refilling (measured as the magnitude of the subsequent NA-induced contraction) was slowed and the extent to which the store could be refilled was markedly inhibited (Figure 4). In contrast, nifedipine alone (in the EGTA-PSS) had no effect on the subsequent NA-induced contraction (Figure 4).

When tissues with full Ca^{2+} stores were exposed to cromakalim (10 μM), subsequent responses to NA in the continuing presence of cromakalim were significantly inhibited (t test, $P < 0.05$, $n = 4$), whereas exposure to nifedipine (0.3 μM) alone

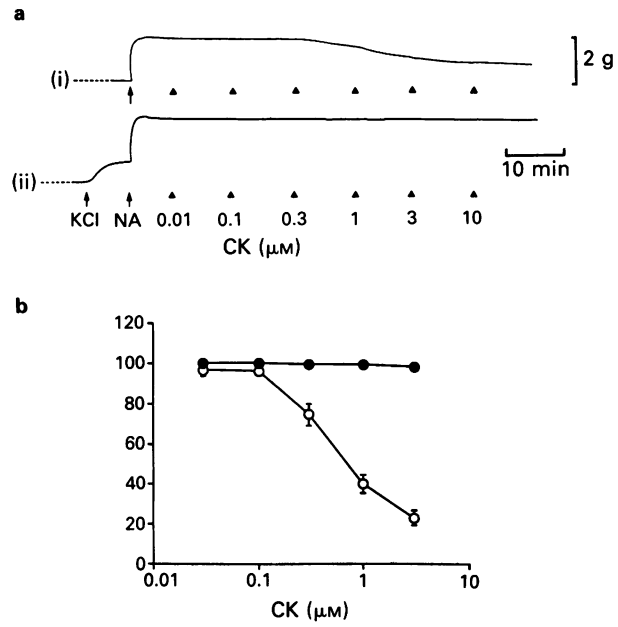


Figure 3 (a) Effects of cromakalim (CK) on the established tonic contraction produced by noradrenaline (NA; 0.3 μM) in (i) normal PSS and (ii) PSS containing KCl 40 mM in a single experiment. (i) The tissue was exposed to nifedipine (0.3 μM) for 55 min followed by NA (0.3 μM for 8 min. CK was then added in the continuing presence of nifedipine + NA using a cumulative protocol. (ii) A separate tissue was exposed to nifedipine (0.3 μM) for 40 min followed by KCl (40 mM) for 15 min and NA (0.3 μM) for 8 min. CK was then added in the continuing presence of nifedipine + KCl + NA using a cumulative protocol. (b) Summary of data derived from the experiments described in (a) in normal PSS + nifedipine (○) and in PSS containing KCl 40 mM + nifedipine (●). Each point is the mean of 4–6 observations; vertical lines shown s.e.mean values.

was again without effect (control (no nifedipine), 108.0 ± 4.3%; control (with nifedipine), 105.9 ± 3.6%; cromakalim, 83.9 ± 3.1%).

Effects on $^{42}K^+$ efflux

Cromakalim (0.3 μM) produced a small but significant increase in the efflux of $^{42}K^+$ from rabbit aorta which was maintained during the period of drug exposure (Figure 5a). Higher concentrations of cromakalim (1 and 10 μM) produced a rapid concentration-dependent increase in the efflux of $^{42}K^+$ which

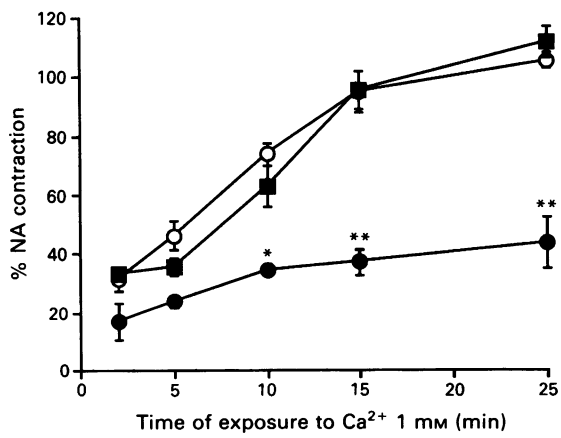


Figure 4 Effect of cromakalim or nifedipine on the time-dependent refilling of noradrenaline (NA)-sensitive Ca^{2+} stores. Ordinate scale: % of contraction to NA (1 μM) in EGTA-PSS. Tissues were exposed to 1 mM Ca^{2+}-PSS containing either ethanol (control, ○), nifedipine 0.3 μM (■), or cromakalim 10 μM + nifedipine 0.3 μM (●) for various exposure times (2, 5, 10, 15, or 25 min). Each point is the mean derived from 4 experiments with s.e.mean shown by vertical bars. Significant differences between test and control using two-factor Analysis of Variance: * $P < 0.05$; ** $P < 0.01$.

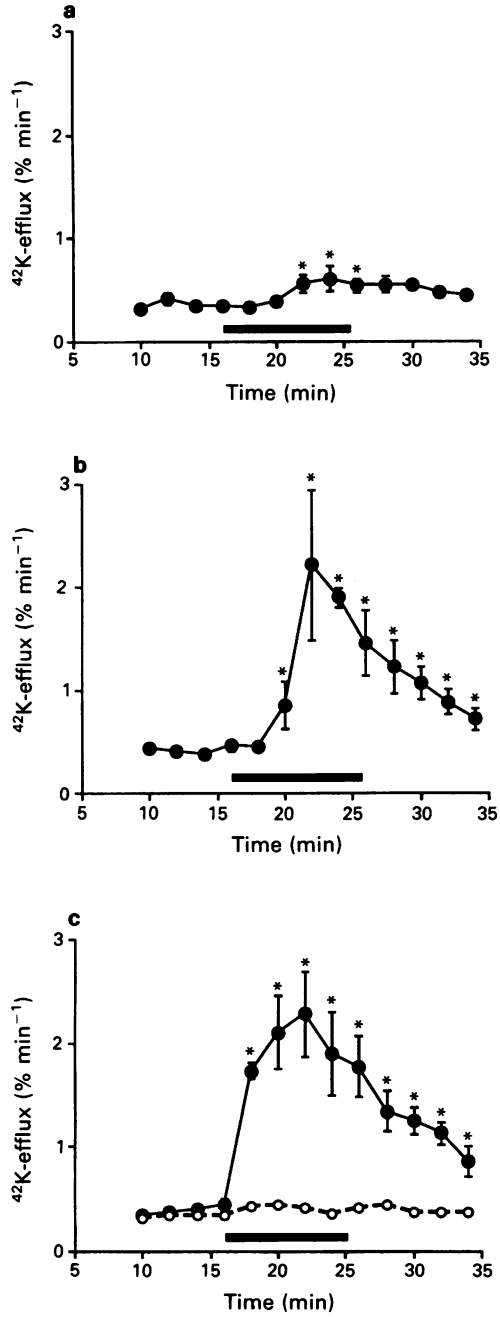


Figure 5 Effect of cromakalim on the loss of $^{42}K^+$ from rabbit aortic strips. Ordinate scale: $^{42}K^+$ efflux rate coefficient expressed as % min^{-1}. Abscissa scale: time (min) after start of efflux period. (a–c) Tissues were exposed to cromakalim 0.3 μM, 1 μM and 10 μM respectively between the 16th and 26th min of the efflux period (■). Broken line and open symbols in (c) show $^{42}K^+$ efflux from control tissues. Each point is the mean derived from 8 experiments (4 animals): vertical bars show sample s.e.mean values. Significant differences between test and control $^{42}K^+$ efflux using two-factor Analysis of Variance; * $P < 0.05$.

was not well maintained but remained significantly above control values during exposure to cromakalim (Figures 5b,c). NA (0.3 μM) also produced an increase in the loss of $^{42}K^+$ from rabbit aorta, an action that was unaffected by pre-incubation with nifedipine (0.3 μM) (Figure 6).

Effects on membrane potential

Insertion of a microelectrode into the smooth muscle cells of rabbit aorta through the internal elastic lamina (IEL) was quite difficult and the electrode often broke before the smooth muscle layer was reached. However, when the IEL had been successfully penetrated, stable recordings of several minutes' duration were possible (Table 1). On exposure to cromakalim (1–10 μM), a slowly-developing, concentration-dependent hyperpolarization was recorded and there was no sign of 'fade' during the continuing presence of cromakalim. No significant change in membrane potential was produced by cromakalim (0.3 μM) (Table 1). Exposure to NA (0.03–3 μM) during a successful impalement always dislodged the electrode within less than 1 min. Although mechanical changes were not monitored during intracellular recording, it was assumed that muscle contraction resulted in cell/electrode damage. By use of a serial pentration technique (see Methods), no significant change in membrane potential in the presence of NA (0.03–3 μM) was detected (Table 1). In some experiments, tissues were exposed to cromakalim in the presence of NA. With the serial penetration method, cromakalim (0.3 μM) did not produce membrane hyperpolarization whereas cromakalim (3 μM) increased membrane potential to a value similar to that seen in the absence of NA (Table 1). In the presence of raised [K^+] (20 or 80 mM), a marked membrane depolarization was recorded (Table 1).

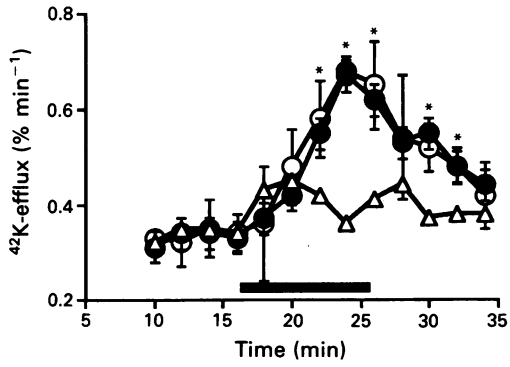


Figure 6 Effect of noradrenaline (NA) in the presence or absence of nifedipine on the efflux of $^{42}K^+$ from rabbit aorta. Ordinate scale: $^{42}K^+$ efflux rate coefficient expressed as % min^{-1}. Abscissa scale: time (min) after start of efflux period. Tissues were exposed to either vehicle control (△), NA 0.3 μM (○) or NA 0.3 μM + nifedipine 0.3 μM (●) between the 16th and 26th min of the efflux period (■). Each point is the mean derived from 8 experiments (4 animals): vertical lines show sample s.e.mean values. Significant differences between test and control $^{42}K^+$ efflux using two-factor Analysis of Variance; * $P < 0.05$.

Table 1 Effect of cromakalim (CK), noradrenaline (NA) and potassium chloride (KCl) on membrane potential in rabbit aorta

Treatment	*Membrane potential* (mV)	
Control	−51 ± 1	(130:26)
CK 0.3 μM	−54 ± 3	(6:4)
CK 1 μM	−64 ± 3	(6:4)
CK 3 μM	−71 ± 2	(7:5)
CK 10 μM	−80 ± 3	(6:5)
NA 0.03 μM	−53 ± 1	(16:4)
NA 0.1 μM	−53 ± 3	(8:2)
NA 0.1 μM + CK 0.3 μM	−54 ± 3	(8:2)
NA 0.3 μM	−50 ± 2	(25:5)
NA 0.3 μM + CK 0.3 μM	−51 ± 4	(25:5)
NA 0.3 μM + CK 3 μM	−68 ± 5	(10:3)
NA 3 μM	−49 ± 3	(20:4)
NA 3 μM + CK 0.3 μM	−50 ± 5	(20:4)
NA 3 μM + CK 3 μM	−71 ± 7	(20:4)
KCl 20 mM	−44 ± 2	(20:4)
KCl 80 mM	−29 ± 3	(20:4)

Figures in parentheses show number of determinations: number of preparations. Recordings in the presence of NA or KCl were made 5–15 min after initial exposure of these spasmogens.

Discussion

In rabbit aorta the characteristics of NA-induced contractions have been widely studied and it is currently believed that the increase in tension in normal PSS comprises two distinct phases. During the initial exposure to NA the initial phasic contraction results from intracellular Ca^{2+} release following an increase in the turnover of phosphatidylinositol (Villalobos-Molina *et al.*, 1982) and the production of inositol 1,4,5-trisphosphate. In the continuing presence of NA, contraction is maintained and this phase is associated with Ca^{2+}-influx through pathways insensitive to Ca^{2+}-entry blocking drugs (Lukeman & van Breemen, 1985). Neither of the contractile phases is accompanied by depolarization (Cauvin *et al.*, 1984).

In the present study, neither cromakalim nor nifedipine significantly modified the cumulative dose-response relationship to NA. In such cumulative experiments, the contractile response largely comprises the phasic mechanical component believed to be associated with intracellular Ca^{2+} release. Thus, neither a K^+-channel opening drug like cromakalim (Hamilton & Weston, 1989) nor a Ca^{2+}-entry blocking drug like nifedipine would be expected to modify this phase of contraction. In contrast, when the contraction to NA was allowed to develop into its sustained (tonic) phase, cromakalim produced a concentration-dependent relaxation of NA-induced tone, whereas nifedipine was still without effect. Similar effects have been reported by Cook *et al.* (1988). The threshold inhibitory concentration of cromakalim was approximately 0.3 μM and exposure to this concentration produced a slowly-developing and complete inhibition of the NA-induced contraction within approximately 75 min. The concentrations of nifedipine used in this study should have produced selective blockade of L-type voltage-operated Ca^{2+}-channels (Bean, 1985) and these results thus suggest that Ca^{2+}-influx through L-type voltage-operated Ca^{2+}-channels is not required for the maintenance of NA-induced tone in rabbit aorta.

In the presence of glibenclamide, the relaxant effects of cromakalim on the maintained phase of NA-induced contractile responses were inhibited, suggesting that the relaxation was associated with the opening of K^+-channels. In rat portal vein glibenclamide antagonizes the relaxant responses to minoxidil sulphase and diazoxide (Newgreen *et al.*, 1990), agents which have previously been shown to open K^+-channels in vascular smooth muscle (Bray *et al.*, 1988a; Meisheri *et al.*, 1988). In the present study the threshold concentration of glibenclamide required for antagonism was 0.1–0.3 μM. This agrees with earlier reports which have also found antagonism of cromakalim by glibenclamide in vascular smooth muscle (Quast & Cook, 1988; Wilson, 1988; Winquist *et al.*, 1989).

Although potency differences exist between the concentrations of glibenclamide used in this study and those which have been shown to block the ATP-dependent K^+-channel in pancreatic β-cells (subnanomolar to nanomolar, Schmid-Antomarchi *et al.*, 1987) the identification of an ATP-dependent K^+-channel in smooth muscle (Standen *et al.*, 1989) which, in the presence of ATP is opened by cromakalim, strengthens the supposition that cromakalim may modulate the opening of an ATP-dependent K^+-channel in rabbit aorta.

To provide further evidence for the importance of K^+-channel opening in the cromakalim-induced inhibition of the NA-induced tonic contractions, preparations were depolarized by increasing the extracellular K^+ concentration to 40 mM. Although membrane potential recordings were not performed with this specific concentration of KCl, it can be presumed that the membrane potential was reduced to the region between -44 mV and -29 mV (see Table 1). Under these conditions cromakalim lost its ability to inhibit the sustained phase of NA-induced contractions. Since E_k under these conditions is in the potential range in which L-type Ca^{2+}-channels are activated (Bolton *et al.*, 1984), a K^+-channel opener like cromakalim is unable to relax a tissue by the indirect closure of such channels (Hamilton *et al.*, 1986). Such data further suggest that the opening of K^+-channels is involved in the inhibition by cromakalim of the tonic phase of NA-induced contractions.

To determine more directly the importance of hyperpolarization in the cromakalim-induced relaxation of the sustained phase of NA contractions, the NA-cromakalim interaction was also studied by both microelectrode and $^{42}K^+$ efflux techniques. Exposure to NA alone produced no detectable change in membrane potential, supporting the previous findings of Cauvin *et al.* (1984). Such a result suggests that L-type Ca^{2+}-channels are not involved in this phase of NA contractions. It is also consistent with the findings of the present study that nifedipine was unable to modify the tonic component of the NA contraction. The reason for the lack of depolarization is unclear, but may reflect the fact that in large tonically active arteries such as rabbit aorta any NA-induced activation of channels mediating inward current may be minimized by an outwardly-directed Ca^{2+}-dependent K^+-current activated by the increased cytosolic levels of Ca^{2+} initially observed following receptor stimulation. This is supported by the observation that NA increased $^{42}K^+$ efflux from aortic segments, an effect that was not antagonized by nifedipine.

Failure to observe NA-induced depolarization in the present study and in that of Cauvin *et al.* (1984) contrasts with the observations of Haeusler & De Peyer (1989). The reasons for this discrepancy are unclear. However, in the present study, exposure to NA for 5–15 min failed to produce detectable membrane potential changes in tissues in which the electrical effects of cromakalim or KCl had been successfully determined. This suggests that poor tissue viability or recording technique did not contribute to the lack of NA-induced membrane potential changes.

Cromakalim itself produced a concentration-dependent hyperpolarization of the smooth muscle membrane with a threshold concentration in the range 0.3–1 μM. In separate experiments cromakalim (0.3–10 μM) produced a significant increase in $^{42}K^+$ efflux. Cromakalim (0.3 μM) produced a slowly-developing full relaxation of NA contractions at a concentration that did not cause membrane hyperpolarization, but at which there was a small increase in the efflux of $^{42}K^+$. The relaxant response produced by low concentrations of cromakalim was inhibited by glibenclamide and by raising extracellular $[K^+]$ to 40 mM, consistent with a cromakalim-induced opening of K^+-channels. The inability to detect significant hyperpolarization in the presence of cromakalim (0.3 μM) cannot be satisfactorily explained at present. It could indicate an insensitivity in the microelectrode recording technique or the simultaneous opening of an ion channel carrying inward current. Further experiments are required to resolve this paradox.

In order to characterize further the inhibitory action of cromakalim, a series of experiments in Ca^{2+}-free PSS was undertaken. Removal of Ca^{2+} from the PSS with the addition of EGTA to chelate any residual Ca^{2+} is a widely-adopted procedure to assess the role of extracellular Ca^{2+} in agonist-induced responses. However, such a procedure produces membrane instability leading to a marked increase in K^+ permeability (Martin & Gordon, 1983; Kreye & Weston, 1986). A marked membrane depolarisation also occurs (Weston, unpublished data). Addition of Mg^{2+} to the PSS greatly reduces membrane excitability (Martin & Gordon, 1983) and this procedure was thus adopted for experiments in which Ca^{2+}-free conditions were employed.

In Ca^{2+}-free (Mg^{2+}-rich) PSS, NA produced a transient increase in tension which was taken as an index of intracellular Ca^{2+} release. On repeated exposure to NA, the magnitude of this contraction decreased rapidly, suggesting that the Ca^{2+} store was of limited size and that re-sequestration of released Ca^{2+} was relatively inefficient (van Breemen *et al.*, 1972; Devine *et al.*, 1972). Re-exposure of tissues with empty Ca^{2+} stores to Ca^{2+} resulted in a time-dependent increase in the response to NA in Ca-free PSS. If, however, store refilling was carried out in the presence of cromakalim, the magnitude of

the NA response was reduced. Additionally when the store was refilled in the presence of cromakalim the magnitude of subsequent NA contractions was significantly less that that observed in control experiments. These data collectively suggest that cromakalim is capable of markedly inhibiting store refilling and/or Ca^{2+} sequestration with some additional inhibition of Ca^{2+} release from a full store. Further experiments currently in progress suggest that this effect of cromakalim on the refilling of NA-sensitive Ca^{2+} stores is dose-dependent with a threshold concentration of 0.3 μM.

An action of cromakalim on Ca^{2+} sequestration could be exerted intracelluarly at the store itself. In skeletal muscle Fink & Stephenson (1987) demonstrated that reducing K^+ conductance in the sarcoplasmic reticulum, either directly or indirectly, increased the ability of the sarcoplasmic reticulum to load Ca^{2+}. Thus it is possible that reversal of this intracellular process, namely increasing the K^+-conductance of the sarcoplasmic reticulum (with cromakalim) may reduce its ability to load Ca^{2+}, if it is assumed a similar process exists in smooth muscle. In other tissue preparations, such as liver cells (Muallem *et al.*, 1985) and brain microsomes (Shah & Pant, 1988), a role of K^+ has been demonstrated during the Ca^{2+} loading and release processes, with K^+ acting to compensate charge flow to allow Ca^{2+} uptake/release into/from Ca^{2+} stores. A K^+-channel opener such as cromakalim may, therefore, increase the permeability of the smooth muscle sarcoplasmic reticulum to K^+, making it leaky and thus compromising the ability of the sarcoplasmic reticulum to load Ca^{2+}.

The results of the present study have clearly shown that cromakalim, in contrast to nifedipine, can inhibit NA-induced contractions in rabbit aorta. These effects were antagonized by glibenclamide and by raising extracellular $[K^+]$, events consistent with cromakalim-induced K^+-channel opening. However, the slowly developing inhibitory effects of low concentrations of cromakalim were not accompanied by membrane hyperpolarization. Since the results of the present study have indicated that cromakalim can retard and inhibit the refilling of intracellular Ca^{2+} stores, it is tempting to speculate that such an action could be responsible for at least some of the cromakalim-induced inhibition of NA tonic contractions. If the smooth muscle Ca^{2+} store does resemble that in skeletal muscle, cromakalim may exert some of its effects on the Ca^{2+}:K^+ exchange mechanism within that store (Fink & Stephenson, 1987). In this way a small increase in $^{42}K^+$ efflux from the cell might be produced without any change in plasmalemmal K^+ permeability.

In the concentration range 1–10 μM, cromakalim produced a marked membrane hyperpolarization with a corresponding increase in $^{42}K^+$ efflux. However, over its spasmogenic concentration-range, NA produced no change in membrane potential. It thus seems unlikely that a cromakalim-induced hyperpolarization with consequential closure of L-type Ca^{2+} channels (Hamilton & Weston, 1989) plays a significant role in the observed relaxation in rabbit aorta precontracted with NA. Furthermore, the failure of nifedipine to modify any phase of the NA-induced contraction emphasizes that dihydropyridine-sensitive Ca^{2+}-channels play no role in this mechanical response. It is thus suggested that cromakalim closes the pathway involved in supplying Ca^{2+} from the extracellular fluid to refill the intracellular Ca^{2+} store and to maintain the NA-induced contraction. Further studies are in progress to test this possibility.

This study was supported by Rhone-Poulenc (J.L.). K.M.B., S.D. and D.T.N. were in receipt of SERC Case Awards. G.E. was supported by Pfizer Central Research and J.L. by Rhône-Poulenc.

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(Received February 14, 1990
Revised September 19, 1990
Accepted September 25, 1990)

Br. J. Pharmacol. (1991), **102**, 345–350 © Macmillan Press Ltd, 1991

Pharmacokinetic characterization of phosphatidylserine liposomes in the rat

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1 The plasma decay, tissue uptake and biotransformation of radiolabelled phosphatidylserine (PS) liposomes have been investigated in rats following bolus i.v. injection (2 mg kg^{-1}).

2 PS plasma concentration showed a biexponential decay with half-lives of 0.85 and 40 min. The following interpretation of the biphasic decay is proposed: (1) The rapid initial decline is due to the irreversible uptake of PS liposomes by the mononuclear phagocyte system, as demonstrated by the almost exclusive accumulation of PS in liver and spleen. (2) The slow decay phase reflects the elimination of that fraction of PS that has been incorporated into high density plasma lipoproteins (HDL). A kinetic model has been developed to describe these phenomena and a good agreement has been observed between experimental data and theoretical values.

3 Evidence has been obtained that a large fraction of PS is hydrolyzed at the injection site, probably by phospholipase A_2 and other hydrolytic enzymes released by platelets. Hydrolysis at the injection site has also been observed following intraperitoneal and intramuscular injections.

4 As shown by the comparative analysis of the biotransformation products found in tissues after administration of either [^{3}H]-glycerol-PS or [^{14}C]-serine-PS, parenterally administered PS follows two distinct metabolic pathways: (1) decarboxylation to phosphatidylethanolamine and (2) extensive hydrolytic degradation with release of the individual components of the molecule. These pathways probably reflect the two main mechanisms of PS uptake, incorporation into the plasma membrane and internalization by endocytosis, respectively.

Introduction

Our initial studies (Bruni *et al.*, 1976; Bigon *et al.*, 1979a,b) have shown that parenteral administration of phosphatidylserine (PS) to rodents induces complex pharmacological effects. These are believed to reflect a pathophysiological role of PS and its lysoderivative in the immune and the inflammatory responses (Mietto *et al.*, 1987). Consistently, macrophages, lymphocytes and mast cells have been shown to be target cells for serine phospholipids *in vivo* and *in vitro*. The interaction of PS with macrophages appears to be mediated by the scavenger receptor (Nishikawa *et al.*, 1990). As a consequence, cells exposing PS at the external membrane surface are internalized and eliminated by macrophages (Tanaka & Schroit, 1983; Schroit *et al.*, 1985). PS uptake is not without consequences for macrophages as it has been demonstrated that ingestion of liposomes containing this phospholipid reduces the microbicidal activity against intracellular leishmanias (Gilbreath *et al.*, 1986). In lymphocytes, PS is incorporated by an energy-dependent process which might involve endocytosis, internalization by the translocator for the aminophospholipids or a deacylation-reacylation cycle (Mietto *et al.*, 1989). Decreased secretion of interleukin-2 follows PS incorporation (Ponzin *et al.*, 1989). Unlike macrophages and lymphocytes, mast cells undergo activation after the interaction with PS and lysoPS. The secretion of mast cell mediators is the result of phosphoinositidase C activation (Bellini *et al.*, 1988; 1990). The *in vivo* disposition of phospholipid liposomes supports the possibility of an action of PS on the immune system. Several studies have indeed shown that parenteral administration of these lipid structures is followed by their accumulation in organs (liver, spleen, lungs) which are rich in immunocompetent cells (reviewed by Poste, 1983). However, the pharmacokinetic properties of liposomes have been thus far investigated in relation to their use as drug carriers. For this reason only the disposition of long-lived liposomes, such as those containing phosphatidylcholine (PC), sphingomyelin (SPH) or cholesterol as basic components, has been extensively studied (Gregoriadis & Neerunjun, 1974; Allen & Everest, 1983; Gabizon & Papahadjopoulos, 1988 and references therein). In spite of their intrinsic pharmacological activity, liposomes made up exclusively of PS have not yet been characterized from a pharmacokinetic point of view. The purpose of this study is to provide essential kinetic data pertaining to the intravenous administration of PS liposomes in the rat.

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Methods

Phosphatidylserine

Conventional preparations of bovine brain PS (Bigon *et al.*, 1979a) were further purified on DEAE-cellulose to remove a small amount of phosphatidylinositol (Rouser *et al.*, 1969). The phospholipid was stored at −20°C as a solution in chloroform-methanol (2:1, v/v) at a concentration of 10–20 mg ml^{-1}, quantified by periodic phosphorus determinations. Phosphatidyl-[^{14}C]-serine (160 mCi mmol^{-1}) contained 4% of hydrosoluble radioactivity and 1% of lysoPS (Orlando *et al.*, 1980). Experiments were also done with a preparation of [^{3}H]-glycerol-PS (13 mCi mmol^{-1}), kindly donated by Dr P. Orlando, Catholic University, Rome. The radiochemical purity was 96%. The desired amounts of unlabelled and labelled PS were mixed in organic solvent and taken to dryness under a stream of nitrogen. Hydration of PS in the form of multilamellar vesicles was obtained by the addition of 50 mM Tris HCl, pH 7.8 (50 mM HEPES KOH or 10 mM phosphate buffer were equivalent). The liposome size was decreased by 4 cycles of sonication (30 s each, tip probe sonicator). The average size of the vesicles was 140 nm, when tested by dynamic light scattering using a nano sizer apparatus (Coulter Electronics). PS vesicles were then used immediately to avoid aggregation.

Experimental protocol

Male albino rats (Charles River, Sprague-Dawley of 250–350 g) were used for this study. In most experiments the PS

dispersion was injected into the exposed jugular vein as a bolus dose of 2 mg kg^{-1}, after pentobarbitone anaesthesia (45 mg kg^{-1}, i.p.). In some experiments PS was also injected into the tail vein without anaesthesia. Heparinized blood samples (0.2 ml) were removed by heart puncture at 0.5, 1.5, 3, 5, 10, 15, 30, 45 and 60 min after injection. Blood sampling was started as early as 0.5 min after injection since preliminary experiments revealed that the disappearance of PS from plasma was extremely rapid (about 75% removed from plasma within 30 s) and the initial decay phase would have been largely missed if sampling were started at a later time.

The organs were sampled, weighed and extracted 60 min after PS injection. Since at this time only a minimal amount of PS was present in the circulation no attempts were made to remove the blood from tissues completely. Correction of tissue radioactivity levels using the blood background correction factors of Hwang *et al.* (1980) showed that the contribution to tissue radioactivity from residual blood content was in fact negligible.

Phospholipids were extracted from plasma and tissues immediately after sampling, by 20 volumes of chloroform-methanol (2:1, v/v). The filtered lipid extract was washed with 0.2 volumes of 10 mM $CaCl_2$ and resolved by two dimensional thin layer chromatography (Punzi *et al.*, 1986). The radioactivity of the samples was determined in a Packard Tricarb 2200 CA liquid scintillation system, using the computerized automatic external standard for quench correction.

Pharmacokinetic analysis

The plasma decay of PS liposomes after bolus i.v. injection was analyzed by means of the kinetic model depicted in Figure 1. The basic assumption of the model is that exit of PS liposomes out of the plasma compartment is a unidirectional process. With the possible exception of very small unilamellar vesicles (less than 60 nm in diameter) phospholipid liposomes are unable to cross either continuous or fenestrated capillaries (Poste, 1983; Hwang & Beaumier, 1988). Their extravasation is limited to tissues containing sinusoidal capillaries and appears predominantly due to endocytosis by the local macrophages, although fusion with cell membranes, deacylation-reacylation or interaction with the transporter for the amino phospholipids may also be operative for PS (Bruni & Palatini, 1982; Mietto *et al.*, 1989). After internalization by endocytosis, phospholipid vesicles are degraded by lysosomal enzymes, whereas the other pathways result in the incorporation of liposomal phospholipids into the phospholipid pool of the cellular membrane. A significant incorporation of liposomal phospholipids occurs also in plasma by high density lipoproteins (HDL) (see, e.g. Scherphof *et al.*, 1978; Vidal *et al.*, 1984; William & Tall, 1988). When cholesterol is not included in liposomes (Damen *et al.*, 1981), or the concentration of HDL phospholipid is in excess over that of liposomes (Bienvenue *et al.*, 1985) phospholipid transfer from liposomes to HDL is an irreversible process, the rate of which depends solely on the liposomal phospholipid concentration. Phospholipids incorporated into HDL are then removed from plasma by reactions involving the uptake of the whole lipoprotein or the independent movement of the lipid moiety (Eisenberg, 1984). A metabolic elimination route from the plasma compartment is also included in the model, since PS was found to be hydrolyzed in the plasma of rats to lysoPS and hydrosoluble compounds (mainly serine). The model shown in Figure 1 is described by the following differential equations:

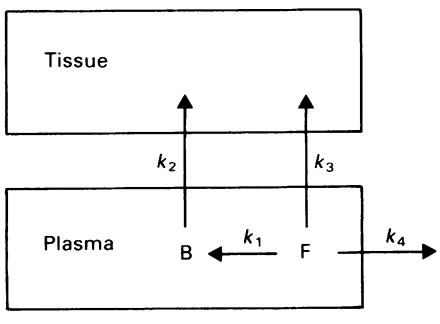


Figure 1 Pharmacokinetic model for phosphatidylserine (PS) disposition. F and B designate free PS (intact liposomes) and high density plasma lipoproteins (HDL)-bound PS, respectively. All rate processes represented are assumed to be first order. k_1 to k_3 are the apparent rate constants for incorporation of PS into HDL, elimination from plasma of HDL-bound PS and tissue uptake of PS liposomes, respectively; k_4 is the sum of all apparent rate constants for the formations of PS metabolites in plasma.

$$\frac{d[F]}{dt} = -K[F] \quad (1)$$

where $K = k_1 + k_3 + k_4$, and F designates free PS (intact liposomes).

$$\frac{d[B]}{dt} = k_1[F] - k_2[B] \quad (2)$$

where B indicates PS bound to HDL.

The solution for equation 1 is:

$$[F] = C_0 e^{-Kt} \quad (3)$$

where C_0 is the zero-time concentration of PS. Substituting equation 3 into equation 2 and solving for [B] yields

$$[B] = \frac{C_0 k_1}{K - k_2}(e^{-k_2 t} - e^{-Kt}) \quad (4)$$

The total concentration of PS in plasma, C = [F] + [B], is given by

$$C = C_0\left(\frac{K - k_1 - k_2}{K - k_2} e^{-Kt} + \frac{k_1}{K - k_2} e^{-k_2 t}\right) \quad (5)$$

PS plasma concentration-time data were fitted to equation 5 by a weighted ($1/C^2$), computer-assisted, non-linear least squares regression analysis.

Results

Kinetics of phosphatidylserine

Figure 2 illustrates the time course of the plasma concentrations of PS (a) and its degradation products (b) following bolus intravenous injection. The concentration of PS showed a biphasic decline with a very rapid initial decay (Figure 2a). Assuming a plasma volume of 40 ml kg^{-1} (Hwang *et al.*, 1980) it could be calculated that 93% of the injected phospholipid was removed from plasma within 3 min. Separate controls showed that there was not significant PS uptake by the blood cells, thereby making it unlikely that the rapid decline was due to adsorption of PS vesicles to the erythrocyte surface or uptake by circulating monocytes. The decay curve of the total liposoluble radioactivity was virtually superimposable to that of PS (results not shown), indicating no phospholipid loss during the chromatographic procedure. The main kinetic parameters of PS, calculated according to the model shown in Figure 1, are given in Table 1. The zero-time concentration, C_0, was lower than the expected value of $175.1 \pm 15.7 \times 10^3$ d.p.m. ml^{-1} for a compound that is not reversibly distributed outside the plasma. The data of Figure 2b may provide an explanation for this apparent discrepancy. It can be seen that the concentrations of lysoPS and hydrosoluble metabolites, abruptly increased at 30 s, decreased thereafter very quickly, in spite of the concurrent rapid disappearance of PS. This does not appear consistent with a continuous generation of metabolites, but is suggestive of a virtually instantaneous degrada-

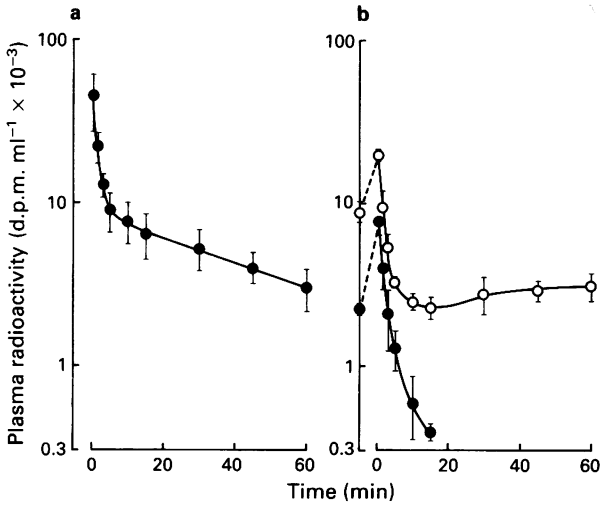


Figure 2 Plasma decay curves after bolus intravenous injection of 2 mg kg^{-1} of phosphatidyl-[^{14}C]-serine (3.5 × 10^6 d.p.m. mg^{-1} of PS). PS liposomes were injected into the jugular vein of anaesthetized rats (45 mg kg^{-1} of pentobarbitone, i.p.). Each point represents the mean from three rats; vertical bars show s.d. (a) Radioactivity associated with phosphatidylserine (PS). The continuous line represents the non-linear least squares regression fit to the data, based on equation 5. (b) Total hydrosoluble radioactivity (○); radioactivity associated with lysophosphatidylserine (●). Concentration data for lysoPS are shown up to the last measurable concentration. Since the injected [^{14}C]-serine-PS contained 4% of hydrosoluble radioactivity and 1% of radioactive lysoPS (see Methods), their initial concentrations, calculated by assuming a plasma volume of 40 ml kg^{-1} (Hwang *et al.*, 1980), are also indicated (points on the ordinate axis).

tion of PS at the injection site. The observation that the total radioactivity level after 30 s (PS plus metabolites = 73.4 ± 18.7 × 10^3 d.p.m. ml^{-1}) was higher than the zero-time concentration of PS (C_0) confirms this hypothesis, thereby explaining the unusually rapid initial disappearance of PS from plasma. A similarly rapid initial decay of plasma PS concentration has been previously observed by Kao & Loo (1980). Although the injected vesicles also contained cholesterol which has a stabilizing effect, 90% of PS was found to be removed from plasma within 4 min. Since the surgical exposure of the jugular vein might favour platelet aggregation with release of hydrolytic enzymes, the phospholipid was also injected into the tail vein. By this route similar kinetic constants for PS were obtained. The initial accumulation of metabolites was somewhat reduced, but not abolished (e.g., radioactivity associated with lysoPS at 30 s was 5.5 ± 3.0 × 10^3 d.p.m. ml^{-1}, in comparison to 7.9 ± 1.4 × 10^3 d.p.m. ml^{-1} after injection into the jugular vein; means ± s.d. from 4 and 3 rats, respectively). *In situ* degradation of PS was also observed after intraperitoneal or intramuscular (posterior leg) injection.

Table 1 Kinetic parameters of phosphatidylserine-[^{14}C]-serine (PS) after bolus i.v. administration of 2 mg kg^{-1}

C_0 (d.p.m. ml^{-1} × 10^{-3})	52.90 ± 15.24
K (min^{-1})	0.85 ± 0.21
$t_{1/2K}$ (min)	0.85 ± 0.23
k_2 (min^{-1})	0.017 ± 0.002
$t_{\frac{1}{2}k_2}$ (min)	40.00 ± 6.10
k_1 (min^{-1})	0.11 ± 0.02

Parameters were obtained by fitting plasma concentration-time data to equation 5 (see Methods). Values are expressed as mean ± s.d. for three rats.
C_0 = zero-time concentration of PS; $K = k_1 + k_3 + k_4$, is rate constant for the disappearance of free PS from plasma; k_2 = rate constant for the elimination from plasma of HDL-bound PS; $t_{1/2K}$ and $t_{\frac{1}{2}k_2}$ are the corresponding half-lives; k_1 = rate constant for the incorporation of PS into HDL.

In either case lysoPS constituted 5–10% of the liposoluble radioactivity present at the injection site. Since degradation of PS at the injection site results in a decrease of the dose effectively administered and, consequently, in an overestimation of clearance and volume of distribution, these parameters were not shown in Table 1.

Tissue uptake and biotransformation

Table 2 shows the content of liposoluble and hydrosoluble radioactivity in plasma and five major organs 1 h after i.v. administration of PS. It can be seen that the largest excess of liposoluble radioactivity was present in liver and spleen, suggesting that, at least in these organs, unmodified PS was prevalently taken up. The liver accumulated the largest fraction of radiolabelled vesicles (95% of the amount recovered). Although the absolute amount of radioactivity in the spleen was low, due to the small size of this organ, its concentration was remarkably high, as previously observed with other types of liposomes (Allen & Everest, 1983). When the composition of the liposoluble radioactivity was examined (Table 3) it was found that PS constituted the largest fraction of the labelled phospholipids, followed by phosphatidylethanolamine (PE). This provided the first indication that part of the incorporated PS underwent decarboxylation (see below). By contrast, only negligible sphingomyelin labelling was observed, suggesting a minor uptake of free [^{14}C]-serine released in plasma from the injected PS. It is indeed known that serine is not incorporated exclusively into PS, but also into sphingolipids. Traces of lysoPS originating from PS hydrolysis and a small amount of lysoPE were also found in plasma. To exclude further a major contribution of free serine in the appearance of labelled PS or PE in tissues, control experiments were done with PS containing [^{3}H]-glycerol instead of [^{14}C]-serine (Table 4). The accumulation of liposoluble radioactivity in liver and spleen (45–66% of the dose) was similar to that found after administration of phosphatidyl-[^{14}C]-serine, confirming that it was due to uptake of the unmodified PS molecule. However, the ratio of the liposoluble to the hydrosoluble radioactivity was 7–10 times higher, indicating a decreased amount of labelled hydrosoluble compounds. The analysis of the composition of the liposoluble radioactivity (Table 5) provided an explanation for this difference. It can be seen that the fraction containing labelled neutral lipids became predominant in plasma, thus indicating that [^{3}H]-glycerol released by hydrolytic degradation of PS was readily removed from the hydrosoluble pool by incorporation into lipoproteins. The high radioactivity in plasma neutral lipids (74–88%) caused the fraction containing labelled PS to become smaller than that detected after administration of [^{14}C]-serine-PS. By contrast, the PS

Table 2 Distribution of radioactivity 60 min after bolus i.v. injection of phosphatidyl-[^{14}C]-serine

	d.p.m. g^{-1} wet wt or ml^{-1} × 10^{-3}		
Organ	*Liposoluble*	*Hydrosoluble*	*Ratio*
Plasma	5.6 ± 1.0 (1.5)	1.8 ± 0.2 (0.5)	3
Liver	200.9 ± 6.9 (68.8)	23.1 ± 1.9 (7.9)	8.7
Spleen	61.1 ± 6.4 (0.8)	9.4 ± 0.8 (0.1)	6.5
Kidneys	19.2 ± 3.0 (0.6)	9.3 ± 0.7 (0.3)	2.3
Lungs	14.6 ± 3.3 (0.5)	3.6 ± 0.3 (0.1)	4
Brain	1.6 ± 0.2 (0.05)	1.4 ± 0.2 (0.04)	1.1

PS 2 mg kg^{-1} (14.6 × 10^6 d.p.m. kg^{-1}) was injected into the jugular vein of 6 rats (316 ± 11 g). Plasma (heart puncture) and organs were sampled, weighed and extracted 60 min later. Data are expressed as d.p.m. g^{-1} of wet tissue (or ml of plasma) and as percentage of the administered dose that is recovered in the whole organ (figures in parentheses). To calculate the recovery, the following values were used: plasma 4% of body weight; brain 0.45%; liver, 5%, spleen, 0.2%; kidneys, 0.47%; lungs, 0.5%. Data from our determinations and the Tables of Charles River (1975). Means ± s.e.mean from 6 rats.

Table 3 Composition of liposoluble radioactivity extracted 60 min after i.v. administration of phosphatidyl-[^{14}C]-serine

	% of liposoluble d.p.m.				
Lipid	*Plasma*	*Liver*	*Spleen*	*Kidneys*	*Lungs*
PS	39.3 ± 1.7	63.7 ± 4.2	68.6 ± 3.8	61.1 ± 3.3	66.3 ± 3.7
PE	12.2 ± 0.5	21.3 ± 1.2	14.8 ± 2.9	26.6 ± 2.2	16.7 ± 1.8
PC	2.1 ± 0.3	1.0 ± 0.1	0.2 ± 0.0	0.9 ± 0.4	1.1 ± 0.2
SPH	1.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0
lysoPS	3.6 ± 0.9	1.5 ± 0.5	1.4 ± 0.3	1.1 ± 0.3	1.8 ± 0.5
lysoPE	11.8 ± 1.3	2.6 ± 0.6	2.5 ± 0.4	3.2 ± 0.7	6.3 ± 1.2
lysoPC	3.3 ± 0.8	ND	ND	ND	ND
Neutral lipid (front)	6.8 ± 1.1	1.4 ± 0.1	1.5 ± 0.2	1.3 ± 0.3	2.3 ± 0.4

The liposoluble radioactivity of Table 2 was resolved by two dimensional thin layer chromatography. The amount of radioactivity not accounted for by the indicated lipids was distributed at the origin or in unidentified spots. PS denotes phosphatidylserine, PE phosphatidylethanolamine, PC phosphatidylcholine, SPH sphingomyelin. ND, not detected. Means ± s.e.mean from 6 rats.

fraction increased in liver and spleen, due to a decrease in PE labelling.

From a comparison of the amounts of PE recovered after the administration of [^{14}C]-serine-PS and [^{3}H]-glycerol-PS (Tables 3 and 5) it can be estimated that uptake of free [^{14}C]-serine accounts approximately for 10–13% of the liposoluble radioactivity found in spleen and liver after the administration of [^{14}C]-serine-PS.

Discussion

In this study the disposition of intravenously injected PS vesicles has been investigated to see whether a relationship exists with the pharmacological actions observed in rodents after parenteral administration. Previous investigations on phospholipid vesicles of various composition have shown two main patterns of interaction with tissue and plasma constituents: (1) uptake by the Kupffer cells of liver and the macrophages of spleen, lungs and bone marrow (reviewed by Poste, 1983) and, (2) interaction with HDL (Scherphof *et al.*, 1978; Vidal *et al.*, 1984; William & Tall, 1988). The latter causes vesicle rupture and reorganization into a lipoprotein complex able to be taken up by the liver parenchymal cells (Scherphof *et al.*, 1978; William & Tall, 1988). Qualitatively, these events are independent of liposome charge, structural class (mono- or multi-lamellar) and composition (Poste, 1983). However, they are amplified when the vesicle size is large (Juliano & Stamp, 1975; Patel & Ryman, 1981) or certain functional groups, such as the phosphorylserine group, are present. Recent reports have indeed shown that this functional group is specifically recognized by the mononuclear phagocyte system (Tanaka & Schroit, 1983; Allen *et al.*, 1988) and accelerates the rate of liposome interaction with HDL (Bienvenue *et al.*, 1985). Consistently, we observed a rapid disappearance of PS vesicles from plasma and accumulation in liver and spleen. Since these organs are rich in immunocompetent cells (macrophages, lymphocytes), these findings are compatible with the results of pharmacodynamic studies pointing to these cells as the pharmacological targets of PS (Schroit *et al.*, 1985; Mietto *et al.*, 1989; Ponzin *et al.*, 1989).

Table 4 Distribution of radioactivity 60 min after bolus i.v. injection of [^{3}H]-glycerol-phosphatidylserine

	d.p.m. g^{-1} wet wt or $ml^{-1} \times 10^{-3}$		
Organ	*Liposoluble*	*Hydrosoluble*	*Ratio*
Plasma, rat 1	5.8 (0.5)	1.4 (0.1)	4.1
rat 2	7.4 (0.7)	1.4 (0.1)	5.4
Liver, rat 1	593.2 (66.4)	6.4 (0.7)	92.1
rat 2	407.9 (45.6)	6.7 (0.7)	60.7
Spleen, rat 1	131.1 (0.8)	1.9 (0.01)	70.5
rat 2	116.3 (0.7)	2.4 (0.01)	49.1

Two rats of 260 g were injected with 2 mg kg^{-1} of phosphatidylserine labelled with [^{3}H]-glycerol (44 × 10^{6} d.p.m. kg^{-1}). Plasma, liver and spleen were analyzed 60 min after the injection. Data are expressed as d.p.m. g^{-1} of wet tissue (or ml of plasma) and as percentage of the administered dose that is recovered in the whole organ (figures in parentheses).

Table 5 Composition of liposoluble radioactivity extracted 60 min after i.v. administration of [^{3}H]-glycerol-phosphatidyl-serine

	% of liposoluble d.p.m.					
	Plasma		*Liver*		*Spleen*	
Lipid	*rat 1*	*rat 2*	*rat 1*	*rat 2*	*rat 1*	*rat 2*
PS	5.5	15.3	85.6	84.4	89.1	89.2
PE	1.0	0.7	7.6	8.4	5.9	4.9
PC	1.0	2.0	2.2	2.3	2.7	2.8
Neutral lipids (front)	87.6	73.7	2.1	2.8	1.0	1.0

Abbreviations as in Table 3. Negligible radioactivity was found to be associated with other phospholipids.

The biphasic decay of the plasma concentration of PS, although in principle consistent with the classical two-compartment open model, cannot be interpreted as a rapid reversible distribution phase, followed by elimination. As discussed above, the interaction of PS liposomes with cells is irreversible and the fast decay step, rather than distribution, describes the main elimination route of PS, i.e. uptake by the mononuclear phagocyte system. The slow component of the PS decay curve has a half-life very similar to that of rat HDL. A linear decay with half-life of 47 min was observed when lipoproteins containing radiolabelled phospholipids were injected intravenously into rats (Stein & Stein, 1966). It is therefore suggested that the slow phase of PS decay in plasma is due to its incorporation into lipoproteins, and reflects the elimination of the lipid moiety of these plasma constituents, possibly influenced by the presence of PS, an unusual phospholipid for HDL.

With other types of liposomes the biphasic clearance from plasma may reflect vesicle size heterogeneity, the larger vesicles being cleared faster than the smaller ones (Juliano & Stamp, 1975; Patel & Ryman, 1981). With PS-containing liposomes, however, the disappearance from plasma has been shown to be independent of the vesicle size (Juliano & Stamp, 1975). This has been attributed to the strong tendency of the smaller PS vesicles to coalesce in the presence of plasma Ca^{2+} to form a rather homogeneous population of larger vesicles (Juliano & Stamp, 1975; Kao & Loo, 1980). A biphasic decline of intravenously injected liposomes may also reflect the presence of two uptake pathways, one of which (uptake by the mononuclear phagocyte system) is saturable (Gregoriadis & Neerunjun, 1974; Hwang & Beaumier, 1988). However, evidence of saturation has been obtained at doses (around

100 mg kg^{-1}) far higher than those employed in this study (2 mg kg^{-1}). In the case of PS, saturation at this dose would imply a very low capacity uptake by the macrophage scavenger receptor that is expected to mediate the endocytosis of vesicles containing this phospholipid (Nishikawa *et al.*, 1990).

The combined use of [^{3}H]-glycerol-PS and [^{14}C]-serine-PS enabled us to outline the metabolic fate of injected PS. Extensive hydrolysis occurs at the injection site yielding, in addition to hydrosoluble metabolites, the pharmacologically active compound lysoPS. The production of lysoPS is most likely due to the phospholipase A_2 released by the platelet aggregating at the site of the perforated vessel. Rat platelets are known to be rich in phospholipase A_2 acting on PS (Horigome *et al.*, 1987). Formation of lysoPS at the injection site, although in small amounts, is also observed after intramuscular or intraperitoneal injection. These observations indicate that there might be a variable contribution of lysoPS to the pharmacological actions of PS, depending on the route of administration.

After incorporation into tissues, PS follows two metabolic pathways which reflect the two main mechanisms of uptake. One involves decarboxylation to PE of a minor fraction (8% in the liver) of the incorporated PS. Since this is the physiological pathway of PS metabolism, it most probably involves that fraction of PS that mixes with the phospholipid pool of the plasma membrane. The other, predominant, metabolic pathway proceeds to the complete hydrolysis of PS to the individual components of the molecule as testified by the detection in tissues of labelled glycerol and serine. This sequence is to be expected if the phospholipid vesicle is internalized by endocytosis and hydrolyzed after fusion of the endocytic vacuole with lysosomes (Dijkstra *et al.*, 1985). Consistently, PS uptake is predominant in organs (liver and spleen) where endocytosis by the local macrophage population is especially active.

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(Received July 6, 1990
Revised September 17, 1990
Accepted October 15, 1990)

Br. J. Pharmacol. (1991), **102**, 351–354 © Macmillan Press Ltd, 1991

Yohimbine increases submaxillary kallikrein release into the saliva in dogs: evidence for α_2-adrenoceptor-mediated inhibition of cholinergic pathways

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1 The effects of the α_2-adrenoceptor antagonist, yohimbine (0.5 mg kg^{-1}, i.v.) on basal, sympathetic and parasympathetic stimulation-induced submaxillary kallikrein release were investigated in the anaesthetized dog. Kallikrein was measured by its kininogenase activity before and after trypsin activation which also allowed a study of the proportion of active to total enzyme.

2 Yohimbine induced a rapid, three fold increase in basal kallikrein release correlated with an increase in salivary flow rate which lasted for 60 min following injection.

3 Sectioning the chorda tympani did not affect basal kallikrein release but abolished yohimbine-induced rise in salivary kallikrein secretion.

4 Parasympathetic stimulation alone induced a 3 to 4 fold increase in basal kallikrein release correlated with an increase in salivary flow rate. Yohimbine induced a significant additional increase in parasympathetic-stimulated kallikrein release.

5 When the cervical sympathetic nerve was sectioned the basal kallikrein release decreased by 30 to 40%.

6 Sympathetic stimulation alone also induced a 3 to 4 fold increase in basal kallikrein. This was not correlated with the salivary flow and unaffected by yohimbine.

7 The results indicate that yohimbine increases submaxillary kallikrein release into the saliva by inhibition of presynaptic α_2-adrenoceptors located on the chorda tympani nerve endings.

Introduction

One of the major actions of glandular kallikrein is to release bradykinin, a potent vasodilator peptide. The kallikrein-kinin system has been localized in several glands such as pancreas, kidney and salivary glands and various biological activities for it have been proposed: e.g. in the regulation of both local blood flow and in the secretion of electrolytes and water secretion from these glands (Fuller & Funder, 1986; Carretero & Scicli, 1989). In particular, large amounts of glandular kallikrein have been detected in the salivary glands (Orstavik, 1978); salivary secretion of kallikrein may be mediated by β-adrenoceptors in cat (Gautvik *et al.*, 1974) and in rat (Orstavik & Gautvik, 1977). Moreover, in the rat, the release of submandibular kallikrein into blood paralleled that into saliva and its secretion was largely enhanced by α-adrenoceptor stimulation (Rabito *et al.*, 1983; Berg *et al.*, 1985). These results suggest a possible independent regulation of salivary flow and endocrine enzyme secretion.

We recently demonstrated that the α_2-adrenoceptor antagonist, yohimbine, increases submaxillary salivation in dogs (Montastruc *et al.*, 1989) and in man (Chatelut *et al.*, 1989). It has been proposed that the release of kallikrein by production of an associated kinin may regulate the ionic composition of saliva (Carretero & Scicli, 1989; Fuller & Funder, 1986). Thus, the aim of the present study was to evaluate the effect of yohimbine on the secretion of kallikrein into saliva during parasympathetic or sympathetic stimulation.

Methods

Animals and general procedure

Dogs of either sex (7 to 15 kg) were prepared as recently described (Montastruc *et al.*, 1989). Animals were anaesthetized with a mixture of chloralose (40 mg kg^{-1}, i.v.) plus urethane (500 mg kg^{-1}, i.v.). To avoid the potential α_2-adrenoceptor antagonist properties of high doses of urethane (Moore *et al.*, 1984), low sub-anaesthetic concentrations were used. Body temperature was kept constant at around 38.5°C, arterial pH was 7.42. Blood pressure and heart rate were monitored with a Statham P23 Id pressure transducer connected to a Honeywell Bull recorder and a tachocardiometer triggered by electrocardiogram (lead 2) linked to a chart recorder.

Protocol for salivary secretion study

Following anaesthesia, Wharton's duct was catheterized by means of a polyethylene tube (Montastruc *et al.*, 1989), three separate experimental protocols were conducted.

Experiment 1: effects of yohimbine on spontaneous kallikrein release Yohimbine (0.5 mg kg^{-1}, i.v. 3 ml, $n = 6$) or saline vehicle (0.9% NaCl 3 ml, in sham experiments, $n = 6$) were injected and, after a resting period of at least 15 min, salivary secretion from both submaxillary glands was collected every 15 min for 90 min.

Experiment 2: effects of yohimbine on parasympathetic kallikrein release In the first experimental sub-group, the left chorda tympani nerve was dissected (Montastruc *et al.*, 1989) and sectioned before starting the experiment. The effect of yohimbine (0.5 mg kg^{-1}, i.v.) was investigated on the spontaneous release of kallikrein from the intact (right) side and compared simultaneously with the release observed from the sectioned (left) side. In the second sub-group of experiments, the effect of yohimbine was investigated during electrical stimulation of the peripheral cut end of the chorda tympani nerve. The parameters of stimulation used (15 s trains, supramaximal intensity, 0.1 ms, 10 Hz every 15 min, once before and six times after yohimbine or saline (3 ml) administration were

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selected to mimic the salivary secretion found with an unstimulated intact nerve (Montastruc *et al.*, 1989). Kallikrein was measured in samples collected during these 15 s periods of stimulation and during the 15 min period following the end of stimulation.

Experiment 3: effects of yohimbine on sympathetic kallikrein release The left cervical sympathetic nerve was isolated and cut in the vagal sympathetic trunk: the effects of yohimbine (0.5 mg kg^{-1}, i.v.) on kallikrein release on the (right) intact nerve were compared with the values obtained from the sectioned stimulated (left) side. The parameters of stimulation were 15 s trains, supramaximal intensity, 0.1 ms, 10 Hz every 15 min, once before and six times after yohimbine or saline (3 ml) administration.

Kallikrein measurement

The level of kallikrein in the saliva was measured by the kininogenase activity by use of a kinin radioimmunoassay (Alhenc-Gelas *et al.*, 1981), before and after trypsin activation, with the nonapeptide bradykinin as standard and heat-treated citrated dog plasma as substrate. This crude substrate preparation was devoid of spontaneous kininogenase and kininase activity. The incubation buffer consisted of 0.1 M Tris HCl, pH 8.5, containing 0.3 mM ortho-phenanthroline and 0.3 mM EDTA as kininase inhibitors. Total activatable kallikrein was measured after trypsinization of the samples which activates prekallikrein by liberating an inactive heptapeptide profragment. The proportion of active over inactive kallikrein, expressed as a percentage of the active form, gives complementary information regarding the secretion and activation mechanisms of kallikrein. Samples were incubated for 30 min with 5 μg trypsin in 0.2 M Tris pH 7.8 at 37°C. The action of trypsin was stopped by adding 20 μg of soybean trypsin inhibitor (SBTI). The results are expressed in ng of bradykinin generated per minute in a 15 min salivary volume (ng BK per 15 min).

The possible degradation of kallikrein or the presence of kallikrein inhibitors in the incubation medium was also checked by adding a fixed amount of standard kallikrein purified from dog urine (personal data) and measuring the recovery of activity. In these test experiments, the recovery of purified dog kallikrein was 86 ± 9%.

Statistical analysis

Results are expressed as mean ± s.e.mean ($n = 6$ in each group). Significance was estimated by Student's t test (after one-way analysis of variance). Intact and sectioned sides were compared by use of Student's t test for paired comparison. P values less than 0.05 were considered significant. Correlation studies were carried out with the results of the salivary volume investigation performed in the same dogs (Montastruc *et al.*, 1989).

Results

Yohimbine administration (0.5 mg kg^{-1} i.v.) did not induce any significant variation of blood pressure or heart rate as previously reported (Montastruc *et al.*, 1989).

Experiment 1: effects of yohimbine on spontaneous kallikrein release (Figure 1)

Compared with saline, yohimbine induced a 3 fold increase in the basal kallikrein secretion which reached maximal values 45 to 60 min after administration. The proportion of active to total kallikrein remained unchanged with an average value of 54 ± 9%. The increase in kallikrein secretion was significantly correlated with the rise in salivary volume ($r = 0.88$, $P < 0.001$; $n = 36$).

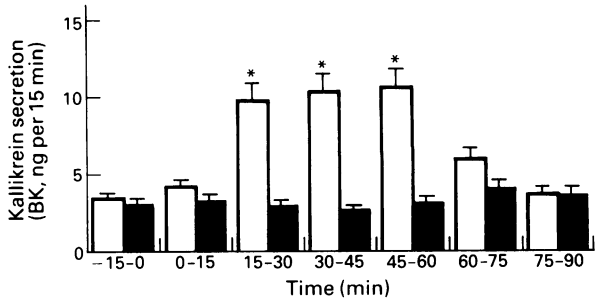


Figure 1 Comparison of the effects of yohimbine (0.5 mg kg^{-1} i.v. injected at time 0: open columns) and i.v. saline vehicle (solid columns) on the release of submaxillary kallikrein measured by the kininogenase activity detected in the salivary volume collected over 15 min from both submaxillary ducts (with intact nerves). * $P < 0.01$. Values are means with s.e.mean shown by vertical bars; $n = 6$.

Experiment 2: effects of yohimbine on parasympathetic kallikrein release

Sectioning of the chorda tympani had no effect on basal kallikrein secretion but abolished the stimulating effect of yohimbine (Figure 2). Stimulation of the parasympathetic chorda tympani nerve induced a 3 to 4 fold rise in kallikrein release which was significantly correlated with the increase in salivary volume ($r = 0.79$, $P < 0.001$; $n = 28$). In fact, the concentration of kallikrein did not change significantly throughout the different periods of stimulation (1.27 ± 0.38 versus 1.1 ± 0.25 ng BK per ml saliva). The increase in kallikrein was due to the enhancement of salivary volume. Yohimbine administration during parasympathetic stimulation induced a further significant increase in kallikrein release (Figure 3).

Experiment 3: effects of yohimbine on sympathetic kallikrein release (Figure 4)

Sectioning of the cervical sympathetic nerve induced a 50 to 70% decrease in basal kallikrein secretion (1.1 ± 0.2 versus 3.4 ± 0.3 ng BK per 15 min, $P < 0.05$). Sympathetic stimulation induced an increase in kallikrein secretion, but this was due to a 3 to 6 fold rise in kallikrein concentration (5.2 ± 0.62 versus 0.91 ± 0.32 ng BK per ml saliva). Furthermore, the increase in kallikrein secretion was not correlated with

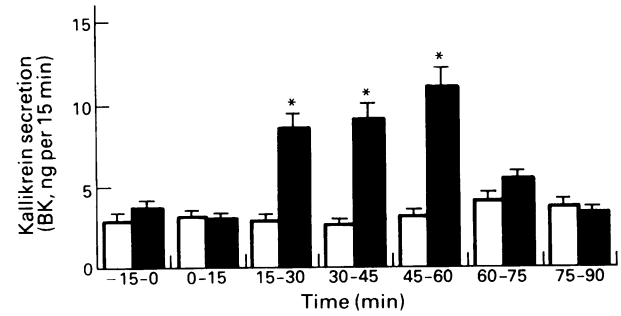


Figure 2 Effect of sectioning the chorda tympani nerve on the effect of yohimbine (0.5 mg kg^{-1} i.v. injected at time 0) on the release of submaxillary kallikrein measured by the kininogenase activity detected in the salivary volume collected over 15 min. * $P < 0.01$. Values are means with s.e.mean shown by vertical bars, $n = 6$; open columns: sectioned side, the nerve was sectioned before starting the experiment; solid columns: intact side.

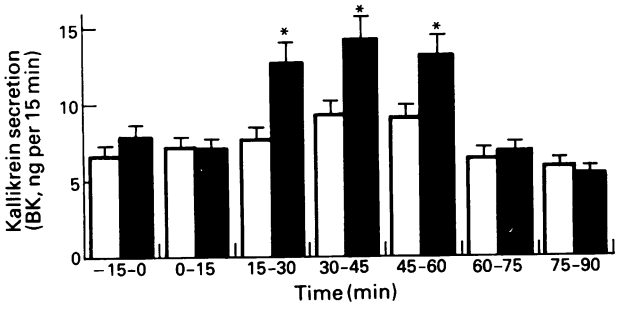


Figure 3 Effect of yohimbine (0.5 mg kg^{-1}, i.v. injected at time 0: solid columns) and i.v. saline (open columns) during parasympathetic stimulation of the peripheral end of the chorda tympani nerve on the release of submaxillary kallikrein measured by the kininogenase activity detected in the salivary volume collected over 15 min. Electrical stimulation began at time −15 min * $P < 0.01$. Values are means with s.e.mean shown by vertical bars. $n = 6$.

changes in salivary volume. The magnitude of kallikrein release after yohimbine administration was similar on the intact and sectioned sides.

In the three experiments, the ratio of active to total kallikrein did not change, indicating that the variations in kallikrein secretion resulted from a proportionate secretion of both the active and inactive forms (data not shown).

Discussion

It has been reported previously that submaxillary kallikrein secretion is mainly under sympathetic control both in cat (Gautvik *et al.*, 1974; Garret *et al.*, 1987) and in rat (Orstavik & Gautvik, 1977; Orstavik *et al.*, 1982a,b; Berg *et al.*, 1985; Rabito *et al.*, 1986). However, a mild parasympathetic stimulating effect was also described (Orstavik & Gautvik, 1977), although its involvement remains controversial (Schachter, 1979). Nevertheless, it should be noted that the release of submaxillary kallikrein has been essentially investigated in blood whereas that in saliva has received less attention. Our data demonstrate that kallikrein release into saliva is also greatly increased by parasympathetic nerve stimulation. Furthermore, the fact that yohimbine, an α_2-adrenoceptor antagonist, induces a significant increase in basal kallikrein secretion, clearly suggests the involvement of an α_2-adrenoceptor in this

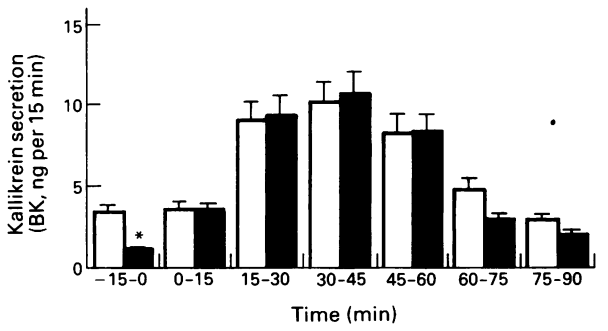


Figure 4 Effect of yohimbine (0.5 mg kg^{-1}, i.v. injected at time 0) during stimulation of the superior cervical nerve on the release of submaxillary kallikrein measured by the kininogenase activity detected in the salivary volume collected over 15 min. Sectioning the superior cervical nerve induced a decrease in kallikrein secretion: period −15–0 min, * $P < 0.05$ when the intact (right) side (solid columns) was compared with the sectioned (left) side (open columns). Electrical stimulation began at time 0 and restored the basal secretion of kallikrein during the period 0–15 min. The effects of yohimbine on the intact (right) side (solid columns) were compared with those obtained from stimulation of the sectioned (left) side (open columns). The magnitude of kallikrein release after yohimbine administration (periods 15–30, 30–45 and 45–60 min) was similar on the intact and sectioned sides. Values are means with s.e.mean shown by vertical bars. $n = 6$.

effect. The present study demonstrates that yohimbine not only increases salivary water content (Montastruc *et al.*, 1989) but also maintains the levels of kallikrein, a physiological factor normally present in the saliva. Several reports have already demonstrated the involvement of α_2-adrenoceptors in the control of salivation in the rat (Bylund & Martinez, 1980; 1981) and in the dog (Montastruc *et al.*, 1989). The stimulating effect of yohimbine on kallikrein release is also dependent on the integrity of parasympathetic innervation: it disappears after sectioning the chorda tympani but is restored during stimulation of the distal end of the nerve. Thus, the present data demonstrate the existence of α_2-adrenoceptors located on the chorda tympani nerve endings which inhibit the basal secretion of kallikrein.

The present data also emphasize the importance of α_2-adrenoceptors in the secretory control of salivary glands especially in the regulation of cholinergic (but not sympathetic) transmission during submaxillary salivation as has already been suggested (Green *et al.*, 1979). The reasons why previous reports have failed to demonstrate such an important activating effect of parasympathetic stimulation may be related to the anaesthetics used since we observed that under chloralose, urethane or sodium thiopentane anaesthesia, yohimbine remained ineffective on salivary secretion (Montastruc *et al.*, 1989). Anaesthetics interfere with α_2-adrenoceptors (Moore *et al.*, 1984). Parasympathetic nerve impulses are however required for normal synthesis of submaxillary kallikrein (Garrett *et al.*, 1987); the submaxillary glands, 3 weeks after chorda tympani excision, showed a very weak light staining due to kallikrein-like activity as revealed by cryostat sections using histochemical methods.

In addition to the indirect sympathetically-induced cholinergic inhibition of kallikrein secretion mediated by α_2-adrenoceptors, the influence of sympathetic nerves *per se* was also confirmed. Sectioning of the superior cervical sympathetic nerve reduced the spontaneous secretion of kallikrein. This result probably indicates that basal kallikrein secretion is under tonic sympathetic influence. Sympathetic stimulation also induced a significant increase in kallikrein above the basal levels. However, this increase is not linked to a rise in salivary secretion and results from an increased concentration of kallikrein.

It appears likely that in the salivary gland, kallikrein release into saliva is under the control of both sympathetic and parasympathetic nerves through two different mechanisms. The main result of the present study is to demonstrate that parasympathetically-induced kallikrein secretion in saliva is under a tonic α_2-adrenoceptor inhibitory control. Therefore, α_2-adrenoceptor blockade induces a stimulation of kallikrein secretion in saliva. The stimulation reflects a true increase in kallikrein secretion since the proportion of active to total kallikrein remained unchanged. It is worth pointing out that this increase in kallikrein secretion is significantly correlated with an enhancement of the salivation rate recently described during parasympathetic stimulation (Montastruc *et al.*, 1989). In contrast to the parasympathetic control of kallikrein secretion, which parallels that of salivation, the sympathetic innervation exerts a tonic effect: its stimulation results in the release of high concentrations of kallikrein independently of salivary secretion. Finally it seems that α_2-adrenoceptors may control in an opposite way blood and salivary release of submandibular kallikrein: stimulation of α_2-adrenoceptors increased the secretion of kallikrein into blood (Rabito *et al.*, 1983) whereas α_2-adrenoceptor blockade enhanced the release of the enzyme into the saliva.

The physiological significance of kallikrein in the submaxillary gland is still questionable. However, the release of kallikrein into the circulation may play a role in the regulation of blood flow and in vasodilatation after treatment with converting enzyme inhibitors or after sympathetic stimulation (Orstavik *et al.*, 1982a,b; Berg *et al.*, 1989). Whether the release of kallikrein into the saliva is involved in the increase of salivary secretion remains to be determined.

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(Received April 10, 1990
Revised October 11, 1990
Accepted October 15, 1990)

Br. J. Pharmacol. (1991), **102**, 355–362 © Macmillan Press Ltd, 1991

Electrogenic uptake contributes a major component of the depolarizing action of L-glutamate in rat hippocampal slices

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1 A grease-gap technique has been used to measure d.c. potentials, in response to the application of excitatory amino acids and electrical stimulation of the Schaffer collateral-commissural pathway, in the CA1 region of rat hippocampal slices. The actions of L-glutamate (L-Glu) have been quantified and compared to those of structurally related compounds.

2 Perfusion of L-Glu (90 s applications) depolarized the tissue with a threshold of approximately 50 μM and a maximum response in excess of 10 mM. L-Aspartate (L-Asp) produced a similar dose-response relationship. By comparison N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) were more potent excitants, producing dose-dependent depolarizations over the range 2–50 μM.

3 Application of the agonists depressed the amplitude of electrically-evoked synaptic responses; an effect that presumably reflects depolarization of neuronal tissue. However, for a given agonist-induced d.c. potential, L-Glu or L-Asp caused smaller depressions of synaptic responses than did either NMDA or AMPA.

4 The combined application of 50 μM D-2-amino-5-phosphonopentanoate (AP5) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) substantially depressed synaptic responses and antagonized responses to NMDA and AMPA producing mean ($\pm$s.e.) dose-ratios of 12.2 $\pm$ 1.2 and 7.0 $\pm$ 0.8, respectively. However, these compounds produced minimal antagonism of responses to L-Glu and L-Asp (dose-ratios of 1.5 $\pm$ 0.1 and 1.5 $\pm$ 0.2, respectively).

5 Responses to the stereoisomers of homocysteate (HCA) were compared over the range 50 μM to 10 mM. D-HCA was approximately 3.6 times more potent than L-HCA and was antagonized to a greater extent by the combined application of 50 μM AP5 and 10 μM CNQX; the dose ratios were 8.7 $\pm$ 0.8 and 5.1 $\pm$ 0.9 for the D- and L- isomers, respectively.

6 The application of high doses of an excitant (e.g., 50 mM L-Glu or 5 mM D-HCA) caused an irreversible loss of sensitivity to NMDA and AMPA and abolished synaptic transmission. Responses to the other excitants were depressed by this excitotoxic lesion in the following order: D-HCA > L-HCA > L-Glu = L-Asp. In slices treated in this manner, L-Glu, L-Asp and L-HCA produced very similar dose-response curves.

7 Some slices were unresponsive to NMDA, AMPA and electrical stimulation from the onset of the experiment but had sensitivity to L-Glu, L-Asp and L-HCA similar to that of slices that had received an excitotoxic lesion.

8 Slices that were experimentally lesioned, such that they became unresponsive to synaptic stimulation, AMPA and NMDA, were depolarized by a variety of compounds when tested at 5 mM. The D- and L- enantiomers of Asp and Glu produced similar responses and were slightly more active than quisqualate and threo-hydroxy-L-aspartate (THLA). Glycine, L-serine, D-serine, L-alanine and 4-aminobutanoate (GABA) elicited responses 15–30% of the size, while L-lysine, α-amino-isobutanoate and L-proline produced depolarizations of less than 10% of the size of those induced by 5 mM L-Glu. NMDA and dihydrokainate were either inactive or induced small negative shifts in the d.c. potential.

9 We conclude that when hippocampal slices are perfused with L-Glu (or L-Asp) the main source of the depolarization is due to an interaction with an electrogenic carrier system.

Introduction

L-Glutamate (L-Glu) is considered to be the major excitatory neurotransmitter in the vertebrate central nervous system. Transmission at presumed glutamatergic synapses seems to be mediated, predominantly at least, via N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (previously termed quisqualate) receptors (Collingridge & Lester, 1989). For example, synaptic transmission in the Schaffer collateral-commissural pathway of the hippocampus can be abolished by a combination of NMDA antagonists, such as D-2-amino-5-phosphonopentanoate (AP5), and AMPA antagonists, such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Davies & Collingridge, 1989; Andreasen *et al.*, 1989). However, responses to L-Glu in the central nervous system are relatively insensitive, compared to exogenous agonists such as AMPA, quisqualate, kainate and NMDA, to excitatory amino acid antagonists (Luini *et al.*, 1981; Teichberg *et al.*, 1984; Davies & Watkins, 1985; Surtees & Collins, 1985; Davies *et al.*, 1988). This has led to the suggestion that L-Glu may have additional actions mediated via other receptors and/or cellular processes.

In the present study we have used a grease-gap method (Blake *et al.*, 1988) in an attempt to identify these additional actions of L-Glu when applied to the CA1 region of rat hippocampal slices. We suggest that a major component of the depolarizing responses induced by L-Glu can be accounted for by an electrogenic uptake process.

Methods

Experiments were performed on 400 μm transverse rat hippocampal slices, following removal of the CA3 region, by use of

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a grease-gap method as described previously (Blake et al., 1988) (Figure 1). In brief, the alveus was partially separated from the grey matter and the cut (subicular) end was placed under a Ag/AgCl wick electrode. The alveus/electrode assembly was covered by an insulating layer of grease (40:60 liquid paraffin:petroleum jelly) and d.c. potentials were recorded between this electrode and a similar one placed in the perfusing medium. A bipolar metal electrode was placed in stratum radiatum to deliver single shocks at 0.033 Hz. The slice was then partially covered with absorbant paper and perfused at approximately 1.5 ml min^{-1} with a solution at 28°C comprising (mM): NaCl 124, $NaHCO_3$ 26, KCl 3, $CaCl_2$ 2, $MgSO_4$ 1 and D-glucose 10 (bubbled with 95% O_2/5% CO_2).

All drugs were added to the perfusate (i.e., no substitutions were made). At the standard dose used (5 mM) osmotic effects and changes in electrode junction potentials were not significant, since effects were dependent upon the chemical structure of the applied drug and in the case of homocysteate, its depolarizing action was stereoselective. Further experiments, to determine the contribution made by junction potentials to the measured responses, were performed with absorbant paper in place of the slice; junction potentials of sufficient size to affect the measurements significantly were not observed at concentrations of L-Glu < 10 mM.

It should be noted that the alveus serves as an extension of the wick electrode and measures the potential at the grease barrier with reference to the perfusate. The d.c. potential does not depend upon axons projecting along the alveus since the alveus can be substituted by absorbant paper. Thus any cells, not just pyramidal neurones, within the vicinity of the grease barrier can contribute to the d.c. potentials recorded.

Data are presented as means ± s.e. with number of slices in parentheses.

Drugs

L-Glutamate, D-glutamate, L-aspartate, D-aspartate, L-homocysteate, D-homocysteate, L-alanine, L-lysine, L-proline, L-serine, D-serine, glycine, 4-aminobutanoate, α-amino-isobutanoate, N-methyl-D-aspartate and kainate were obtained from Sigma. D-2-Amino-5-phosphonopentanoate, 6-cyano-7-nitroquinoxaline-2,3-dione, α-amino-3-hydroxy-5-methyl-4-

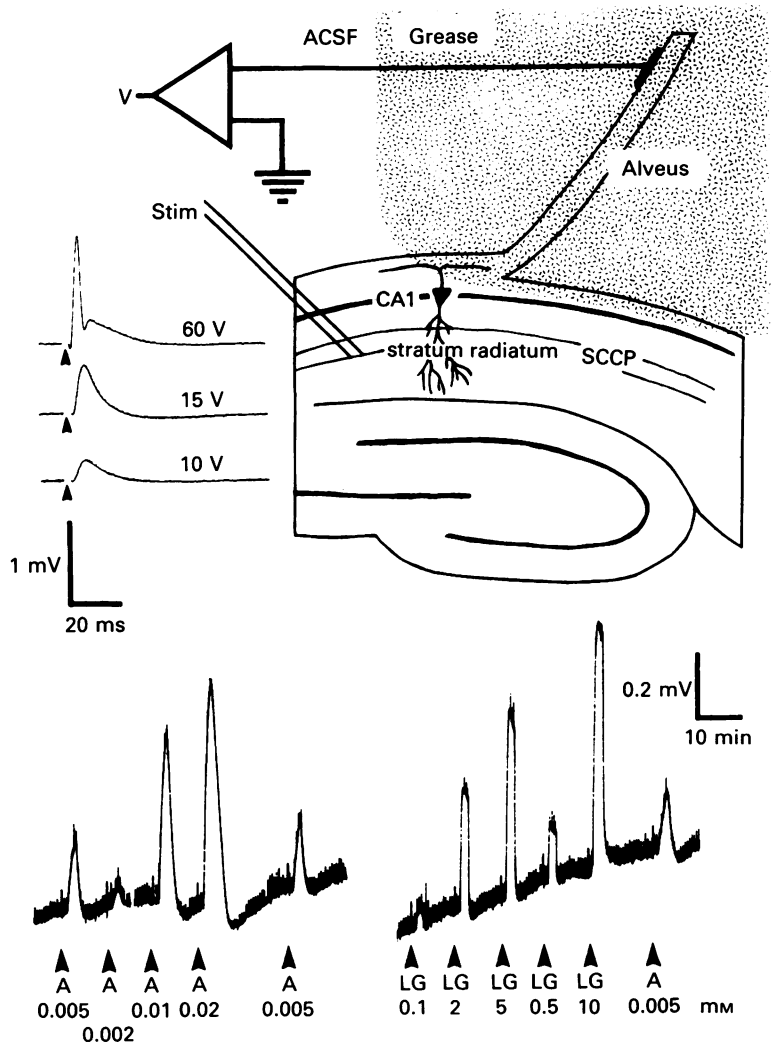


Figure 1 Grease-gap recordings from rat hippocampal slices. The experimental arrangement is shown diagrammatically and representative computer-averaged synaptic and d.c. chart recordings are illustrated. A stimulating electrode (Stim) was placed in stratum radiatum to activate the Schaffer collateral-commissural pathway (SCCP) and d.c. potentials were recorded across the grease barrier with respect to an electrode placed in the artificial cerebrospinal fluid (ACSF). In this and subsequent figures, synaptic records are averages of 3–5 successive records and stimulus artefacts have been replaced by arrowheads. The waveform is a field excitatory postsynaptic potential (e.p.s.p.) which increases in amplitude and slope with increasing stimulus intensity (10–60 V) and at the highest intensity illustrated a small population spike is present. The chart records show typical dose-dependent responses induced by 90 s applications of agonists. In this and subsequent figures, the time at which an agonist was administered is indicated by an arrowhead (and sometimes also by a small dark upward deflection on the chart record). The other upward deflections are filtered versions of the synaptic responses. L-Glutamate (LG); α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (A). The numbers refer to the agonist concentrations (in mM).

isoxazolepropionate, L-2-amino-4-phosphonobutanoate, D-2-amino-4-phosphonobutanoate, quisqualate, dihydrokainate and threo-hydroxy-L-aspartate were gifts from Professor J.C. Watkins (Department of Pharmacology, University of Bristol).

Results

Responses to L-glutamate

L-Glu elicited dose-dependent depolarizations with a threshold of 50 μM and a maximum in excess of 10 mM. The effects of L-Asp were indistinguishable from those of L-Glu. In contrast, both AMPA and NMDA were considerably more potent. In addition, AMPA and NMDA could also be distinguished from L-Glu and L-Asp on the basis of the shapes of the depolarizing responses; those to the latter being more rapid in onset and offset. Representative responses to L-Glu and AMPA are illustrated in Figure 1.

These differences between NMDA/AMPA versus L-Glu/L-Asp could be due to different mechanisms which underlie their depolarizing actions. The following series of experiments was designed to investigate these differences.

Effects of agonists on synaptic transmission

The amplitude of electrically-evoked synaptic responses was depressed by the agonists, as expected on the basis of their depolarizing actions. However, the size of the depression did not correlate to the magnitude of the depolarizations, in that L-Glu and L-Asp produced smaller synaptic depressions than did AMPA and NMDA when applied in doses that elicited comparable depolarizations (Figure 2). This suggests that at least part of the L-Glu (and L-Asp)-induced depolarizations are mediated via a population of cells that are not involved in the generation of these electrically-evoked synaptic responses.

One possibility is that all four agonists are acting on the same receptors but that L-Glu and L-Asp are having most of their effect on surface cells because their diffusion through the slice is limited by uptake processes (Garthwaite, 1985), whereas, in view of the absence of uptake processes for NMDA and AMPA, these agents would be expected to distribute more evenly and hence a greater proportion of their depolarizing action, compared to L-Glu and L-Asp, would be mediated by deeper cells. Since the deeper cells would be expected to receive the synaptic innervation, this differential distribution could, in theory, account for the relative differences between the two sets of compounds. To examine this possibility we applied a combination of antagonists that would be expected to distribute evenly throughout the slice and block NMDA and AMPA receptors uniformly.

Effects of antagonists on agonist-induced responses

A combined application of 10 μM CNQX and 50 μM AP5 virtually abolished synaptic transmission and antagonized responses to AMPA and NMDA, yielding dose-ratios for AMPA of 7.0 $\pm$ 0.8 (6) and for NMDA of 12.2 $\pm$ 1.2 (4). In contrast, L-Glu- and L-Asp-induced responses were minimally antagonized by this treatment; their respective dose-ratios were 1.5 $\pm$ 0.1 (7) and 1.5 $\pm$ 0.2 (3). In these experiments, the responses to 5 mM L-Glu and L-Asp were depressed to 80 $\pm$ 3% (6) and 80 $\pm$ 12% (3) of control, respectively. An example of the differential antagonism of L-Glu and L-Asp compared to AMPA and NMDA is illustrated in Figure 3. In two of these experiments, increasing the concentration of both antagonists (2 or 5 fold) produced little or no further antagonism of the responses to L-Glu and L-Asp (not illustrated).

Thus, it can be concluded that only a small proportion of the depolarizing responses induced by L-Glu and L-Asp was mediated via AMPA and/or NMDA receptors. Other possibilities to explain the relative resistance to the two antagonists include actions on other classes of excitatory amino acid receptor or an electrogenic uptake system into neurones and/or glia. In order to examine the contribution of post-synaptic neuronal elements to the responses to L-Glu we have applied high doses of agonist to effect an acute excitotoxic lesion of neurones (Garthwaite & Garthwaite, 1989). To examine the role of uptake we have applied homocysteate (HCA) since this shows stereoselectivity for certain uptake processes (Balcar & Johnston, 1972b; Cox *et al.*, 1977).

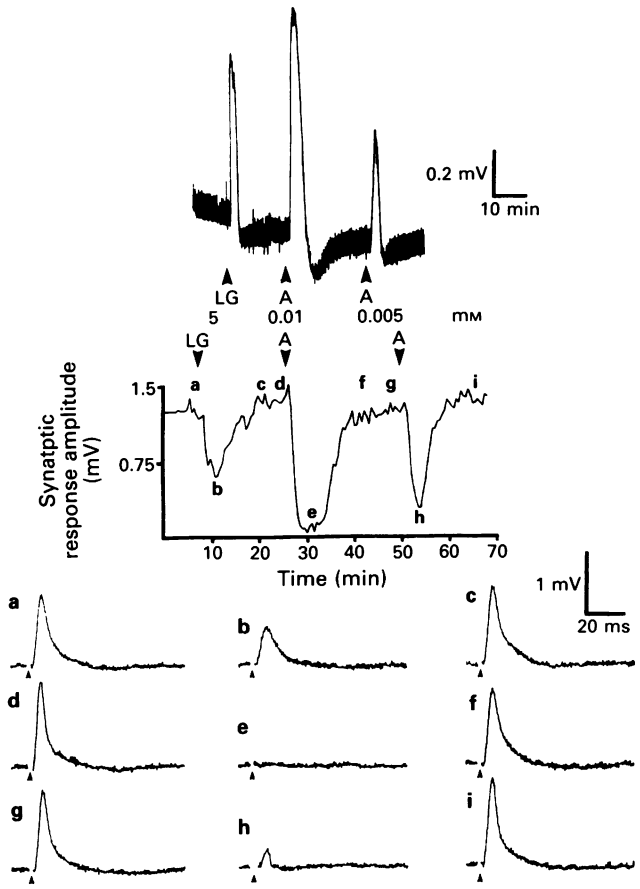


Figure 2 Dissociation between depolarizing and synaptic depressant activity of L-glutamate (LG), compared to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (A). The upper trace is the chart record of d.c. polarity showing responses to LG and A. The plot below shows the amplitudes of the synaptic responses evoked during this period. Averages of 3 synaptic records are shown for the times indicated by (a)–(i).

The effects of homocysteate and excitotoxic lesions

The stereoisomers of HCA were intermediate between AMPA and L-Glu in depolarizing potency. Their rank order of potency was AMPA > NMDA > D-HCA > L-HCA > L-Glu = L-Asp (Figure 4). In these experiments D-HCA was 3.6 $\pm$ 0.4 (11) times as potent as L-HCA. In addition to the potency difference, D-HCA could be distinguished from L-HCA on the basis of the time-course of their responses (D-HCA more AMPA/NMDA-like, L-HCA more L-Glu/L-Asp-like).

Application of either 5 mM D-HCA or 50 mM L-Glu (1–4 applications, each of 90 s separated by 10–30 min) abolished synaptic responses and severely depressed or abolished responses induced by AMPA, NMDA, and D-HCA, for the remainder of the experiment (Figure 4). Responses induced by 50 μM kainate and 50 μM quisqualate were also greatly attenuated by this treatment, to $<$20% (2) and $<$10% (2) control, respectively. In contrast, responses to L-Glu (Figure 4) and L-Asp were only partially reduced by this treatment. The size

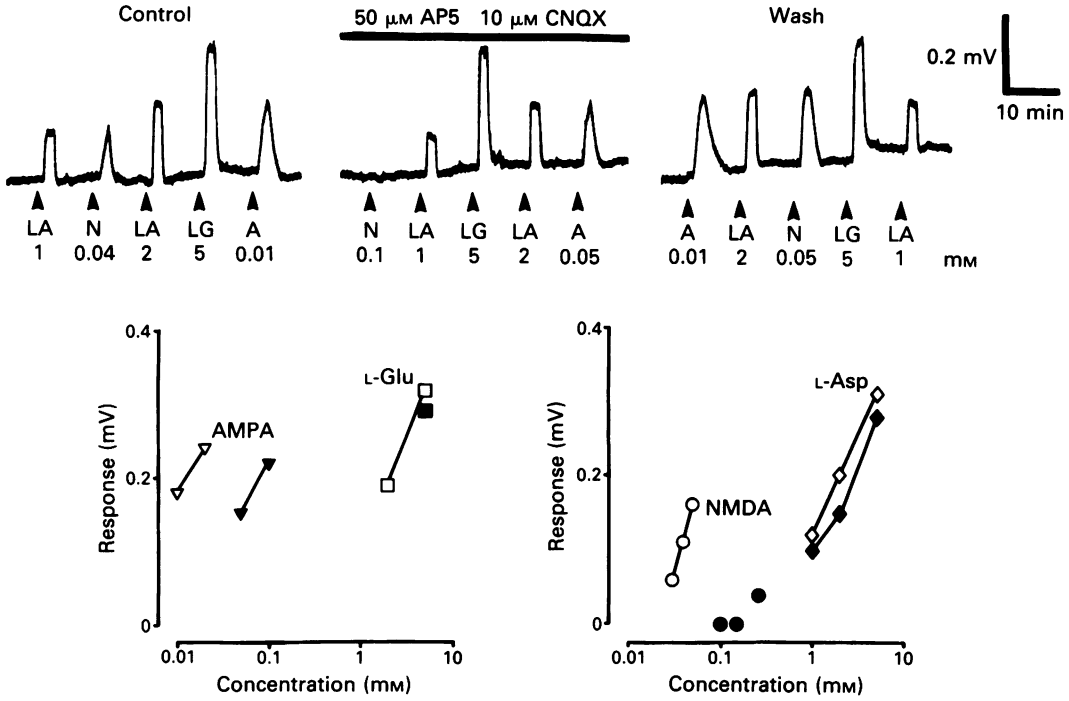


Figure 3 Minimal antagonism of responses to L-glutamate (L-Glu) and L-aspartate (L-Asp). Excerpts from the chart record illustrating reversible antagonism of responses to N-methyl-D-aspartate (NMDA; N), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA; A), L-Glu (LG) and L-Asp (LA). The graphs show dose-response relationships before (open symbols) and during (filled symbols) the application of antagonists. Since the recovery plots overlapped the controls these have been omitted for purposes of clarity. The dose-ratios were for L-Glu, 1.3; L-Asp, 1.5; AMPA, 6.3; NMDA, 9.1. AP5 = D-2-amino-5-phosphonopentanoate; CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione.

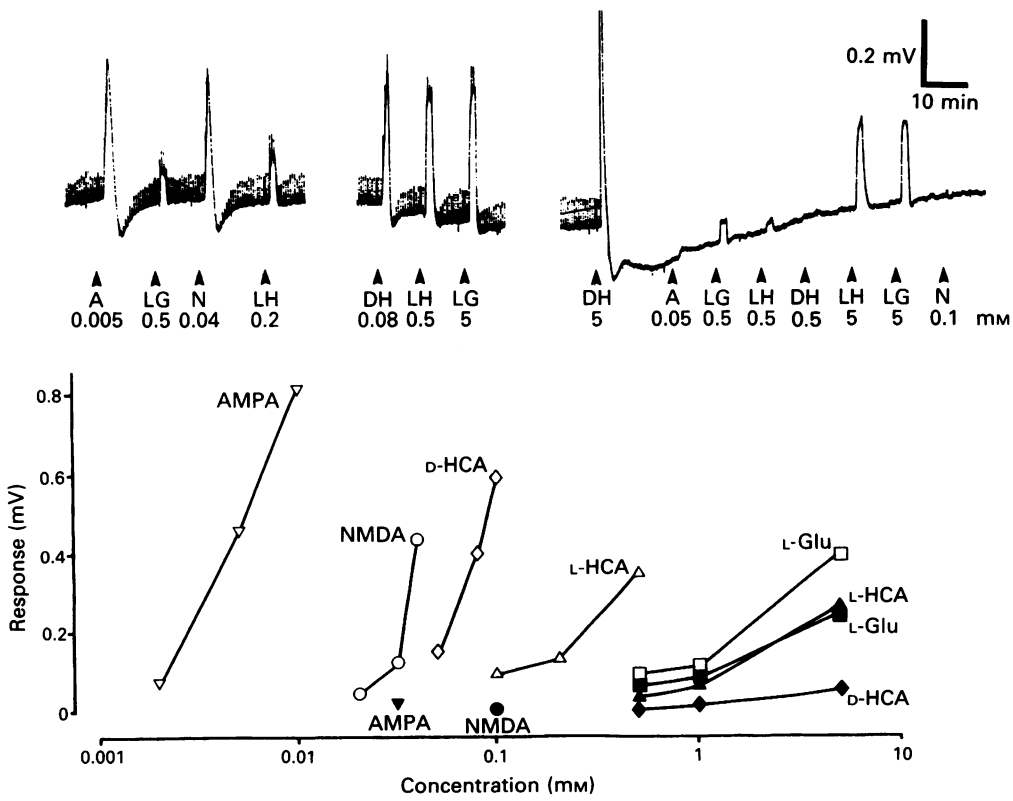


Figure 4 Effects of an acute excitotoxic lesion. Responses to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA; A), L-glutamate (L-Glu; LG), N-methyl-D-aspartate (NMDA; N), L-homocysteate (L-HCA; LH) and D-homocysteate (D-HCA; DH) before (open symbols on graph) and after (filled symbols) an acute excitotoxic lesion effected by 5 mM D-HCA are illustrated. Note the reversal in the potency order of the enantiomers of HCA following the lesion and the abolition of the synaptic responses.

of responses to 5 mM L-Glu and L-Asp in lesioned slices, expressed as percentages of their respective controls, were 57 ± 4 (10) and 58 ± 6 (5), respectively. The reduction of responses to 5 mM L-Glu caused by the lesion was significantly greater ($P < 0.001$; unpaired t test) than that produced by the combined application of CNQX and AP5.

Responses to L-HCA were depressed to an intermediate degree, such that its potency became equivalent to that of L-Glu (and L-Asp). On occasion, slices were from the onset of the experiment synaptically unresponsive and did not respond to AMPA or NMDA, yet responded to L-Glu, L-Asp and L-HCA in a manner qualitatively similar to that of the lesioned slices. A comparison of responses to L-Glu, L-Asp, L-HCA and D-HCA in such a slice is shown in Figure 5. Note that in these (and lesioned) slices doses of excitants (e.g., 5 mM D-HCA), which profoundly alter the responsiveness of synaptically viable slices, do not condition the agonist induced responses (Figure 5).

The greater depression of D-HCA versus L-HCA responses by the excitotoxic lesion implies that in untreated slices D-HCA is having a greater proportion of its effects via receptors on postsynaptic neurones. Accordingly, it might be expected that AP5 and CNQX should antagonize D-HCA to a greater extent than L-HCA. This was found to be the case, since 10 μM CNQX plus 50 μM AP5 produced dose-ratios of 8.7 ± 0.8 and 5.1 ± 0.9 for D-HCA and L-HCA, respectively (paired t test; $P < 0.05$; $n = 5$).

Dose-response curves for L-glutamate

Figure 6 shows sequential dose-response curves for L-Glu before and following an excitotoxic lesion, which was effected by 2 applications of 50 mM L-Glu. The lesion caused a decrease in the maximum, estimated from the logistic fit, of 56% but little alteration in the threshold response. The precise shape and actual maximum response could not be determined experimentally since high concentrations of L-Glu induced junction potentials (see methods).

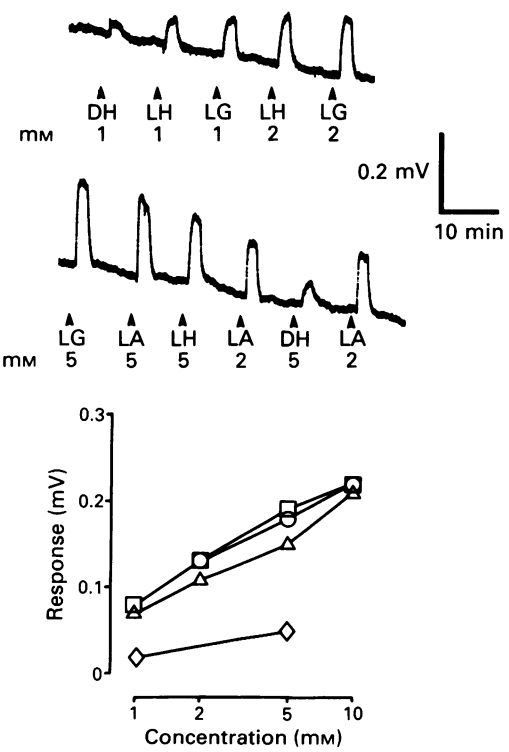


Figure 5 Agonist-induced responses in a synaptically-non viable slice. This slice did not respond to synaptic activation or to N-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) at any time. The chart records show depolarizations induced by L-glutamate (L-Glu, LG), L-aspartate (L-Asp, LA), L-homocysteate (L-HCA, LH) and D-homocysteate (D-HCA, DH). The dose-response plot illustrates the similarities between L-Glu (□), L-Asp (○) and L-HCA (△) and their difference from D-HCA (◇). Note the similarity to the post-lesioned responses in Figure 4.

Effects of other amino acids

The differential properties of D- and L-HCA described above suggested uptake may be important in the generation of AP5/CNQX-resistant responses. Therefore, we examined further the possibility that the residual depolarizations to L-Glu are mediated by an electrogenic uptake process by testing the effects (at 5 mM) of other substrates and inhibitors for L-Glu uptake systems. Those compounds that invariably depolarized lesioned slices are listed in Table 1, which also gives their potency relative to that of 5 mM L-Glu. Of note, D-Glu and D-Asp were of similar potency to L-Glu (Figure 7a), and quisqualate and threo-hydroxy-L-aspartate (THLA) were only slightly less effective (Figure 7b). In addition, several neutral amino acids, such as glycine, D-serine, L-serine and 4-aminobutanoate (GABA) were moderately active (Figure 7) and the basic amino acid L-lysine displayed some activity.

In addition to the compounds listed in Table 1 the following substances were tested, on at least 4 separate preparations, and were found to have different effects: kainate invariably induced a depolarization (18–32% of that of L-Glu) that was followed by a long-lasting negative shift in the d.c. potential (Figure 7a). AMPA produced either similar effects to kainate, (Figure 7b), a small depolarization or a small fast negative shift in the d.c. potential. NMDA either had no effect or induced a small negative shift in the d.c. potential (Figure 7b). The effects of the stereoisomers of AP4 were variable, ranging from small depolarizations (Figure 7b) to fast negative d.c. shifts. Dihydrokainate induced fast negative d.c. shifts (not illustrated).

Discussion

In the present study we have identified, in the CA1 region of rat hippocampal slices, a depolarizing effect of L-Glu that is not generated by actions on AMPA and NMDA receptors. This conclusion is based in part on the relative resistance of responses of L-Glu and L-Asp to antagonism by CNQX and

Table 1 A comparison with L-glutamate (L-Glu) of the activity of amino acids at depolarizing hippocampal slices following an acute excitotoxic lesion

Compound	*Mean ± s.e.*	*Range*	n
L-Glu	100		13
D-Glu	118 ± 13	95–141	3
D-Asp	108 ± 14	84–132	3
L-HCA	105 ± 7	90–114	3
L-Asp	102 ± 3	93–114	9
Quisqualate	82 ± 10	54–100	4
THLA	72 ± 10	58–92	3
Glycine	27 ± 2	23–31	4
L-Serine	25 ± 4	17–40	5
D-Serine	24 ± 4	18–32	3
D-HCA	19 ± 3	14–24	3
L-Alanine	18 ± 1	16–19	4
GABA	17 ± 3	12–24	4
L-Lysine	8 ± 3	3–16	4
AIB	6 ± 1	4–9	4
L-Proline	5 ± 1	3–7	4

The amplitude of the depolarization was expressed as a percentage of that induced by L-Glu in the same slice. n refers to the number of slices tested with each amino acid. All compounds were applied at 5 mM.
Abbreviations: Glu, glutamate; Asp, aspartate; HCA, homocysteate; THLA, threo-hydroxy-L-aspartate; GABA, 4-aminobutanoate; AIB, α-amino-isobutanoate.

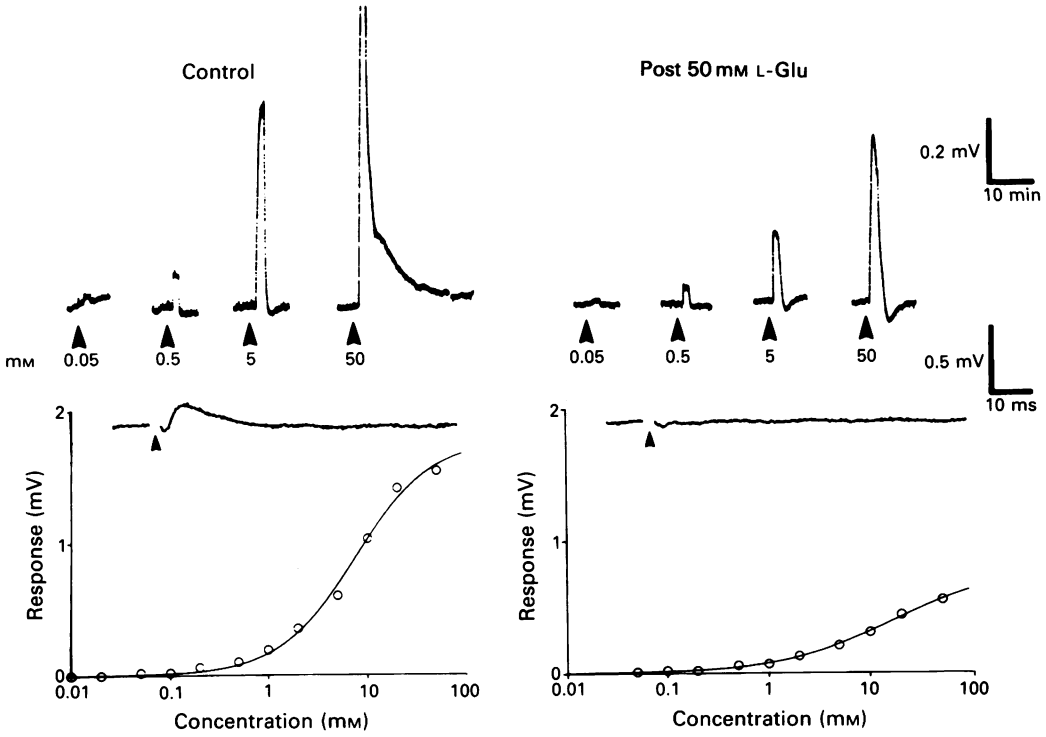


Figure 6 Dose-response curves for L-glutamate (L-Glu) before and following an excitotoxic lesion induced by two applications of 50 mM L-Glu. The first application (partly illustrated) was used in the construction of the first dose-response curve. A second application was required to abolish completely the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-induced responses (not illustrated). Synaptic responses before and following the lesion are illustrated above the respective dose-response plots. The response (Y) was fitted to the concentration (X) of L-Glu using the logistic equation $Y = M(X^p/[X^p + K^p])$ where M is the maximum effect, K is the concentration producing half this effect and the power p determines the slope of the curve (Barlow & Blake, 1989).

AP5. In this respect, it should be noted that since the antagonism is expressed in terms of dose-ratios the resistance to antagonism is unlikely to be attributable to the high agonist concentrations required to elicit the response. This conclusion is supported by the failure of higher antagonist concentrations to produce further significant antagonism. The relative resistance of L-Glu responses to excitotoxic lesions, which abolished responses to AMPA, NMDA and synaptic activation, further supports this conclusion. A major action of L-Glu on other types of excitatory amino acid receptor seems unlikely, since responses to kainate and quisqualate were also greatly reduced by the acute excitotoxic lesion.

An alternative source of depolarization is electrogenic uptake processes into neurones and glia (see Watkins & Evans, 1981). On the basis of radiotracer experiments, uptake of L-glutamate can occur via low affinity ($Km > 100\,\mu M$), high capacity and via high affinity ($Km < 50\,\mu M$), low capacity processes (Balcar & Johnston, 1972b; Logan & Snyder, 1972). These two uptake systems have been distinguished pharmacologically; L-Glu is accumulated in a stereoselective manner by the high affinity uptake system (Balcar & Johnston, 1972a) whereas L-Glu and D-Glu are accumulated similarly by the low affinity process (Balcar & Johnston, 1972b; Levi *et al.*, 1967). In contrast, neither isomer of HCA is believed to be accumulated appreciably by the high affinity process, whereas L-HCA (but not D-HCA) is a good substrate for the low affinity system (Cox *et al.*, 1977). In view of the concentrations of L-Glu required to depolarize the tissue ($EC_{50} > 1$ mM) and the findings that, in lesioned slices, Glu is not stereoselective and that L-HCA (but not D-HCA) is an effective depolarizing agent indicates that these residual responses are due to the low affinity uptake process. In which case, low affinity uptake is electrogenic in nature.

Alternatively, high affinity uptake, which is known to be electrogenic (Brew & Attwell, 1987; Schwartz & Tachibana, 1990), may be responsible for the depolarizations in lesioned slices. Thus, it could be argued that the uptake dose-response curve is, like the receptor-mediated dose-response curve (Garthwaite, 1985), distorted by the uptake process, in terms of the agonist concentration needed to generate a signal. For example, the cells deep in the tissue would receive a low concentration of L-Glu and this could generate the signal via the high affinity site. In this scheme, the signal generated by D-Glu would have to be explained by a non-specific effect on the high-affinity site, which might be possible if these cells experienced a much higher concentration of D-Glu than of L-Glu. However, this would assume that the cells experiencing high concentrations of L-Glu (i.e., surface cells) could establish a concentration-gradient through the slice without contributing a signal. It would also assume that, contrary to radiotracer experiments, D-Glu is not accumulated by a low affinity process. The stereoselectivity of L-HCA is also difficult to explain on the basis of high affinity uptake, since this compound is almost exclusively a substrate for the low affinity process (99–100% at 0.1–2.0 mM, ~80% at 1 μM (Cox *et al.*, 1977)). In addition, other amino acids that are reasonable substrates for low affinity uptake processes, such as glycine, L-serine and L-alanine (Logan & Snyder, 1972; Levi *et al.*, 1967), are also effective agents in lesioned slices. In summary, although a role of high affinity uptake in the generation of these signals cannot be excluded, we favour an electrogenic process, with the characteristics of the low affinity uptake system described in radiotracer experiments.

The L-Glu-induced depolarization could be generated by any cells in electrical contact (via intra- and extra-cellular pathways) with the tissue at the grease barrier (Evans, 1989); this could include CA1 neurones, the axons and terminals of CA3 and other neurones, and glial cells. In lesioned slices it is most probable that the majority of CA1 neurones, if not dead, are functionally inactive. However, glial cells and presynaptic elements are unlikely to be damaged by the acute excitotoxic lesion and are thus candidates for the source of the signal. It is conceivable that, in non-lesioned slices (and *in situ*), an uptake process could also accumulate L-Glu into postsynaptic neuro-

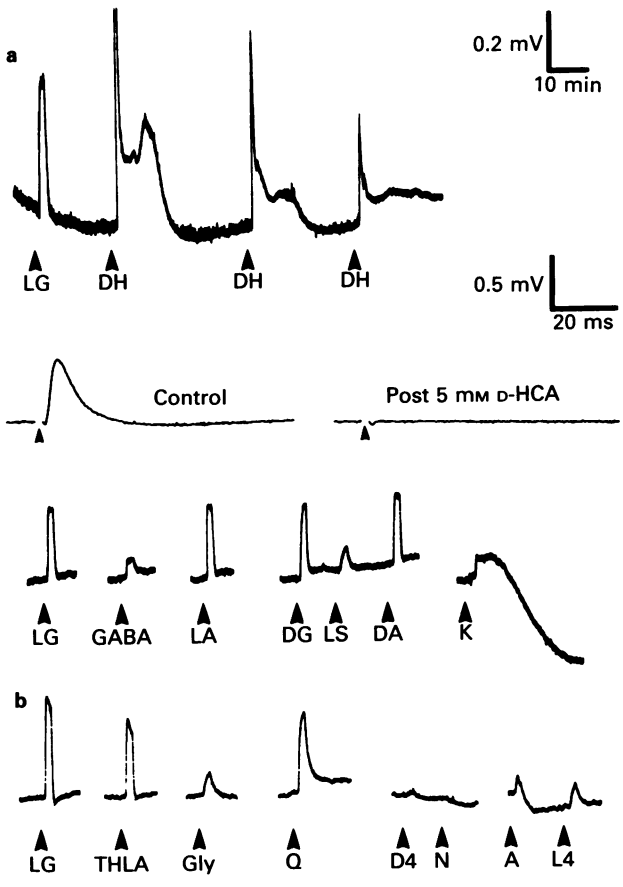


Figure 7 Effects of amino acids (tested at 5 mM) on lesioned slices. The chart record in (a) shows responses to L-glutamate (LG) before and following the acute excitotoxic lesion, which was induced by the three illustrated applications of D-homocysteate (DH; D-HCA). The traces interposed between the chart records are averages of 5 successive synaptic responses obtained prior to the application of any agonist (Control) and following the D-HCA-induced lesion. Excerpts from the chart record also illustrate responses obtained from this lesioned slice to 4-aminobutanoate (GABA), L-aspartate (LA), D-glutamate (DG), L-serine (LS), D-aspartate (DA) and kainate (K). In (b) are excerpts from the chart recording of a slice following a D-HCA-induced lesion to compare the effects of L-Glu with those of threo-hydroxy-L-aspartate (THLA), glycine (Gly), quisqualate (Q), D-2-amino-4-phosphonobutanoate (D4), N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (A) and L-2-amino-4-phosphonobutanoate (L4).

nal elements and thereby depolarize neurones (Watkins & Evans, 1981). If this were the case, it could explain why the L-Glu excitation of single neurones is sometimes reported to be less sensitive to the actions of excitatory amino acid antagonists than are the excitations induced by, for example, NMDA, kainate and quisqualate (Davies & Watkins, 1985; Davies *et al.*, 1988).

Assuming that the depolarization in lesioned slices represents uptake, then this preparation can be used to examine the activity of compounds as substrates for this process. It is likely that the effects measured correspond to activity at a variety of different transport processes. Thus, at least six low affinity transport sites for amino acids have been detected in brain tissue; transporters for GABA, small neutral, large neutral, small basic, large basic and acidic amino acids (Lerner, 1978). In this regard, in the present study L-Glu, D-Glu, L-Asp, D-Asp and L-HCA were indistinguishable and considerably more active than the other amino acids tested; an effect which correlates with the greater effectiveness of the acidic amino acid carrier (Lerner, 1978). A compound that generates a moderate response is glycine; such an action might contribute to the excitatory effects of high doses of glycine in the central nervous system. In this context, it is interesting that serine had a similar potency to glycine but that, in contrast to actions at the allosteric site on the NMDA receptor (Lodge & Johnson, 1990), serine was not stereoselective. THLA, a potent competitive inhibitor of high affinity (Balcar & Johnston, 1972a) and low affinity (Johnston *et al.*, 1980) uptake and quisqualate, a substance believed to be accumulated by a low affinity process (Lodge *et al.*, 1980), also generated fairly large responses.

In conclusion, a major part of the depolarizing action of exogenously administered L-Glu in hippocampal slices is due to an electrogenic uptake process. This component therefore needs to be considered when attempting to estimate the contributions of receptors to the depolarizing actions of L-Glu and other compounds that are substrates for uptake processes.

We are most grateful to Professor J.C. Watkins for generous gifts of compounds and Drs R.H. Evans and R.B. Barlow for valuable discussions. This work was supported by the MRC (J.F.B.) and a Glaxo research studentship (B.G.F.).

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(Received July 23, 1990
Revised September 25, 1990
Accepted September 27, 1990)

Br. J. Pharmacol. (1991), **102**, 363–368 © Macmillan Press Ltd, 1991

Effect of thyrotropin releasing hormone (TRH) on acetylcholine release from different brain areas investigated by microdialysis

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1 The effect of thyrotropin releasing hormone (TRH) administration upon acetylcholine (ACh) release in freely moving rats was investigated by means of transversal microdialysis coupled to h.p.l.c. TRH administered either s.c. or via local perfusion increased the ACh release from the cortex and hippocampus but not from the striatum. The increase in ACh release was maintained after 7 days of s.c. administration of TRH.

2 After s.c. injection of the neuropeptide, the increase in ACh release was dose-dependent and reached a maximum at 40 min after administration. The maximal percentage increases were 18, 52, 66 and 89% at doses of 1, 2.5, 5 and 10 mg kg^{-1} and 35, 48 and 54% at doses of 2.5, 5 and 10 mg kg^{-1} in the cortex and hippocampus, respectively. The effect of TRH was dependent on neuronal activity since it was completely inhibited by perfusion with tetrodotoxin (TTX), 5×10^{-7} M.

3 Perfusion with TRH, 2.5 μg μl^{-1}, caused 198% and 150% increase in ACh release 60 and 80 min after the beginning of the perfusion in the cortex and hippocampus, respectively. After this initial peak, a 100% increase in ACh release persisted throughout the perfusion.

4 Systemic TRH administration was followed by marked hyperactivity and stereotyped behaviour that showed a time course shorter than that of the increase in ACh release.

5 These findings demonstrate that TRH exerts a strong stimulant action on cortical and hippocampal cholinergic pathways.

Introduction

Thyrotropin-releasing hormone (L-pyro-glutamyl-L-histidyl-L-prolineamide, TRH) is present not only in the hypothalamus where it participates in the process of releasing thyrotropin and prolactin from the anterior pituitary (Boler *et al.*, 1969) but is also localized in neurones of the septal nuclei, preoptic area (Brownstein *et al.*, 1974), raphe nuclei of the medulla oblongata (Palkovits *et al.*, 1984) and spinal cord (Harkness & Brownfield, 1985). TRH is synthesized in neuronal cell bodies as a high molecular weight precursor which is then processed via post-translational modifications (Lechan *et al.*, 1986) to make the mature peptide ready to be released from the nerve endings where it is contained in large dense core vesicles (Johansson *et al.*, 1980). Mendez *et al.* (1987) demonstrated that TRH is released from slices of different brain structures in response to depolarizing stimuli. TRH receptors have been found in many areas of the CNS, the highest densities being present in limbic structures such as the amygdala, hippocampus and hypothalmus, and lower densities in the brain stem and cerebellum (Pilotte *et al.*, 1984; Simasko & Horita, 1982). These findings suggest that TRH, in addition to its hormonal activity, plays a role as a neurotransmitter and/or modulator of other neurotransmitter agents (Horita *et al.*, 1986). In particular TRH stimulates the turnover of acetylcholine (ACh) in the parietal cortex of freely moving rats (Malthe-Sorenssen *et al.*, 1978), and in the hippocampus of pentobarbitone-anaesthetized rats (Brunello & Cheney, 1981). Since intraseptal injections of TRH antagonize pentobarbitone-induced depression of ACh turnover and the anaesthetic action of this barbiturate (Brunello & Cheney, 1981), it appears that TRH would stimulate the activity of forebrain cholinergic neurones to elicit behavioural arousal (Horita *et al.*, 1986). A role of cholinergic mechanisms in the behavioural effect of TRH is also indicated by the finding that the TRH-induced arousal in pentobarbitone-anaesthetized rats is blocked by atropine (Kalivas & Horita, 1983). Furthermore, a TRH analogue reverses the learning deficits and hippocampal decrease in high affinity choline uptake induced by lesioning the medial septal cholinergic neurones (Horita *et al.*, 1989).

In the present study we investigated whether the action of TRH on cholinergic neurones was equally present in various brain areas and its characteristics in terms of dose-dependence, time-course and reproducibility. We studied the release of endogenous ACh, which is a reliable index of the functional activity of cholinergic neurones (Pepeu, 1973). ACh release was investigated by the intracranial dialysis technique (Ungerstedt, 1984) in freely moving rats in order to correlate behavioural changes with those in ACh release. TRH was administered either systemically or by intracerebral perfusion.

Methods

Animal housing and surgery

Male adult Wistar rats (Charles River, Italy) weighing 250–300 g were used. They were housed in groups of three with free access to food and water and kept on a 12 h light/dark cycle. The rats were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and placed in a stereotaxic frame (Stoelting, Stellar). Microdialysis tubes were inserted transversely in the parietal cortex, dorsal hippocampi and both striata following the procedure described by Wu *et al.* (1988). The microdialysis tubing (AN 69 membrane, Dasco, Italy; of 220 μm internal diameter and 310 μm external diameter, molecular weight cut-off >15,000 Da) was covered with Super-Epoxy glue along the whole of its length except for a region corresponding to the brain areas to be studied (8 mm for the parietal cortex, 6 mm for the dorsal hippocampus and two sections of 3.5 mm separated by a glued central zone 2.5 mm long for the striata). The coordinates used for the implantation of the microdialysis tubing were as follows (Paxinos & Watson, 1982): for the parietal cortex, AP – 0.5 mm and H 2.5 mm from bregma; for the dorsal hippocampi, AP – 3.3 mm and H 3.4 mm from bregma; for the striata, AP 0.0 mm and H 5 mm from bregma.

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Microdialysis procedure

One day after surgery each rat was placed in a Plexiglas cage and the inlet of the microdialysis probe was connected to a microperfusion pump (Carnegie Medicine, mod. CMA/100, Sweden) while the outlet was inserted into a 200 μl test tube containing 5 μl of 0.5 mM HCl to prevent hydrolysis of ACh. The microdialysis tubing was perfused at a constant flow rate (2 μl min^{-1}) with Ringer solution (composition mM: NaCl 147, $CaCl_2$ 3.4, KCl 4.0, pH 7.0) containing physostigmine sulphate, 7 μM. The molecular weight cut-off of the membrane allows low molecular weight solutes, such as choline (Ch) and ACh, to cross the dialysis membrane according to their concentration gradients and to be collected at the outlet for quantitative analysis.

After 1 h settling period the perfusate was collected at 20 min intervals and directly assayed for ACh and Ch. After collecting the first three samples to measure the basal release of ACh and Ch, TRH (dissolved in normal saline) was injected subcutaneously (1–10 mg kg^{-1}) into a group of rats. In another group, 2.5 μg μl^{-1} TRH (dissolved in Ringer solution containing 7 μM physostigmine sulphate) was perfused through the dialysis tubes. In a third group of rats TRH (5 mg kg^{-1}, s.c.) was administered once daily for 6 days; on the 7th day, the microdialysis membrane was implanted in the parietal cortex after TRH administration. On the following day the release of ACh and Ch was measured before and after the last injection of TRH (5 mg kg^{-1}).

Histological control

At the end of the experiment the rats were anaesthetized with urethane (1.2 g kg^{-1}, i.p.) and killed by decapitation. The brain was rapidly removed and placed in a vial containing 10 ml of 9% phosphate buffered formaldehyde solution. Two or three days later the brain was frozen with liquid CO_2 and coronal slices were cut with a freezing microtome. The 50 μm thick slices were observed under light microscopy (Leitz, Dialva 20 EB) to verify the positioning of the dialysis membrane. Data obtained from rats in which the membrane tubing was not correctly positioned were discarded.

In vitro *recovery experiment*

In order to evaluate the recovery of ACh and Ch through the microdialysis tubing *in vitro* recovery experiments were performed as follows. Dialysis tubing of varying exposed length (prepared as described under 'surgery') was placed in a glass beaker containing Ringer solution with Ch and ACh at concentrations similar to those found in the cortex, hippocampus or striatum of rats killed by microwave irradiation (Spignoli *et al.*, 1986). The membrane was then perfused at room temperature for 20 min with Ringer solution at a flow rate of 2 μl min^{-1}. The content of ACh and Ch of perfused samples and of standard Ringer solution was then measured. Average recovery from two tubes of equal length was then calculated, during three 20 min periods, as a percentage of the amount of ACh and Ch present in the standard solution. The recovery of ACh and Ch from the dialysis tubing was: 56% ± 3.3 and 61% ± 3.4 for the tubing used for the cortex, 56% ± 0.6 and 60% ± 0.7 and 58% ± 2.9 and 64% ± 3.2 (mean ± s.e. mean) for the tubing used for the hippocampus and striatum, respectively. ACh and Ch values reported here were not corrected for recovery.

Assay of acetylcholine and choline in the dialysate

ACh and Ch were directly assayed in the dialysate by a high performance liquid chromatography (h.p.l.c.) method with an electrochemical detector as described by Damsma *et al.* (1987a). The h.p.l.c. apparatus consisted of a Perkin Elmer Series 10 pump (Perkin Elmer, Norwalk, CT, U.S.A.), a presaturation column (Chromspher 5 C18, 100 × 3 mm, Chrompack, Middleburg, The Netherlands), an injector (Rhehodyne 7125, Rhehodyne, Cotati, U.S.A.) equipped with a 32 μl loop, a guard column (reverse phase) and an analytical column (Chromspher 5 C18, 100 × 3 mm, Chrompack), an enzyme reactor (10 × 2.1 mm, Chrompack) containing Lichrosorb-NH_2 activated with glutaraldehyde to which the enzymes acetylcholinesterase (AChE, E.C. 3.1.1.7) and choline oxidase (E.C. 1.1.3.17) were covalently bound, an electrochemical detector (BioAnalytical System, Electroanalytical Instruments) and a Perkin Elmer chart recorder. The mobile phase consisted of 0.1 M phosphate buffer (pH 8.0) with 5 mM KCl added; the solution was filtered through a 0.45 μm nylon membrane (Supelco Inc., Bellefonte, PA, U.S.A.) and degassed by ultrasonic vibration under vacuum. The flow rate was 0.6 ml min^{-1}.

ACh and Ch were separated on a cation exchange column prepared by loading the reverse phase column with sodium lauryl sulphate (0.5 mg ml^{-1}) as described by Damsma *et al.* (1987b). ACh was hydrolyzed by AChE to acetate and Ch in the postcolumn enzyme reactor; Ch was then oxidized by choline oxidase to produce betaine and hydrogen peroxide. Hydrogen peroxide was electrochemically detected by a platinum electrode at +250 mV.

For the quantitative analysis of ACh and Ch we constructed a calibration curve by spiking the Ringer solution with standard ACh and Ch in the concentration-range we expected to find in the dialysates. Three or four concentrations for each calibration curve were then injected at the beginning and the end of the analysis and the heights of the recorded peaks were then plotted against the concentrations. A regression line was calculated and quantitation of unknown samples was carried out by the method of inverse prediction. Under these experimental conditions the sensitivity limit (s/n ratio 3/1) was 30 fmol μl^{-1} and 15 fmol μl^{-1} for ACh and Ch, respectively.

Chemicals

All reagents were of analytical grade. Physostigmine sulphate, AChE (E.C. 3.1.1.7., grade VI-S) and choline oxidase (E.C. 1.1.3.17) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TRH (bitartrate salt) was kindly supplied by Cyanamid Italia (Catania).

Statistical analysis

ACh and Ch release rates were expressed as percentage variation over basal output which in each group of experiments was the mean (± s.e.mean) of all pre-drug determinations. Significant differences were evaluated on the percentage values by use of one-way analysis of variance, followed by Dunnett's test. P values of less than 0.05 were considered significant.

Results

Single administrations

The release of ACh and Ch at rest in freely moving rats is shown in Table 1. There was no significant difference in ACh release between the cortex and the hippocampus, while the

Table 1 Acetylcholine (ACh) and choline (Ch) release from different brain regions in freely moving rats at rest

	Cortex	*Hippocampus*	*Striatum*
ACh	4.4 ± 0.24 (40)	4.5 ± 0.24 (48)	10.4 ± 0.63* (17)
Ch	111.8 ± 4.8 (32)	114.7 ± 5.1 (41)	105.9 ± 5.7 (12)

Release expressed as pmol/20 min ± s.e.mean. Number of determinations in parentheses. *$P < 0.01$ with respect to the cortex and hippocampus determined by Student's t test.

release from the striatum was significantly larger. No significant differences were found among the release of Ch from these three brain structures. The subcutaneous administration of TRH at doses of 1, 2.5, 5 and 10 mg kg^{-1} brought about an increase in ACh release from the cerebral cortex and hippocampus (but not from the striatum) 1 h after the beginning of sample collection. Ch efflux was not modified, regardless of the doses used and the regions investigated. Figure 1 shows the effect of TRH on cortical release of ACh. The curves represent the time-course of the increase for each dose tested, expressed as percentage changes from the means of three collection periods before TRH administration, taken as controls. TRH elicited a dose-dependent increase in ACh release that peaked 40 min after its administration and returned to basal values within 100 min. While 1 mg kg^{-1} was ineffective, the peak response to the 2.5, 5 and 10 mg kg^{-1} administration was significantly different from controls. The largest effect (+87 ± 20%) was observed following 10 mg kg^{-1} TRH. Figure 2 shows the effect of TRH on the release of ACh from the hippocampus. The maximal increase in ACh release occurred between 40 and 60 min after TRH administration, and was statistically significant for all doses of TRH used (2.5, 5 and 10 mg kg^{-1}). Altogether, the increases observed in the hippocampus were lower than those observed in the parietal cortex (see Figure 1) and saturated at 5 mg kg^{-1} TRH. Injection of the vehicle alone (normal saline, 1 ml kg^{-1}) did not change the release of ACh from the cortex and hippocampus, ruling out an effect upon ACh release due to the stress of the injection. Finally, at a dose of 5 mg kg^{-1} TRH did not change the release of either ACh or Ch from the striatum (−4.5 ± 2%; $n = 3$).

A few minutes after a s.c. injection of TRH, stereotyped behaviours such as wet-dog shaking, sniffing, forepaw licking, tail elevation, face washing and hyperactivity were observed. Although no quantitative analysis of the behavioural changes was made, it was apparent that these effects developed within the first 20 min after TRH administration and then gradually disappeared.

It is well established that locally applied tetrodotoxin (TTX) blocks the release of ACh (Damsma *et al.*, 1988). In the present experiments we studied whether TRH (5 mg kg^{-1}) could reverse the action of TTX on the ACh release from the cerebral cortex. Figure 3 shows that following local application of TTX (5×10^{-7} M) via the dialysis tubing the s.c. injection of TRH (5 mg kg^{-1}) did not enhance the release of ACh which gradually fell to undetectable values.

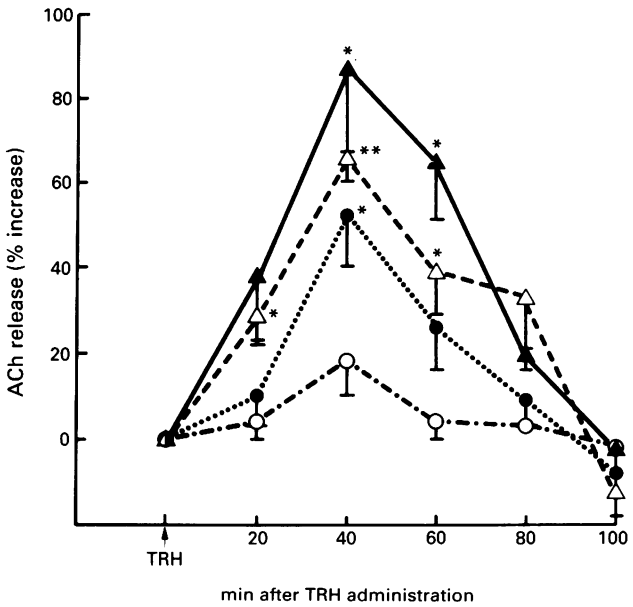


Figure 1 Effect of s.c. administration of thyrotropin releasing hormone (TRH) on the release of acetylcholine (ACh) from the parietal cortex expressed as percentage variation over basal output: TRH, 1 mg kg^{-1} (○); 2.5 mg kg^{-1} (●); 5 mg kg^{-1} (△); 10 mg kg^{-1} (▲). Each point represents the mean of at least 4 independent experiments; s.e.mean shown by vertical bars. Significant differences were evaluated by comparing the percentage variation vs the mean ± s.e.mean of all pre-drug determinations. *$P < 0.05$; **$P < 0.01$, Dunnett's test.

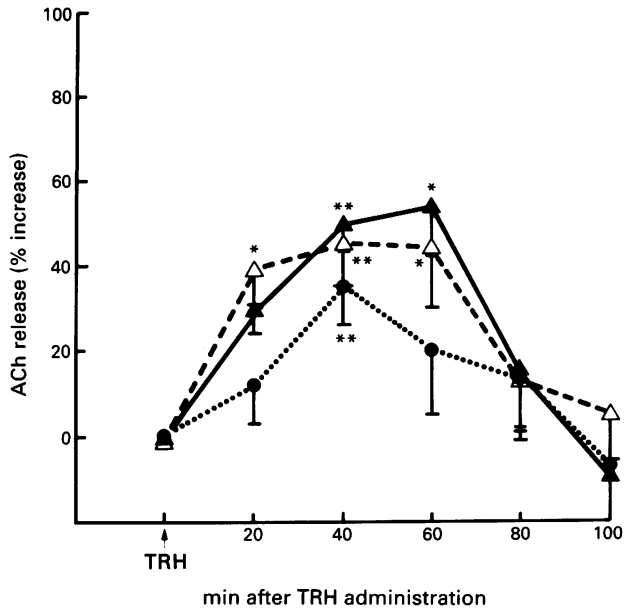


Figure 2 Effect of s.c. administration of thyrotropin releasing hormone (TRH) on hippocampal acetylcholine (ACh) release expressed as percentage variation over basal output: TRH 2.5 mg kg^{-1} (●); 5 mg kg^{-1} (△); 10 mg kg^{-1} (▲). Each point represents the mean of at least 4 independent experiments; s.e.mean shown by vertical bars. Significant differences were evaluated by comparing the percentage variation vs the mean ± s.e.mean of all pre-drug determinations. *$P < 0.05$; **$P < 0.01$, Dunnett's test.

Repeated administration

In order to verify whether tolerance may develop after repeated administration of TRH, cortical ACh release was investigated in rats injected daily with TRH (5 mg kg^{-1} s.c. for 7 days). On day 6 the microdialysis tubing was implanted and on day 7 release of ACh and Ch was investigated before and after the last administration of 5 mg TRH. The basal ACh and Ch outputs from the parietal cortex were similar to those of untreated animals. The size and time-course of the increase in ACh release were not significantly different from those observed in untreated animals (+78 ± 14%; $n = 4$).

Intracerebral perfusion

Figure 4 shows the effect of perfusing 2.5 μg μl^{-1} TRH through the dialysis tubing on ACh release from the cortex, hippocampus and striatum. Each curve represents the time-course of the increase, expressed as percentage change from the mean of three collection periods before TRH. TRH perfusion started 60 min after the beginning of the collection period and lasted throughout the experiment. TRH perfusion was accompanied by a significant increase in ACh release from the cortex and hippocampus but not from the striatum. The maximal increase of 200% for the cortex and 150% for the hippocampus occurred 60 and 80 min, respectively, after the beginning of TRH administration. Both in the cortex and the hippocampus, the increase was significantly different from the mean of the basal values at 40 min after the beginning of TRH perfusion (Dunnett's test, $P < 0.05$). After a peak value, the increase in ACh release settled at about 100% rise which persisted throughout the perfusion period. In three experiments a dose of 0.5 μg μl^{-1} of TRH was used. This lower dose

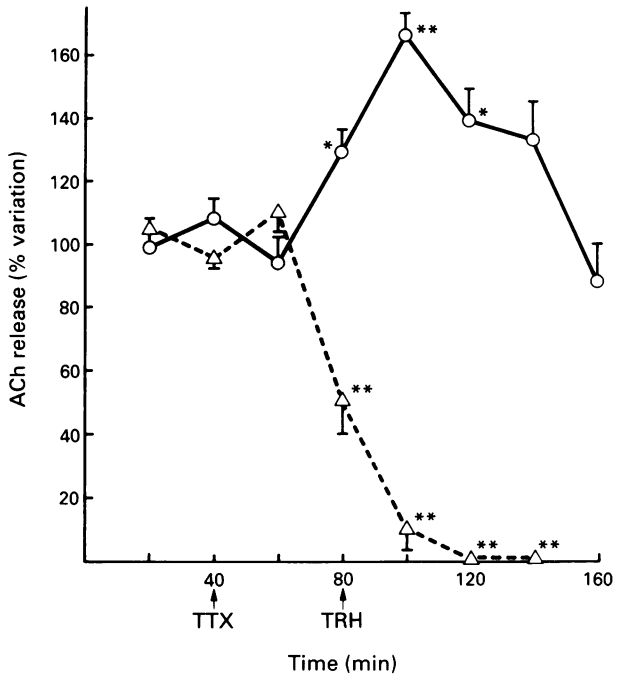


Figure 3 Effect of tetrodotoxin (TTX) perfusion on acetylcholine (ACh) release elicited by s.c. thyrotropin releasing hormone (TRH) administration to the cortex: (○) 5 mg kg^{-1} TRH only; (△) 5 mg kg^{-1} TRH administered 20 min after the beginning of 5 × 10^{-7} M TTX perfusion. Each point represents the mean of at least 3 independent experiments; s.e.mean shown by vertical bars. Significant differences were evaluated by comparing the percentage of variation vs the mean ± s.e.mean of all pre-drug determinations. *$P < 0.05$; **$P < 0.01$, Dunnett's test.

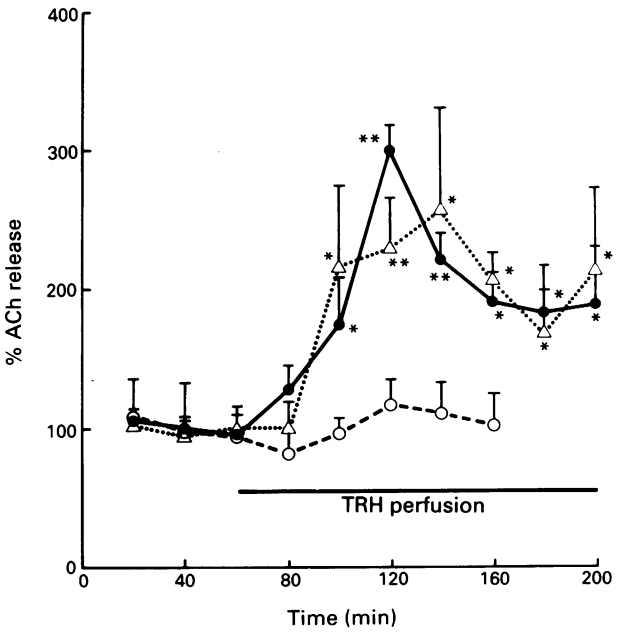


Figure 4 Effect of thyrotropin releasing hormone (TRH) perfusion (2.5 μg μl^{-1}) on acetylcholine (ACh) release from the cortex (●), hippocampus (△) or striatum (○). TRH perfusion started after three basal values had been collected and lasted throughout the experiment. The three individual basal values are shown and expressed as percentage values ± s.e.mean. Each point represents the mean of at least 4 independent experiments; s.e.mean shown by vertical bars. Significant differences were evaluated by comparing the percentage variation with the mean ± s.e.mean of all pre-drug determinations. *$P < 0.05$; **$P < 0.01$, Dunnett's test.

elicited a +66 ± 20% increase in the release of ACh from the parietal cortex, showing a dose-dependence in the effect.

Stereotyped behaviours were not seen after local perfusion of TRH.

Discussion

The technique of microdialysis *in vivo* has enabled us to evaluate directly the effects of TRH on the release of endogenous ACh from the brain of freely moving rats. Such a release is considered to be a reliable index of the functional activity of central cholinergic neurones (Pepeu, 1973).

Most importantly, our results demonstrate that TRH selectively activated the cholinergic neurones of the cortex and the hippocampus, while being ineffective on those of the striatum, as shown by the marked and long lasting increase in the release of endogenous ACh from the first two brain areas. These differences in regional cholinergic activation may be related to the uneven distribution of TRH receptors in the central nervous system (Simasko & Horita, 1982; Pilotte *et al.*, 1984) with the striatum showing the lower density among the three brain regions under investigation (Ogawa *et al.*, 1983). Our results provide direct evidence that TRH modulates central cholinergic transmission and accord with other circumstantial evidence in favour of it. In fact, intraseptal injection of TRH increases the turnover of ACh in the hippocampus of pentobarbitone-anaesthetized rats (Brunello & Cheney, 1981) and i.c.v. administration of TRH increases the turnover of ACh in the parietal cortex of freely moving rats but not in other areas investigated (Malthe-Sorennsen *et al.*, 1978). Muscarinic antagonists also block some central effects induced by TRH, including hypothermia and reversal of the hypnotic action of barbiturates (Breese *et al.*, 1975; Kalivas & Horita, 1979). No direct demonstration of the ability of TRH to enhance ACh release from the central nervous system has been reported before. In this respect, Hedley & Vaughan (1985) failed to demonstrate any effect of TRH on ACh release from rat nucleus accumbens and septal *in vitro* cubes both in the presence and absence of depolarizing concentrations of K^+. One can only surmise that these areas are, like the striatum, comparatively insensitive to TRH.

It has been shown that TTX infusion (10^{-6}–10^{-7} M) through the microdialysis probe causes a drop in the output of basal ACh to undetectable values (Damsma *et al.*, 1988). In our experiments with local application of TTX (5 × 10^{-7} M) the s.c. injection of TRH (5 mg kg^{-1}) was unable to enhance the release of ACh which gradually fell to undetectable values. This result indicates that the effect of TRH depends on propagated electrical activity of the cholinergic neurones themselves or of afferent fibres that in turn activated the cortical cholinergic pathways. The possibility that TRH may exert an indirect action on cortical neurones is supported by the findings of Burov *et al.* (1989) who showed that while systemic administration of TRH induces an increase of the electrical activity of sensorimotor cortical neurones, microiontophoretic application of TRH causes a decrease in the spike rate of most of them. They assumed that the excitation of cortical neurones by systemic administration of TRH was due to activation of extracortical neurones exerting a stimulatory effect on the cortex. On the other hand, direct stimulation by TRH of rat cholinergic neurones such as motoneurones has been demonstrated (Takahashi, 1985; Lacey & Nistri, 1989).

In our experiments after one week of daily administration of TRH (5 mg kg^{-1}) no tolerance to its effects was observed. On the contrary, Ogawa *et al.* (1983) found a down regulation of TRH receptors in the cortex, hippocampus and hypothalamus of rats treated daily for 14 days with TRH (6 mg kg^{-1}, i.p.). It appears therefore that in our experiments either the duration of treatment was too short and/or the dose used too low to develop tolerance to the effect of TRH. It might also be the case that a down regulation of TRH recep-

tors develops, but that it is too small to influence the release of brain ACh if the system possesses adequate spare capacity.

Systemic administration, but not local infusion, of TRH was followed by marked behavioural effects, such as wet-dog shaking, sniffing, forepaw licking, tail elevation, face washing and hyperactivity. These effects reached a maximum 20 min after TRH administration and then gradually disappeared, showing a shorter time-course than the increase in the release of ACh. Thus, the different time-course of the biochemical and behavioural effects and the lack of the latter during the increase in ACh output elicited by local TRH infusion indicate that the two effects are not simply related to each other. Although Sills *et al.* (1988) reported that the wet-dog shaking caused in the rat by an analogue of TRH (MK-771) is blocked, in a noncompetitive manner, by atropine or scopolamine, other evidence indicates that the stereotyped behaviour and the locomotor effects elicited by TRH rely also on other neurotransmitter systems, particularly the dopaminergic system of the nucleus accumbens (Roth *et al.*, 1976; Kalivas *et al.*, 1987). In this respect, TRH stimulates the release of [^{3}H]-dopamine from slices of rat nucleus accumbens but not nucleus caudate *in vitro* (Kerwin & Pycock, 1979); bilateral injections of TRH into the nucleus accumbens of rats increase locomotor activity and promote behavioural activities similar to those produced by activation of the dopaminergic system (Heal & Green, 1979; Heal *et al.*, 1981).

Since in the present study no close correlation between increased release of brain ACh and stereotyped behaviour was observed, the question arises as to whether the enhanced activity of cholinergic neurones may have other functional consequences. The recent demonstration that an analogue of TRH can reverse learning deficits caused by cholinergic impairment (Horita *et al.*, 1989) might indicate that cognitive rather than motor behaviour is more susceptible to the interaction between ACh and TRH.

This research was partly funded by the NATO Grant N. 0388/88 and CNR Grant N. 89.02737.04. We thank Mr C. Susini for his technical assistance.

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(Received June 22, 1990
Revised September 14, 1990
Accepted October 2, 1990)

Br. J. Pharmacol. (1991), **102**, 369–372 © Macmillan Press Ltd, 1991

Secretion of growth hormone elicited by intravenous desipramine in the conscious, unrestrained rat

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1 Acute intravenous administration of either clonidine (Clon) ($50\,\mu g\,kg^{-1}$) or desipramine (DMI) ($5\,mg\,kg^{-1}$) elicited a pulse of growth hormone (GH) and corticosterone secretion in conscious, unrestrained rats.

2 The responses to DMI were similar to those with Clon, except that the GH pulse following DMI was delayed and was not dose-dependent.

3 The GH response to DMI was inhibited by prior administration of idazoxan ($1\,mg\,kg^{-1}$) or yohimbine ($0.5\,mg\,kg^{-1}$), but not by atropine ($10\,\mu g\,kg^{-1}$), sulpiride ($5\,mg\,kg^{-1}$) or prazosin ($1\,mg\,kg^{-1}$).

4 The corticosterone secretion following DMI was not altered by prior atropine, sulpiride or prazosin, but was augmented by idazoxan ($1\,mg\,kg^{-1}$).

5 GH secretion was not influenced by atropine, sulpiride, prazosin or idazoxan given alone. Idazoxan or yohimbine given alone elicited significant secretion of corticosterone.

6 It is concluded that i.v. DMI caused an activation through indirect mechanisms of α_2-adrenoceptors specifically involved in hypothalamic-pituitary regulation of GH release and also a distinct, independent and transient generalized activation of the pituitary-adrenal axis.

Introduction

The functional state of specific neurotransmitter receptors may be monitored by the measurement of anterior pituitary hormone secretion elicited by acutely administered agonist drugs (Muller, 1979). Plasma concentrations of growth hormone (GH), prolactin and thyroid-stimulating hormone have proved useful in monitoring the responsiveness of receptors involved in the neurohypothalamic-pituitary axis (Muller, 1989). Among the most extensively studied of specific responses is the prompt pulse of GH secretion elicited by administration of clonidine (Clon) in rats (Durand *et al.*, 1977; Eden & Modigh, 1977). The rat pituitary response of GH secretion following intravenous Clon has been firmly identified as a primary neural effect brought about by activation of hypothalamic α_2-adrenoceptors (Krulich *et al.*, 1982) which are located post-synaptically (Eriksson *et al.*, 1982).

GH secretory responses to Clon, similar to those found in rats, have also been demonstrated in man and have been employed as probes of α-adrenoceptor functional status in disease states including endogenous depression (Checkley *et al.*, 1984). Intramuscular or oral administration of the tricyclic antidepressant desipramine (DMI) has also been shown to cause an acute rise in plasma GH in healthy human subjects, an effect inferred, via the blockade of noradrenaline uptake, to have been elicited by the action of increased endogenous neurotransmitter on α_2-receptors (Laakmann *et al.*, 1986). It has been claimed that this response is also blunted in those diagnosed as suffering clinically significant depression of the endogenous type (Laakmann, 1980; Sawa *et al.*, 1982). Acutely administered DMI has a number of actions in addition to inhibition of noradrenaline reuptake, i.e. high-affinity binding to α_1-adrenoceptors and muscarinic cholinoceptors with lower affinity for 5-hydroxytryptamine (5-HT) and dopamine receptors (Hall & Ogren, 1981). In view of these effects of DMI and its reported efficacy in mimicking the effect of Clon in man, an investigation was made of the actions of DMI compared to Clon on GH secretion by rat pituitary.

The experiments were performed on conscious, unrestrained animals subjected to the minimum attainable degree of disturbance since both anaesthetics (Takahashi *et al.*, 1971) and stress (Schalch & Reichlin, 1968; Terry *et al.*, 1976) are known to influence GH secretion in rats. These objectives were achieved by the use of animals implanted with chronic intravenous cannulae permitting both drug-dosing and blood-sampling in conscious animals with minimum perturbation. In order to assess any effects of pituitary activation, other than specific effects leading to the secretion of GH, plasma corticosterone was assayed in parallel with GH determinations.

Methods

Animals and surgery

Male Wistar rats (300–320 g) were anaesthetized with chloral hydrate ($375\,mg\,kg^{-1}$) and intra-atrial cannulae implanted via the right external jugular vein by a method modified from that of Harms & Ojeda (1974). Briefly, the cannula was filled with heparinised saline, inserted 3 cm into the vein and secured by a cuff to the pectoralis major muscle. The free end of the cannula was then passed under the skin and drawn out at the back of the neck, so that the free end extended 1 cm. The wound was closed and the lumen sealed with a removable wire plug. On completion of surgery animals were housed one per cage in a controlled environment with a 12 h light : 12 h dark cycle (lights on at 08 h 00 min) and given food and water *ad lib.* The animals were deemed to have recovered from surgery when pre-operative weight was regained (3–5 days).

Drug challenge and blood sampling

Both injection of drugs and withdrawal of blood samples were performed via the cannula. One hour before use the cannula was unsealed and a length of polyethylene tubing (PE 50, i.d. 0.58 mm, o.d. 0.96 mm, Portex) sufficient to extend outside the cage was attached to the cannula. Drugs were injected, or blood samples withdrawn, by syringe attached to the Portex tubing. Blood was drawn into syringes rinsed with heparin ($1000\,u\,ml^{-1}$) and transferred to polypropylene centrifuge tubes (Eppendorf tubes, 0.5 ml) for separation of plasma. Cells were resuspended in saline and returned to the animal following removal of the next sample.

Rat GH secretion is pulsatile in nature with a periodicity of about 3 h, the first peak occurring just after 'lights-on' and the first trough beginning 3 h later (Tannenbaum & Martin, 1976).

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Therefore, the first blood sample was withdrawn at 10 h 30 min followed immediately by an injection of saline or antagonist drug. A second pre-challenge sample was withdrawn at 10 h 50 min. Drug challenges were at 11 h 00 min, and 4 post-challenge samples were taken at 11 h 05 min, 11 h 20 min, 11 h 35 min, and 11 h 50 min. All blood samples were of 0.4 ml volume. animals were rested for 3 days before being challenged again.

Growth hormone and corticosterone assays

All plasma samples were stored frozen (−20°C) in Eppendorf tubes prior to assay. Rat GH was assayed by radioimmunoassay (RIA) with materials supplied by Dr A.F. Parlow and the National Institute for Diabetes and Digestive, and Kidney Diseases (NIDDK). Purified rat GH (NIADDK-rGH-1-5) was used for labelling with ^{125}I. The NIADDK reference preparation rGH-RP-2 was used for standards, and the antiserum used was NIADDK-anti-rGH-S-5. Addition of label was delayed by 2 h; this non-equilibrium protocol increased the sensitivity of the assay (Rodbard *et al.*, 1971). Separation of antibody-bound from free hormone was by addition of Protein A (Sigma Chemical Co.). The intra-assay and inter-assay variations were 6.9 and 11.6%, respectively.

Plasma corticosterone was routinely assayed by a modification of the chloroform extraction and ethanol : sulphuric acid fluorescence technique (Glick *et al.*, 1964). To a plasma sample (0.1 ml) in a polypropylene tube (Eppendorf tube 1.0 ml) was added 0.6 ml chloroform. Corticosterone was extracted by vortex mixing. The chloroform phase (0.5 ml) was removed to a similar tube containing 0.4 ml ethanol : sulphuric acid (35 : 65) and shaken to re-extract adrenocortical steroids and develop fluorescence. The fluorescence of the acid phase was determined at excitation wavelength 474 nm, emission 518 nm. Appropriate standards and blank were processed in parallel. The validity of the fluorimetric assay was checked against an RIA employing ^{3}H-labelled corticosterone, and rabbit anti-corticosterone 21-thyroglobulin serum. The fluorimetric method correlated well with the RIA ($r = 0.93$, $n = 24$), and plasma from animals 14 days post adrenalectomy showed no detectable corticosterone in the fluorimetric assay. The intra- and inter-assay variances were 8.3 and 11%, respectively, for the fluorescence method.

Analysis of data

GH concentrations were measured in ng ml^{-1} and are presented as median ± interquartile ranges (Clarke & Cooke, 1983). The data were not normally distributed and therefore non-parametric tests were used for group comparisons. Post-treatment levels were compared with the immediately pre-treatment levels by Wilcoxon's matched-pairs signed-rank test, each animal acting as its own control. For comparisons between treatments, differences of post-treatment values from baseline were calculated and peak changes (ΔGH) for each animal were plotted. Results were expressed as median values and the significance of any differences between medians was assessed by the Mann-Whitney U-test. Plasma corticosterone levels (μg dl^{-1}) were normally distributed and so these were expressed as mean value for a group of animals. Significance of differences between pre- and post-treatment corticosterone concentrations was assessed by paired Student's *t* test, while the unpaired *t* test was used for between-treatment comparisons.

Drugs

Chloral hydrate was purchased from BDH Chemicals, desipramine HCl, clonidine HCl, atropine SO_4, and yohimbine HCl were purchased from Sigma Chemical Company. Prazosin HCl was a gift from Pfizer Ltd, idazoxan HCl was a gift from Reckitt and Colman Ltd, and sulpiride was a gift from De La Grange. Chloral hydrate (7.5%) was dissolved in saline, and administered in a vol of 5 ml kg^{-1} i.p. All other drugs were injected i.v. in a volume of 1 ml kg^{-1}. Prazosin HCl (1 mg ml^{-1}) was dissolved in distilled water and sonicated. Sulpiride (5 mg ml^{-1}) was dissolved in distilled water, 1–2 drops of 1 N HCl added and sonicated, and the pH was adjusted to 6–8 with 1 N NaOH. All other drugs were dissolved in sterile saline (0.9%), to give the following concentrations per ml: 0.05, 0.1, 0.25, 0.5, 1.0 and 5.0 mg DMI; 10, 25, 50, and 100 μg Clon; 10 μg atropine SO_4; 1 mg idazoxan HCl; 0.5 mg yohimbine HCl.

Results

Drug challenges

Cannulated rats, when administered saline vehicle at 11 h 00 min, did not show any subsequent significant increase in GH during the sampling period (Figure 1). Clonidine (50 μg kg^{-1}) elicited an increase in plasma GH, which was raised significantly over pre-drug levels at 20 and 35 min post-challenge. At 50 min post-injection GH had subsided to pre-drug values. DMI (5 mg kg^{-1}) also caused a pulse of GH secretion which differed temporally from the pattern after Clon in that a significant rise was achieved only at 35 min following drug challenge, but was similar in that the effect was essentially terminated 50 min post-injection.

Dose-dependence of growth hormone responses

The dependence of the GH secretory response on dose of drug was assessed by use of various dose levels of secretagogues in the standard challenge protocol. Results of these experiments

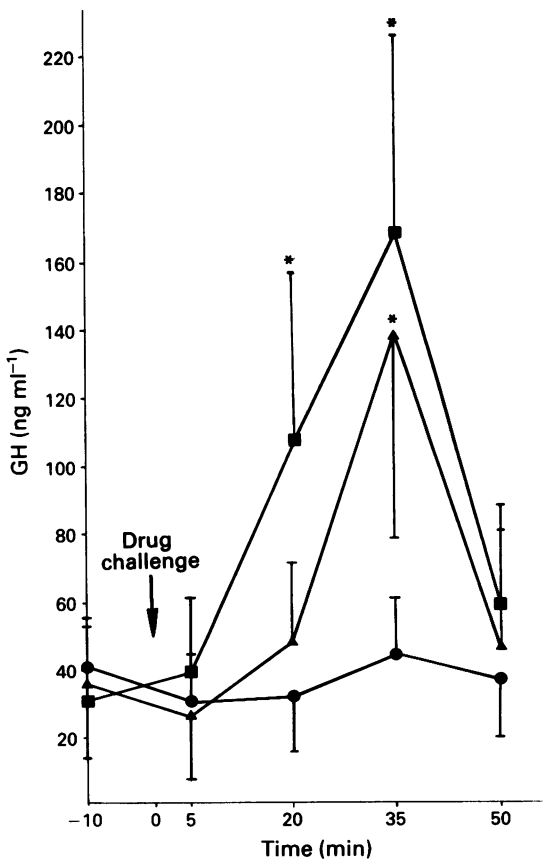


Figure 1 Median (± semi-interquartile range, 1/2{Q3 − Q1}) growth hormone (GH) levels following i.v. saline (●) ($n = 7$), clonidine 50 μg kg^{-1}, (■) ($n = 8$), or desipramine (DMI 5 mg kg^{-1}), (▲) ($n = 8$). *Significantly increased over pre-treatment value at $P < 0.05$, (Wilcoxon's matched-pairs signed-rank test).

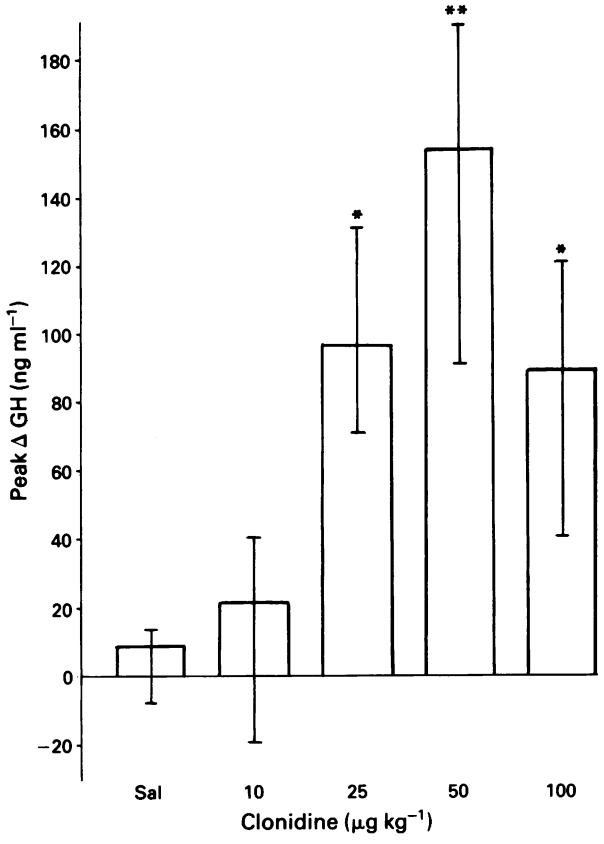


Figure 2 Median growth hormone (GH) responses (± interquartile range) to clonidine ($n = 9$) or saline ($n = 7$) expressed as peak ΔGH. Statistically significant differences from the control group are shown as $*P < 0.005$; $**P < 0.001$ (Mann-Whitney U-test).

are shown in Figures 2 and 3, where the magnitude of secretory response as peak ΔGH is illustrated for the dose-ranges tested. Response to Clon was statistically significant at doses above $10\,\mu g\,kg^{-1}$; responses showed a dose-related increase for doses up to $50\,\mu g\,kg^{-1}$, while the median response to the $100\,\mu g\,kg^{-1}$ dose was reduced from the $50\,\mu g\,kg^{-1}$ level (Figure 2).

Low doses of DMI ($0.05–0.25\,mg\,kg^{-1}$), unlike clonidine at these doses, did not elicit statistically significant GH release. Doses of $0.5\,mg\,kg^{-1}$ and higher doses all yielded significant responses, although no clear dose-response relationship was observed (Figure 3).

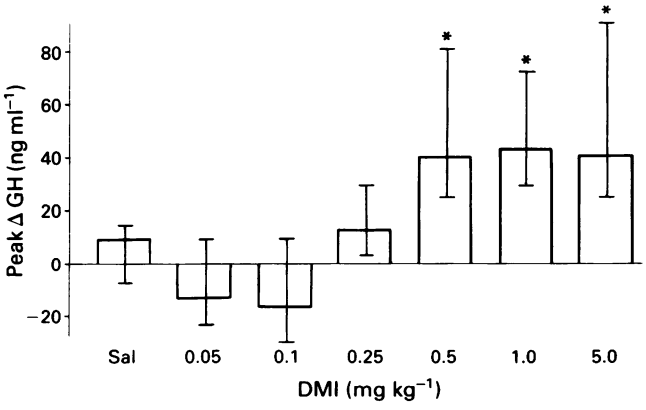


Figure 3 Median growth hormone (GH) responses (± interquartile range) to saline or to desipramine (DMI, $n = 12$ for $5\,mg\,kg^{-1}$ group, $n = 8$ for other doses), expressed as peak ΔGH. *Significantly different from saline controls at $P < 0.05$ (Mann-Whitney U-test).

Effect of receptor antagonists on the response to desipramine

Cannulated rats were given doses, chosen for optimum efficacy, of various receptor antagonists at 30 min prior to a DMI challenge performed as described above. The dose of DMI was $5\,mg\,kg^{-1}$, this being the most consistently effective of the doses of DMI employed. The influence of prior treatment with muscarinic, D_2-dopamine receptor and α_1- or α_2-adrenoceptor antagonists on the subsequent response to a standard DMI challenge was investigated. Both idazoxan ($1\,mg\,kg^{-1}$) and yohimbine ($0.5\,mg\,kg^{-1}$) significantly reduced the GH response to subsequent challenge with DMI ($P < 0.05$ Mann-Whitney U-test). Atropine, sulpiride or prazosin did not attenuate the response. Administered alone at the same doses as used in pretreatment, none of the receptor antagonists had any detectable effect on plasma GH.

Effects of receptor antagonists on plasma corticosterone

Plasma corticosterone concentrations were determined in parallel with plasma GH following the various drug challenges investigated. Animals given saline alone showed no subsequent alteration in plasma corticosterone for the duration of the sampling period. Clon at 10 or $25\,\mu g\,kg^{-1}$ had no significant effect; at 50 or $100\,\mu g\,kg^{-1}$ Clon, corticosterone was elevated at 5 min post-injection. DMI at $5\,mg\,kg^{-1}$ characteristically caused a significant rise in corticosterone levels also evident at 5 min post-injection. In rats treated with idazoxan prior to DMI there was an elevation in corticosterone levels compared to saline controls, but the effect was temporally different from that seen after DMI alone, being manifest at 20 min and sustained until 35 min post-injection (Figure 4).

Idazoxan alone elevated corticosterone at 5 min post-injection, the level falling back to base-line by 20 min. Yohimbine increased corticosterone levels at 20 and 35 min post-injection. As in the case of GH response, none of the other blockers (atropine, sulpiride or prazosin) had any significant effect on plasma corticosterone when administered alone. Animals administered these blockers prior to DMI failed to show a subsequent rise in corticosterone levels, although the concentrations found were not significantly lower than those obtained with DMI alone.

Discussion

The present investigation employed the technique of permanent cannulation of the jugular in order to examine neuroendocrine responsiveness in unanaesthetized, unrestrained,

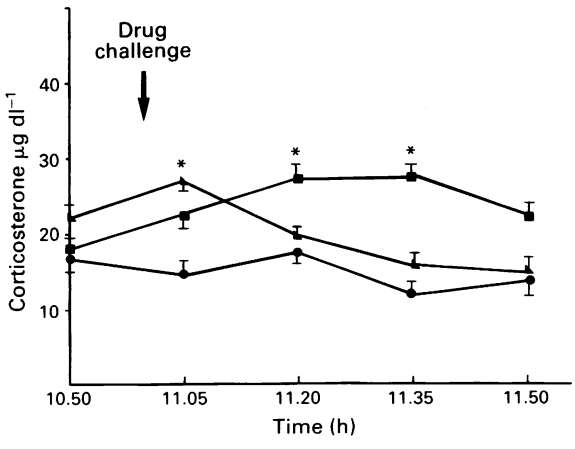


Figure 4 Corticosterone levels (mean with s.e.mean shown by vertical bar) following saline (●) ($n = 7$), desipramine (DMI, $5\,mg\,kg^{-1}$) (◣) ($n = 40$), or idazoxan $1\,mg\,kg^{-1}$ (■) 30 min prior to DMI ($n = 6$). *Significantly different from pretreatment values at $P < 0.05$ (paired Student's t test).

and by implication, unperturbed animals. By the criterion of weight regain animals had recovered from the cannulation surgery within 3–5 days, and the basal levels of both corticosterone and GH, against which drug challenges were evaluated, showed that the period of day chosen was appropriate for the demonstration of subtle responses of the neuro-hypothalamic axis.

The GH response to intravenous Clon was very similar to that reported by Krulich *et al.* (1982). While a degree of dose-dependence was found there was a reduction noted at the highest concentration. Eden & Modigh (1977), using doses higher than $100\,\mu g\,kg^{-1}$, failed to show a dose-response relationship. A falling-off in response may be ascribed to the possibility of α_1-adrenoceptor effects or partial agonist behaviour of Clon at high doses. In the case of DMI, although the GH response was qualitatively similar to that evoked by Clon, two differences were apparent. Firstly, the DMI action was delayed compared to that of Clon. If, as is likely, DMI works indirectly through an increased availability of endogenous transmitter following on inhibition of noradrenaline reuptake, then some delay in onset of action may be expected even after acute intravenous administration of DMI. The second difference in the case of DMI effects was the absence of any clear dose-response relationship. This also may be explained by the indirect nature of the action of DMI on α_2-adrenoceptors.

The response to DMI, although apparently indirectly mediated, was unequivocally blocked by prior treatment with α_2-adrenoceptor antagonists and therefore it may be inferred that the DMI effect is brought about by a final mechanism similar to that with Clon, i.e. activation of α_2-adrenoceptors involved in neuro-hypothalamic regulation. The sensitivity of the GH secretion by DMI to blockade by prior idazoxan or yohimbine and the ineffectiveness of muscarinic, D_2-dopamine or α_1-adrenoceptor antagonists are similar to the findings on GH response to DMI in man (Laakmann *et al.*, 1986); in the rat the GH response to Clon has been shown to be also specifically blocked by α_2-antagonist pretreatment (Eriksson *et al.*, 1982). Other theoretically possible actions of DMI on central receptors, including an action on α_1-adrenoceptors, are not likely to be involved in the GH response. Although Krulich *et al.* (1982) found that α_1-adrenoceptor agonists evoked a prazosin-sensitive lowering of rat plasma GH, in our experiments no effects of DMI consistent with such actions were detected.

The secretion of GH in response to DMI and to Clon was preceded by a brief pulse of corticosterone, indicating that these injected drugs activated the hypothalamic-pituitary-adrenal axis in addition to specifically triggering mechanisms leading to pituitary GH release. The results of prior administration of various blocking agents on the GH and corticosterone responses to DMI showed that the two effects were separable, since both idazoxan and yohimbine reduced the GH response but not the corticosterone secretion following DMI. In contrast, the three blocking agents which failed to alter the GH response had no clear effect on the corticosterone levels following DMI.

We wish to thank the Health Research Board, Ireland for financial support and Dr A.F. Parlow, the NIDDK and NHPP, University of Maryland School of Medicine, U.S.A. for the generous gift of RIA materials.

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(Received May 28, 1990
Revised September 4, 1990
Accepted October 3, 1990)

Br. J. Pharmacol. (1991), **102**, 373–380 © Macmillan Press Ltd, 1991

Radioligand binding to muscarinic receptors of bovine aortic endothelial cells

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1 Muscarinic receptors on endothelial cells of bovine thoracic aorta were characterized by binding assays in which (−)-[^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) was used as radioligand.

2 Binding of [^{3}H]-NMeQNB to crude membranes of freshly isolated endothelial cells was atropine-displaceable and of high affinity (K_D = 0.48 nM) to a single class of sites (maximum binding capacity: 14 ± 3 fmol mg^{-1} protein). Stereospecificity of the binding sites was demonstrated in experiments in which [^{3}H]-NMeQNB binding was inhibited by dexetimide in the nanomolar range (K_I = 0.63 nM) and by levetimide, its stereoisomer in the micromolar range (K_I = 3.2 μM) (selectivity factor: ~5000).

3 Drug competition curves indicated a single class of binding sites for antagonists and the following apparant affinities (K_I, nM): methyl atropine: 1.1; 4-diphenylacetoxy *N*-methyl piperidine methyl bromide (4-DAMP): 3.4; pirenzepine: 16; 11-[2-(diethylamino-methyl)-1-piperidinyl-acetyl]-5,11-dihydro-6H-pyrido(2,3-b)1,4-benzodiazepine-6-one (AF-DX 116): 2.500. Competition of acetylcholine with [^{3}H]-NMeQNB was best described by two affinity sites (or states) (K_H = 0.82 μM, K_L = 1.6 μM). In the presence of guanylimido diphosphate [Gpp(NH)p] (100 μM), acetylcholine affinity (IC_{50}) was slightly, but significantly reduced (factor ~4).

4 Binding of [^{3}H]-NMeQNB to freshly harvested intact cells was also atropine-displaceable, stereospecific (selectivity factor: ~3500) and of high affinity (K_D = 0.35 nM). The maximum binding capacity (9 ± 2 fmol mg^{-1} total cell protein) was comparable to that of membranes and corresponded to ~900 binding sites per endothelial cell. Binding to enzymatically harvested and cultured endothelial cells, or membranes derived therefrom, showed no atropine-displaceable binding.

5 The results suggest that (1) bovine aortic endothelial cells contain muscarinic binding sites with all necessary criteria of functional muscarinic receptors; (2) the receptor most closely corresponds to the M_1 subtype and is of comparatively very low density, and (3) cultured endothelial cells lose their receptors during isolation or culture procedures.

Introduction

In a number of *in vitro* studies, acetylcholine has been shown to dilate blood vessels with intact vascular endothelium, whereas vessels with damaged or denuded endothelium usually contract (Furchgott & Zawadzki, 1980; Busse *et al.*, 1985; Vanhoutte & Miller, 1985; Angus & Cocks, 1989). Both responses are entirely or partially blocked by muscarinic cholinoceptor antagonists, suggesting them to be muscarinic in nature. Vasoconstriction has been attributed to activation of muscarinic receptors localized on smooth muscle cells (Ito *et al.*, 1979) or the release of constrictor prostanoids from endothelial cells which then act on smooth muscle cells (Kashef & Catravas, 1986). The localization of the receptors mediating endothelium-derived relaxing factor (EDRF) release and consequent vasodilatation is unclear. Most authors have tacitly accepted the original suggestion of Furchgott & Zawadzki (1980) that the receptors mediating vasorelaxation are localized on endothelial cell membranes (Hynes *et al.*, 1986). Evidence for the presence of muscarinic receptors on endothelial cells has been accumulating recently. Thus, acetylcholine has been shown to induce hyperpolarization of cultured endothelial cells (Busse *et al.*, 1988; Olesen *et al.*, 1988) and to increase their intracellular calcium concentration six to seven fold (Danthuluri *et al.*, 1989). Muscarinic receptors were found to be dispersed on true capillary endothelium of the gastric mucosa by electron microscopic autoradiography (Nakamura *et al.*, 1984). Conventional autoradiographic data, on the other hand, are ambivalent: Stephenson *et al.* (1988) have found no evidence for muscarinic receptors on endothelial cells of a variety of different vessels while Tsukahara *et al.* (1989), using two different radioligands, have shown the endothelial cells of rabbit thoracic aorta to exhibit some characteristics of muscarinic binding sites: [^{3}H]-propyl benzilylcholine ([^{3}H]-PrBCh) mustard binding increased with time, and [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding was displaced by atropine and pirenzepine. The labelled binding sites were inferred to be muscarinic receptors.

Since no synoptic radioligand binding studies on endothelial muscarinic receptors have yet been reported, we have initiated a detailed study of the binding sites of mechanically-harvested bovine aortic endothelial cells using as many of the receptor criteria as possible. Thus, endothelial cell membranes were labelled with (−)-[^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB), and specificity, saturability, stereoselectivity of binding and the potency of several antagonists in displacing bound radioligand were determined. Some of the characteristics were also tested on intact endothelial cells and the results compared to those of cell membranes and cultured cells. In view of the great progress in the analysis of muscarinic receptor subtypes (review: Mitchelson, 1988) we have also attempted to determine the subtype present on endothelial cells. The results show that freshly harvested bovine aortic endothelial cells do contain binding sites with all the characteristics typical of muscarinic receptors of other tissues but their density is comparatively low.

Methods

Membrane and endothelial cell preparation

Bovine thoracic aortae of about 30 cm length were trimmed of extraneous fat and connective tissue in the local slaughterhouse, placed in ice-cold Tris-HCl-buffered saline (pH 7.4) and transported to the laboratory. The vessels were cut open longitudinally and air-dried for 5 min. The endothelial cells were harvested with a blunt scalpel, placed in modified Krebs-phosphate buffer (mM: NaCl 137, KCl 3, Na_2HPO_4 8,

KH_2PO_4 1, $MgCl_2$ 5, $CaCl_2$ 0.5, sucrose 250, dithiothreitol (DTT) 1, pH 7.4) passed through two sieves (pore size: 1 and 0.2 mm^2) centrifuged at 30,000 g and frozen in liquid nitrogen until use. On the day of the experiment, the cells were washed once at 30,000 g, homogenized at 4–7°C by six sonifications (total 90 s, 100 Watt) (Labsonic 1510, Braun, Melsungen, Germany) and centrifuged at 2000 g for 15 min. The supernatant was then centrifuged at 50,000 g for 45 min. The pellet was resuspended in buffer without sucrose and DTT. All membrane preparations were conducted at 4–7°C. For experiments on intact cells, aortae were prepared as above, the cells suspended in buffer (containing 11 mmol l^{-1} glucose instead of 250 mmol l^{-1} sucrose), centrifuged twice at 500 g and used in binding assays. Cells were counted in a haemocytometer. All preparations contained 3–5 $\times$ 10^7 cells per ml. For purposes of comparison, adventitial connective tissue was harvested from aortic arches, minced with scissors, homogenized with an Ultra-Turrax and processed as endothelial cell homogenates. All protein concentrations were determined by the method of Bradford (1976).

Purity of endothelial cell harvests

Endothelial cells were identified by immunofluorescence essentially as described by Schwartz (1978). Briefly, cells were plated on a microscope slide and fixed with methanol followed by acetone (5 min each, −20°C). Selected areas of the cover slips were overlaid with rabbit antibody globulin to human factor VIII (AHF)- associated protein, incubated at 37°C for 30 min, washed for 45 min, dried and exposed to fluorescein-conjugated second antibody (both Behringwerke AG, Marburg, Germany). Control areas of the slide were exposed to serum of rabbits that had not been immunized with antigen. Fluorescence was observed under a fluorescence microscope. Another aliquot of the preparation was routinely inspected for contamination of endothelial cells with connective tissue or smooth muscle cells under a light microscope. Connective tissue was identified by staining the preparation with orcein which renders connective tissue sharply brick-red. Such connective elements were occasionally observed, but always accounted for far less than 5% of all cells. The presence of smooth muscle cells in an endothelial cell preparation was checked by sectioning (20 μm thickness) the thoracic aortic arch at its upper and lower end and staining the sections with ferro-haematoxylin and picrofuchsin which renders connective tissue a brilliant red and muscle tissue brown. In such sections, brown elements (smooth muscle) were well away from the endothelial cell layer, separated from it by a multilayered band of (red-stained) connective tissue. This band of solid connective tissue was still present after careful removal of the endothelial layer, which explains why no smooth muscle elements were noticeable in the preparation.

Culture of bovine aortic endothelial cells

Endothelial cells were cultured according to Schmidt *et al.* (1989). Briefly, bovine aortae were transported to the laboratory in chilled phosphate buffered saline (PBS) containing 2000 u l^{-1} heparin and endothelial cells isolated immediately. The intimal surface was washed thoroughly with prewarmed PBS and incubated for 10 min at 37°C with a solution of 0.1% collagenase, 0.1% trypsin and 1 mM $CaCl_2$ in PBS. Cells were harvested in PBS, centrifuged for 5 min at 500 g, resuspended in Dulbecco's Minimum Essential Medium, containing 10% newborn calf serum, penicillin (100,000 u l^{-1}), streptomycin (0.1 g l^{-1}) and amphotericin B (1.25 mg l^{-1}), seeded in two plastic Petri dishes and incubated in a humified atmosphere. The medium was first changed the day after seeding and then every three days. Confluence was reached after 3–6 days. The monolayer was then trypsinized for 5 min and the cells were divided in a ratio of 1 : 5 and subcultured over two to three passages. The identity of the endothelial cells was verified by immunofluorescence as described above.

Radioligand binding assay

The incubation medium for all radioligand binding experiments was the modified Krebs-phosphate buffer described above, containing 11 mM glucose (cells) or no sugar (membranes) and 0.5% bovine serum albumin. A volume of 50 μl membrane or cell suspension ($\sim$230 μg membrane protein or 2 $\times$ 10^6 cells per assay), 50 μl [^{3}H]-NMeQNB, 100 μl water or competitor (acetylcholine or antagonists) and 200 μl buffer (total volume: 400 μl) were incubated at 25°C for 100 min. [^{3}H]-NMeQNB was used at 0.09–3 nM (saturation curves) or $\sim$1.4 nM (competition curves). When acetylcholine was used, physostigmine sulphate (final concentration, 2 μM) was added to the membrane suspension to prevent degradation of acetylcholine. In each competition assay, 11 competitor concentrations were used. Specific [^{3}H]-NMeQNB binding was determined experimentally from the difference between radioligand bound in the absence and presence of 10 μM atropine sulphate. Specific binding was 70% at the radioligand concentration used in competition experiments ($\sim$1.4 nM). All measurements were made in triplicate (intact endothelial cells) or quadruplicate (membranes) in 2 to 5 separate experiments, as indicated in the tables. Specific binding never exceeded 2% of added ligand so that the concentration of free radioligand did not change significantly during the experiment, allowing total radioligand concentrations to be taken as free concentrations in all curve fitting analyses.

Binding reactions were terminated by filtering the incubation media over glass fibre filters (Whatman GF/C (membranes); AHF-934 (intact cells)) using a Brandel cell harvester (Gaithersburg, MD, U.S.A.). All filters were presoaked in aqueous polyethylenimine (0.1%). Each filter was rinsed four times with 3 ml ice-cold water (which was found to be equivalent to using 10 mM Tris-HCl buffer or physiological saline), receptor-bound radioactivity retained on the filter was extracted for 2 h with 3 ml of 'Gold' scintillation cocktail (Packard-Canberra Instr.) at 6°C. Radioactivity of each sample was determined by liquid scintillation counting (Packard 4530) at a counting efficiency of at least 50% (background count rate due to all sources except filter-bound radioactivity, 12 c.p.m.).

Data analysis

All experimental saturation and competition binding data were analyzed by the GIPMAX non-linear least-squares regression curve fitting programme. The programme package was obtained from Dr Dierk Brockmeier, Hoechst AG, Frankfurt (Germany) for use with a Hewlett-Packard work station (series 9000, model 310). The data derived from the saturation of [^{3}H]-NMeQNB binding were analyzed with a one-site binding model, competition data with a one-site and two-site binding model according to the Law of Mass Action by non-linear least-squares best fit as described by Feldman (1972). In all cases, the models were fit to individual sets of raw data. In the case of competition experiments, total binding, and in the case of saturation experiments, total plus simultaneously determined non-specific binding were fitted to experimental models to obtain the binding parameters summerized in the tables. Pseudo Hill coefficients (nH) were always derived from specific binding data. The choice of appropriate model for the competitors was based on the residual sum of squares, using the partial F-test as criterion (De Léan *et al.*, 1982). IC_{50} values were converted to K_I values, by the Cheng & Prusoff (1973) equation. Tests of significant differences between means were performed using the t test for paired experiments. All IC_{50}, K_D and K_I values are expressed as geometric means with 95% confidence intervals (CI), calculated as the product of s.e.mean $\times$ Student's t values, B_{max} and nH values were calculated as arithmetic mean $\pm$ s.e.mean (de Léan *et al.*, 1982).

Materials

(−)-[^{3}H]-N-methyl quinuclidinyl benzilate chloride ([^{3}H]-NMeQNB) (specific activity: 70 Ci mmol^{-1}) and (−)-[^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) (specific activity: 33 Ci mmol^{-1}) were purchased from New England Nuclear, Vienna, atropine sulphate, atropine methyl bromide and 5'-guanyl-imidodiphosphate Na [Gpp(NH)p], physostigmine hemisulphate, and dithiothreitol from Sigma, Munich, Germany, acetylcholine chloride from Fluka, Vienna, Austria. The following drugs were kindly donated by the indicated companies and persons: AF-DX 116 (11-[2-(diethylamino-methyl)-1-piperidinyl-acetyl]5,11-dihydro-6H-pyrido(2,3-b)1,4-benzodiazepine-6-one): Dr Thomae, Biberach, Germany; 4-DAMP (4-diphenylacetoxy *N*-methyl piperidine methyl bromide: Dr R.B. Barlow, Univ. Bristol, U.K.; pirenzepine dihydrochloride: Dr R. Hammer, de Angeli, Milano, Italy; dexetimide and levetimide: Janssen Pharmaceuticals, Beerse, Belgium. Dye cocktails for tissue staining were kindly donated by Dr M. Hartmann (Department of Histology, University of Graz).

Results

Validation of binding to endothelial cells

Since the presence of minute amounts of connective tissue (< 5%) could not be excluded in our preparations, binding of [^{3}H]-NMeQNB to homogenates of connective tissue was tested at two ligand concentrations below and above the K_D value for binding to endothelial cells and with two membrane preparations of different quality. With the 50,000 *g* pellet and 0.2 nM radioligand, the specific binding amounted to 34%, and at 0.6 nM, zero % ($n = 4$). With the cruder preparation (2000 *g*), 22 and 4% specific binding, respectively, were observed.

Specificity, saturability and stability of [^{3}H]-*NMeQNB binding*

Saturation experiments of [^{3}H]-NMeQNB binding were performed on endothelial cell membranes and intact cells in the absence (total binding) and presence of 10 μM atropine sulphate to determine non-specific binding (Figures 1 and 2). The saturation isotherms comprised a saturable and a non-saturable component, the latter being linearly related to total radioligand concentration (Figure 1a and 2a). Specific binding amounted to roughly 80% of total binding at low (~0.1 nM) and 45% at saturating radioligand concentrations (~3 nM). The saturation curves of both membranes and cells were best defined by interaction of the radioligand with a single population of saturable high-affinity binding sites (Figure 1b and 2b, Table 1). The equilibrium dissociation constant (K_D) for [^{3}H]-NMeQNB binding to cell membranes prepared from five individual cell harvests was 4.8 (3.8–6.1) × 10^{-10} M (geometric mean ± 95% confidence limits) and for intact cells (three individual harvests) 3.5 (2.2–5.5) × 10^{-10} M. In membranes, the maximal density of binding sites (B_{max}) for [^{3}H]-NMeQNB binding was 14 ± 3 (arithmetic mean ± s.e.mean) fmol mg^{-1} membrane protein, and for cells 9 ± 2 fmol mg^{-1} total cell protein, corresponding to ~900 binding sites per endothelial cell (Table 1).

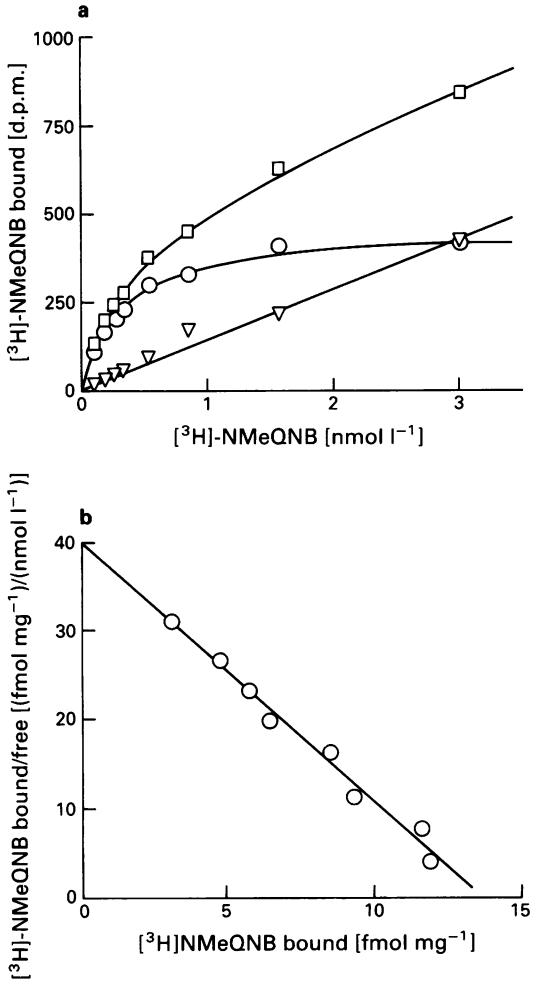


Figure 1 (a) Saturation experiment of [^{3}H]-*N*-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) binding to endothelial cell membranes. Assay conditions were as described under Methods. Membranes (200–250 μg) were incubated in quadruplicate with increasing concentrations of radioligand for 100 min at 25°C in the absence (□, total binding) and presence of 10 μM atropine sulphate (▽, non-specific binding). Specific binding was determined as the difference between total and non-specific binding (○). The K_D and B_{max} value were determined by non-linear least squares regression analysis (see Methods) and were 0.34 nM and 465 d.p.m. per assay, respectively, corresponding to 13 fmol [^{3}H]-NMeQNB specifically bound per mg protein. The parameters derived from five independent experiments are listed in Table 1. (b) Scatchard plot of specific [^{3}H]-NMeQNB binding data.

Table 1 Equilibrium binding parameters for the interaction of [^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) and muscarinic receptors of bovine aortic endothelial cells

Tissue	K_D (M; 95% CL)	B_{max}[a] (fmol mg^{-1} protein)	*Binding sites per cell*
Membranes ($n = 5$)	4.8 (3.8–6.1) × 10^{-10}	14 ± 3	
Intact cells ($n = 3$)	3.5 (2.2–5.9) × 10^{-10}	9 ± 2	863 ± 84

The data represent the affinity estimates obtained from five (membranes) and three (intact cells) experiments which were fitted individually to a one-binding site model as described in Methods. Results are expressed as geometric means with 95% confidence limits (K_D) and as arithmetic means ± s.e. (B_{max}).

The number of receptors per cell was calculated based on three separate determinations of B_{max} (fmol [^{3}H]-NMeQNB per 1.5 × 10^6 cells).

[a] Calculation based on membrane and total cell protein, respectively.

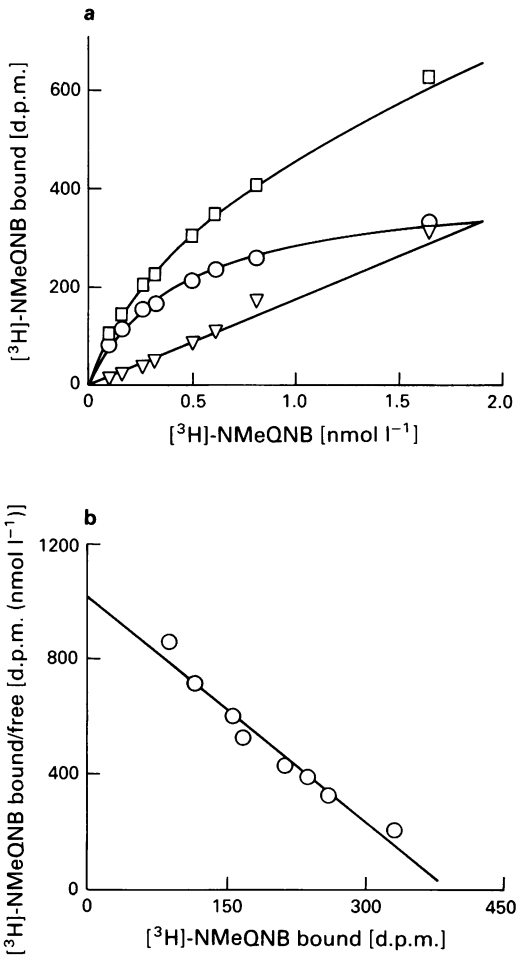


Figure 2 (a) Saturation experiment of [^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) binding to intact endothelial cells: 2×10^6 cells were incubated in triplicate with increasing concentrations of radioligand for 100 min at 25°C in the absence (□, total binding) and presence of 10 μM atropine sulphate (▽, non-specific binding). Specific binding was determined as the difference between total and non-specific binding (○). The K_D and B_{max} values were determined by non-linear least squares regression analysis (see methods) and were 0.42 nM and 402 d.p.m. per assay, respectively, corresponding to 9 fmol [^{3}H]-NMeQNB specifically bound per mg total cell protein or 855 binding sites per cell. The parameters derived from three independent experiments are listed in Table 1. (b) Scatchard plot of specific [^{3}H]-NMeQNB binding data.

Binding of [^{3}H]-NMeQNB or [^{3}H]-QNB to cultured endothelial cells was entirely non-specific. In no case did the specific component exceed ~10% of total binding (data not shown).

Binding of [^{3}H]-NMeQNB to endothelial cell membranes was tested up to 4 h. No reduction of specific binding was observed.

Stereospecificity

The stereospecificity of [^{3}H]-NMeQNB-receptor binding was tested by comparing the inhibitory potencies of stereoisomers of the muscarinic antagonist, benzetimide. The dissociation constant of dexetimide was 6.3 (3.6–11) $\times 10^{-10}$ M (membranes) and 1.4 (0.8–2.4) $\times 10^{-9}$ M (intact cells), that of levetimide, the laevorotatory isomer, 3.2 (2.8–3.8) $\times 10^{-6}$ M (membranes) and 3.9 (3.0–4.9) $\times 10^{-6}$ M (intact cells), respectively (Figures 3 and 4, Table 2). Thus, dexetimide affinity was approximately 5000 fold (membranes) and 3500 fold (cells) higher than that of levetimide.

Displacement of [^{3}H]-NMeQNB binding by agents acting on cholinoceptors

The displacement of [^{3}H]-NMeQNB binding to endothelial cell membrane preparations was examined by adding increasing concentrations of unlabelled compounds acting on cholinoceptors. Figure 5 shows typical competition curves of representative nonselective and subtype-selective muscarinic antagonists. The data of individual competition curves were fitted to a one-site model and affinity constants (K_I) and nH values derived by non-linear least squares computer fitting.

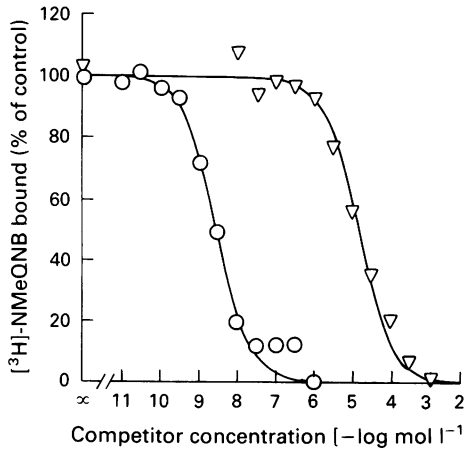


Figure 3 Stereospecificity of binding to muscarinic receptors of endothelial cell membranes. Microsomal membranes were incubated with 1.4 nM [^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) for 100 min at 25°C in the absence (control) and presence of increasing concentrations of dexetimide (○) and levetimide (▽), respectively. Fitting of the data (one individual experiment) to a one-binding site model gave apparent affinity values (K_I) of 0.48 nM (dexetimide) and 3 μM (levetimide), respectively. The K_I values and pseudo Hill coefficients derived from three individual experiments are listed in Table 2.

Table 2 Relative potencies of antagonists in competing for [^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) binding to endothelial cell membranes and intact cells

	Membranes		*Intact cells*	
Antagonist	K_I (M; 95% CL)	*nH* (mean ± s.e.)	K_I (M; 95% CL)	*nH* (mean ± s.e.)
Dexetimide (n = 3)	6.3 (3.6–11) $\times 10^{-10}$	1.04 ± 0.11	1.4 (0.8–2.4) $\times 10^{-9}$	0.87 ± 0.21
Levetimide (n = 3)	3.2 (2.8–3.8) $\times 10^{-6}$	0.98 ± 0.09	3.9 (3.0–4.9) $\times 10^{-6}$	1.10 ± 0.29
Methyl atropine (n = 3)	1.1 (0.7–1.8) $\times 10^{-9}$	0.87 ± 0.10		
4-DAMP (n = 2)	3.4 (1.5–7.9) $\times 10^{-9}$	0.90		
Pirenzepine (n = 4)	1.6 (1.1–2.4) $\times 10^{-8}$	1.22 ± 0.13		
AF-DX 116 (n = 4)	1.8 (1.3–2.3) $\times 10^{-6}$	0.74 ± 0.01**		

The competition curves were fitted to a one-binding site model (K_I) and the Hill model (nH). The results are expressed as geometric means with 95% confidence limits (K_I) or as arithmetic means with s.e. (nH).
** Significantly different from unity ($P < 0.01$); nH: pseudo Hill slope.

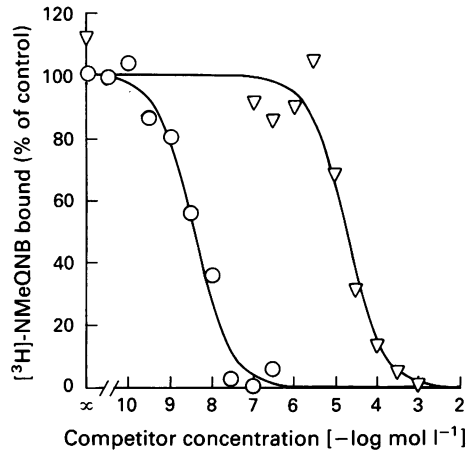


Figure 4 Stereospecificity of binding to muscarinic receptors of intact endothelial cells: 2×10^6 cells were incubated in triplicate with 1.3 nM [^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) for 100 min at 25°C in the absence (control) and presence of increasing concentrations of dexetimide (○) and levetimide (▽), respectively. Fitting of the data (one individual experiment) to a one-binding site model gave K_I values of 0.88 nM (dexetimide) and 4.7 μM (levetimide), respectively. The K_I values and pseudo Hill coefficients of three individual experiments are listed in Table 2.

Methyl atropine was the most effective competitor (K_I = 1.1 nM), followed by 4-DAMP (K_I = 3.4 nM), pirenzepine (K_I = 16 nM) and AF-DX 116 (K_I = 1.8 μM) (Table 2). A pseudo Hill slope not significantly different from unity is consistent with a single population of high affinity receptors. The

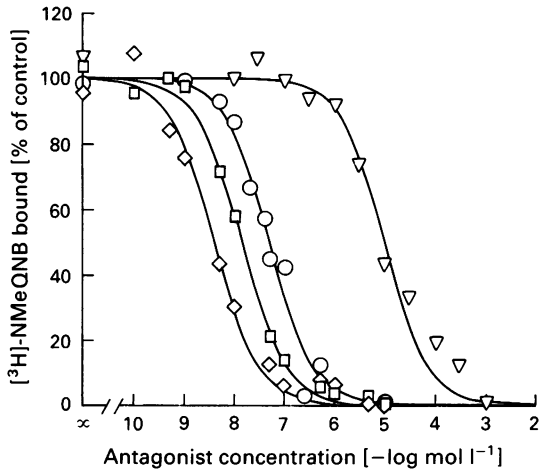


Figure 5 Competition for specific [^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) (1.2–1.4 nM) binding to endothelial cell membranes by the muscarinic antagonists methyl atropine (◇) 4-diphenylacetoxy *N*-methyl piperidine methyl bromide (4-DAMP) (□), pirenzepine (○), and AF-DX 116 (▽). Protein concentration was 200–250 μg per assay. Individual experiments performed in quadruplicate are shown. The K_I value and pseudo Hill coefficients for each drug are shown in Table 2.

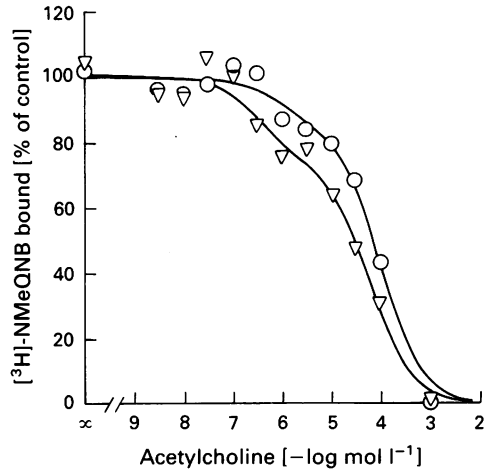


Figure 6 Effect of the guanine nucleotide Gpp(NH)p (100 μM) on acetylcholine competition of [^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) (1.4 nM) binding to endothelial cell membranes. Experimental conditions were as described under Methods. (▽) control; (○) + 0.1 mM Gpp(NH)p. Fitting of the data (one individual experiment, performed in quadruplicate) to a two-binding site model gave the following apparent affinities: K_H 0.16 μM, K_L 18 μM [no Gpp(NH)p] and K_H 0.32 μM, K_L 32 μM [+Gpp(NH)p], respectively. Binding parameters are summarized in Table 3.

AF-DX 116 competition curves were somewhat flatter, but, despite a pseudo Hill coefficient significantly less than unity ($P < 0.01$), still best described by a one-site model ($P < 0.05$) (Table 2).

The competition curves of the agonist acetylcholine and [^{3}H]-NMeQNB were shallow (pseudo Hill coefficients 0.53 ± 0.02) and were best described by a two-site binding model (Figure 6 and Table 3). The high affinity component [K_H 8.2 (3.1–22) × 10^{-7} M] comprised 39 ± 5.5%, the low affinity component [K_L 1.1 (0.4–2.9) × 10^{-4} M] 61 ± 5.5% of specific binding sites. In the presence of 1 mM of the non-hydrolyzable guanine nucleotide, Gpp(NH)p, the competition binding curves were slightly steeper (nH 0.66 ± 0.09), displaced to the right and the IC_{50} value significantly raised from 5.1 (2.6–10) × 10^{-5} M [without Gpp(NH)p] to 2.4 (1.5–3.7) × 10^{-4} M [with Gpp(NH)p].

Discussion

The present study for the first time provides direct evidence for the existence of muscarinic receptors on endothelial cells and supports indirect evidence previously published inferring their existence. Our previous attempts to demonstrate muscarinic receptors with different radioligands were unsuccessful. In contrast, [^{3}H]-NMeQNB binding described in this paper is specific, saturable, stable, reversible and stereospecific. In agreement with all published reports, there is only a single class of high affinity sites for antagonists, while binding of the endogenous agonist acetylcholine is compatible with a two

Table 3 Affinity of acetylcholine for [^{3}H]-N-methyl quinuclidinyl benzilate ([^{3}H]-NMeQNB) binding sites in the absence and presence of 100 μM guanylimido diphosphate (Gpp(NH)p)

	IC_{50} (M; 95% CL)	*nH* (mean ± s.e.)	K_H (M; 95% CL)	K_L (M; 95% CL)	%*H* (mean ± s.e.)
Without Gpp(NH)p	5.1 (2.6–10) × 10^{-5}	0.53 ± 0.02	2.2 (3.1–22) × 10^{-7}	1.1 (0.4–2.9) × 10^{-4}	39 ± 5.5
With Gpp(NH)p	2.4 (1.5–3.7) × 10^{-4}*	0.66 ± 0.09	1.6 (0.4–6.8) × 10^{-6}	2.7 (0.9–10) × 10^{-4}	34 ± 7.2

The parameter values were obtained by computer analysis of competition curves according to a two-site binding model. Results are expressed as geometric with 95% confidence limits (IC_{50}, K_H, K_L) or as arithmetic means with s.e. (nH, %H).
* Significantly different from control ($P < 0.05$); nH: pseudo Hill coefficient; H: high affinity site; L: low affinity site.

binding site model. However, the absolute number of receptors per cell is extremely small (~900) and may well explain previous failures to demonstrate these receptors by binding techniques.

Careful mechanical harvesting of the aortic intimal layer resulted in sufficiently homogeneous preparations of endothelial cells as judged by a combination of immunological and staining techniques. Endothelial cells were identified by human Factor VIII-immunofluorescence and the contaminating cell material by differential staining for connective tissue and smooth muscle cells. Viewed from the lumen to the adventitial side, single smooth muscle cells followed by smooth muscle cell layers occur well away from the intimal cell boundary and were separated from it by dense connective tissue. This explains why no smooth muscle cells were detected in our preparations. Therefore smooth muscle receptors could not have contributed to the observed binding. Connective tissue, on the other hand, was unlikely to contribute to endothelial cell binding since [^{3}H]-NMeQNB binding to several preparations of adventitial connective tissue was almost exclusively nonspecific. At comparable ligand concentrations, binding to endothelial cells was 80% and to connective tissue 0–30% atropine-displaceable. This small percentage of possibly specific binding, together with the feeble contamination (<5%) of endothelial cell preparations with connective tissue fibres, makes it extremely unlikely that non-endothelial tissues account even for a small fraction of the specific binding observed.

Using [^{3}H]-QNB in competition experiments and [^{3}H]-PrBCh mustard in kinetic and autoradiographic experiments, Tsukahara *et al.* (1989) have also demonstrated endothelial muscarinic binding sites on rabbit thoracic aorta. However, these data do not really prove the existence of muscarinic receptors, since no saturation experiments were performed with either ligand nor was the degree of stereospecificity of binding determined. Furthermore, as the K_D value for [^{3}H]-QNB binding to endothelial and smooth muscle binding sites was not determined by these authors, the absolute affinity of atropine and pirenzepine (the only antagonists used) may also not be compared to published values.

The principal binding characteristics of endothelial receptors as characterized in the present paper are similar to those of numerous other tissues (Fields *et al.*, 1978), and isolated or cultured cells (Gossuin *et al.*, 1984). However, due to the very small number of receptors per endothelial cell and the considerable difficulty in obtaining large amounts of cells, some compromises had to be made. Thus, the fraction of nonspecific binding contained in total binding amounted to ~30% at the K_D of the radioligand which is quite high, but still sufficient for a precise determination of binding parameters (see, for instance, Yamanaka *et al.*, 1986). The affinity of [^{3}H]-NMeQNB determined in this study for isolated membranes (0.48 nM) and intact cells (0.35 nM) was in good agreement with the value determined for rat heart (0.15 nM; Brown & Goldstein, 1986; Lee & El-Fakahany, 1988). Although intact cell preparations ensured optimal receptor integrity, they may similarly preserve various secondary cellular processes such as ligand internalization leading to the observed small differences in affinity of [^{3}H]-NMeQNB to receptors of intact cells and homogenates. The less than two fold difference reported herein is strong support for the identity of the receptor labelled on intact cells and cell membranes. Several attempts to use the tertiary [^{3}H]-QNB on intact cells or homogenates gave no atropine-displaceable binding which may be due to the different charge distribution and lipophilicity of this compound compared to the quaternary derivative (Lee & El-Fakahany, 1988). A similar conclusion was reached by Gossuin *et al.* (1984) for the binding of several radioligands to cultured neuroblastoma × glioma hybrid cells. Saturability of specific binding was demonstrated both on intact cells and membranes. Saturation of membrane receptors was attained at 10–15 fmol [^{3}H]-NMeQNB mg^{-1} protein, and of intact cells at 5–10 fmol mg^{-1} total cell protein, corresponding to about 900 receptors per endothelial cell. These B_{max} values are smaller than or similar to those reported for several blood vessels including pig coronary artery (~40 fmol mg^{-1} protein; Rinner *et al.*, 1988; Yamada *et al.*, 1988), bovine aorta (intima + media: 15 fmol mg^{-1} protein; Yamanaka *et al.*, 1986) and bovine coronary artery (15 fmol mg^{-1} protein; Brunner *et al.*, 1990), but 10 to 50 times lower than those reported for cardiac or ileal homogenates (100–500 fmol [^{3}H]-QNB or [^{3}H]-NMeQNB sites mg^{-1} protein; Ehlert *et al.*, 1980; Lee & El-Fakahany, 1988) and even 100 times lower than those reported for several brain areas (0.8–1.5 pmol mg^{-1} protein; Sokolovsky *et al.*, 1983) which are among the most receptor-rich tissues cited in the literature. Likewise, the number of ~900 receptors per endothelial cell is the same as for rabbit pulmonary vascular endothelium determined *in vivo* (~700 receptors per cell; Orphanos & Catravas, 1989). This density is, however, much lower than that reported for most other tissues, e.g., chick heart (~5000 [^{3}H]-NMeQNB sites per cell; Brown & Goldstein, 1986, colonic epithelium (~10,000 [^{3}H]-QNB sites per cell; Zimmerman & Binder, 1982), spleen lymphocytes (~60,000 [^{3}H]-QNB sites per cell; Costa *et al.*, 1988), gastric antrum (~100,000 [^{3}H]-NMS sites per smooth muscle cell; Moummi *et al.*, 1988), the canine stomach (~200,000 [^{3}H]-QNB sites per smooth muscle cell; Collins & Crankshaw, 1986) or lung fibroblasts (~200,000 receptors per cell, labelled with different radioactive antagonists; André *et al.*, 1988).

The stereospecificity of the NMeQNB receptor site was demonstrated by use of the stereoisomers of benzetimide, dexetimide and levetimide. Pharmacologically, the antagonist dexetimide is reported to be about 6000 times more potent than its laevorotatory stereoisomer, levetimide (Soudijn *et al.*, 1973). In the present binding studies, dexetimide was about 3000 to 5000 times more potent than levetimide in competing with [^{3}H]-NMeQNB binding at both cell membranes and intact cells. Hence, even by the most stringent criteria for receptor binding (Laduron, 1984), there can be no doubt about the muscarinic nature of these binding sites on endothelial cells.

Characterization of the binding sites on endothelial cell membranes was completed by competition experiments with the antagonists methyl atropine, 4-DAMP, pirenzepine, AF-DX 116, and the agonist acetylcholine. The potency order dexetimide > methyl atropine ≥ 4-DAMP > pirenzepine > AF-DX 116 ≥ levetimide in displacing a radiolabelled antagonist and the K_I values measured are in agreement with muscarinic acetylcholine receptor properties. Acetylcholine exhibited a heterogeneous interaction with endothelial receptors, as evidenced in the competition curves which significantly deviated from the law of simple mass action (nH less than unity). According to non-linear least squares regression analysis, all curves were accounted for by the presence of a high and a low affinity state, in agreement with published data on nonvascular and vascular tissues (e.g., Yamanaka *et al.*, 1986). In contrast to classical antagonists, the recognition of different affinity states by muscarinic agonists is dependent on assay conditions. In particular, guanyl nucleotides were found to convert some high-affinity sites to low-affinity sites by regulating the interaction of muscarinic receptors with guanosine triphosphate (GTP)-binding proteins. Since the binding of agonists to M_2 receptors is known to be highly sensitive to guanine nucleotides while M_1 receptors are relatively insensitive (Vickroy *et al.*, 1984), we have determined the competition profile of acetylcholine in the presence of 1 mM Gpp(NH)p, a stable analogue of GTP. The nucleotide decreased the agonist's affinity for both the high and low affinity site only slightly, resulting in a displacement of the competition curve to the right and a somewhat steeper pseudo Hill coefficient. The moderate modulation of agonist binding by Gpp(NH)p in endothelial cells of bovine aorta is similar to that observed in several brain regions (Ehlert *et al.*, 1980), rabbit peripheral lung (Bloom *et al.*, 1987) or retina (Dunlap & Brown, 1984). As in these latter examples, inhibition of adenylate cyclase

probably plays no important role in the generation of intracellular signals.

As to the heterogeneity of muscarinic receptors, the original division into M_1 (high affinity for pirenzepine) and M_2 (low affinity for pirenzepine) (Hammer *et al.*, 1980) has recently been extended to comprise the M_1 and at least two peripheral M_2 receptors present in cardiac and smooth muscle, respectively (Mutschler *et al.*, 1988) or in heart and exocrine glands, respectively (Doods *et al.*, 1987). All peripheral M_2 receptors are characterized by one or more relatively selective antagonists and a low affinity for pirenzepine. The present results point to an M_1 receptor on endothelial cells for the following reasons: (1) The K_I value for pirenzepine (16 nM) is the same as the average affinity of pirenzepine at M_1 receptors of cerebral cortex and sympathetic ganglia (5–30 nM; Hammer *et al.*, 1980; Watson *et al.*, 1983); (2) the K_I value for 4-DAMP (3.4 nM) corresponds to its affinity at high affinity [^{3}H]-pirenzepine binding sites in the hippocampus or cortex (1–3 nM; Doods *et al.*, 1987); (3) the affinity estimate (K_I 1.8 μM) for the cardioselective antagonist AF-DX 116 was in reasonable agreement with published values of AF-DX 116 affinity to brain M_1 receptors (0.8 μM; Hammer *et al.*, 1986); and (4) the moderate modulation of agonist binding by Gpp(NH)p observed in endothelial cells is likewise characteristic for the M_1 subtype.

The postulate of M_1 receptors in the cardiovascular system is not unexpected. Using an indicator dilution technique in living rabbits, Orphanos & Catravas (1989) have recently described M_1 receptors on pulmonary endothelial cells. Yamanaka *et al.* (1986), also employing binding analyses, found high affinities (M_1) for pirenzepine to the medial (K_I = 22 nM) and intimal (K_I = 8.7 nM) layers of bovine aortae, while aortae from rabbit and dog contained M_2 receptors. In the cerebral circulation of the newborn pig, M_1 receptors mediate cerebrovascular constriction and increased cerebrospinal fluid prostanoid synthesis (Armstead *et al.*, 1988) and in rabbit saphenous artery, acetylcholine-induced hyperpolarization of endothelial cells is blocked by atropine and pirenzepine at similar concentrations (Komori & Suzuki, 1987), also suggesting M_1 receptors. In the canine saphenous vein, postjunctional receptors belong to the M_1, prejunctional receptors to the M_2 subtype (O'Rourke & Vanhoutte, 1987).

Functional *in vitro* studies with endothelium-preserved vessels have so far yielded mixed results: Tsukahara *et al.* (1989) have concluded that relaxation of rabbit thoracic aorta is mediated by non-M_1 receptors (pA_2 value for pirenzepine given in the text: 6.6) while Eglen & Whiting (1985) have reported a nine times higher affinity (pA_2 7.5) for pirenzepine in the same vessel and Choo *et al.* (1986) a low value (6.75). The latter authors concluded that the vascular receptor was neither of the M_1 nor M_2 subtype as known at that time. In our own studies on bovine coronary artery (unpublished) we have also obtained a similar pA_2 value for pirenzepine (7.38). This value is somewhat lower than functional estimates for M_1 receptors in the central nervous system ($pA_2 \sim 8$), but close to the range of values found for most peripheral tissues ($\sim$8–7.5) (Mitchelson, 1988). The lower pA_2 value of pirenzepine found by Tsukahara *et al.* (1989) may in fact be higher ($\sim$7.2 according to their Figure 1), or/and due to species differences or differences in experimental conditions, especially a much shorter incubation time (15 min) than used in our studies (45 min) which may result in non-equilibrium conditions and underestimation of antagonist affinity (Kenakin, 1980).

In summary, the present work allows several important conclusions: First, radioligand binding studies demonstrate muscarinic binding sites on fresh bovine aortic endothelial cells with all essential criteria for functional muscarinic receptors. Second, endothelial cells which were harvested enzymatically and grown in culture show no specific binding sites. Third, the subtype present on endothelial cells of the bovine thoracic aorta is of the M_1 subtype.

The authors are grateful to Dr Dierk Brockmeier for the supply of the computer programme GIPMAX for the HP 310 computer, to Dr R.B. Barlow for the supply of 4-DAMP, the drug companies di Angeli (Milano) for pirenzepine and Dr Thomae (Biberach) for AF-DX 116, to Dr K. Schmidt and Ms M. Rehn for cell culture, Mr P. Wusser and Ing. M. Marterer for expert preparation and transport of aortae, and Mr Th. Marcovic for photography. The excellent technical assistance of Mrs Heike Stessel is also acknowledged. Supported by the Austrian Research Council (Fonds zur Förderung der wissenschaftlichen Forschung), project Nr. P 6644 M.

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(Received February 24, 1990
Revised August 17, 1990
Accepted October 9, 1990)

Br. J. Pharmacol. (1991), **102**, 381–385 © Macmillan Press Ltd, 1991

The differential contribution of endogenous prostaglandins to the release of acetylcholine from the myenteric plexus of the guinea-pig ileum

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1 Prostaglandin E (PGE) may be essential for maintaining the sensitivity of the myenteric plexus of guinea-pig ileum to nicotine. The contributions of prostaglandins to nervous activity evoked by different stimuli have now been investigated by measuring the amount of acetylcholine (ACh) released from the myenteric plexus of the guinea-pig ileum.

2 The amount of ACh released in response to dimethylphenylpiperazinium (DMPP) or substance P was depressed to about 40% of control by 2.8 μM indomethacin (Ind), whereas the release of ACh induced by 5-hydroxytryptamine (5-HT) was not affected. The inhibitory effects of Ind were overcome by 14.3 nM PGE_2.

3 Mepacrine 5 μM, an inhibitor of phospholipase A_2, depressed the release of ACh in response to DMPP and substance P to the same extent as Ind. These inhibitory effects of mepacrine were overcome by arachidonic acid (10 μM), but not by arachidonic acid plus Ind. The release of ACh evoked by 5-HT or electrical field stimulation (EFS) was also inhibited to about 60% of control by mepacrine but these inhibitions were overcome by arachidonic acid (10 μM) either in the absence or the presence of Ind.

4 The results suggest that endogenous prostaglandins and arachidonic acid contribute to the maintenance of the excitability of the myenteric plexus by DMPP and substance P. By contrast, the release of ACh induced by 5-HT and EFS may be regulated by arachidonic acid and not by prostaglandins.

Introduction

In the myenteric plexus of the guinea-pig ileum, the release of acetylcholine (ACh) evoked by electrical stimulation or a high concentration of K^+, which depolarize the axons or terminals of neurones, respectively, is not affected by 2.8 μM indomethacin (Ind), an inhibitor of cyclo-oxygenase (Yagasaki *et al.*, 1984). Contradictory results have been obtained in studies with nicotine, which has been shown to activate the soma of myenteric neurones (Yagasaki *et al.*, 1984; Wood, 1987). Ind inhibited both the release of ACh from the myenteric plexus and the response of the enteric inhibitory nerves in the taenia coli to nicotine (Okpako & Taiwo, 1984; Yagasaki *et al.*, 1984; Iselin *et al.*, 1988). Such inhibition was overcome by prostaglandin E_2 (PGE_2). In addition, the inhibition of phospholipase A_2 by mepacrine, which decreases the liberation of arachidonic acid, depressed the excitability of cholinergic neurones by nicotine in the same manner as did Ind, and this inhibition was overcome by arachidonic acid (Takeuchi & Yagasaki, 1987). Since the restorative effect of arachidonic acid on the inhibition by mepacrine was partly overcome by Ind, the effect of arachidonic acid was assumed to be due to both arachidonic acid itself and to prostaglandins synthesized from exogeneously added arachidonic acid (Takeuchi & Yagasaki, 1987). These pieces of evidence suggest that the products of the arachidonic acid cascade, in particular arachidonic acid and PGE_2, may contribute to the modulation of the excitability of the myenteric neurones by nicotine.

Substance P and 5-hydroxytryptamine (5-HT) may be present in the myenteric plexus (Llewellyn-Smith *et al.*, 1989; Costa *et al.*, 1982), to excite the somal membranes of enteric neurones (Wood, 1987). The application of these agents to guinea-pig ileum induces the release of ACh via direct stimulation of the cholinergic neurones (Holzer & Lembeck, 1980; Kilbinger & Pfeuffer-Friederich, 1985). However, the contribution of prostaglandins to the release of ACh induced by substance P and 5-HT remains to be examined. In order to determine whether or not a common mechanism, sensitive to the products of the arachidonic acid cascade, is involved in the receptor-mediated release of ACh, we determined the effects of Ind and mepacrine on the release of ACh induced by substance P and 5-HT. The results were compared with the effects of inhibitors of arachidonic acid cascade on the release of ACh induced by the nicotinic stimulant, dimethylphenylpiperazinium (DMPP) (Yokoyama *et al.*, 1986). A summary of this work was published previously (Takeuchi *et al.*, 1987).

Methods

Male guinea-pigs, weighing 300 to 700 g, were killed by a blow on the head and bled. The longitudinal muscle of the ileum with the myenteric plexus attached was prepared as previously described (Yagasaki *et al.*, 1981). The preparation was mounted in an organ bath that contained 3 ml of Tyrode solution of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.6. Physostigmine salicylate (5 μM) and choline chloride (1 μM) were added to the bathing medium, which was kept at 37°C and bubbled with 95% O_2 and 5% CO_2.

Release of acetylcholine induced by various stimuli

Before samples of bathing medium were collected for estimation of the release of ACh, the strips were equilibrated for 15 min by perfusion with Tyrode solution at a rate of 1–2 ml min^{-1}. Thereafter, the perfusion was stopped and the bathing medium was removed and replaced by 3 ml of fresh Tyrode soluton at intervals of 1 min. After two consecutive samples were collected for the measurement of spontaneous release of ACh, the strips were stimulated for 1 min by addition of DMPP (10 μM), substance P (1 μM), 5-HT (2.6 μM), or by electrical field stimulation (EFS). Each stimulus was applied twice with an interval of 19 min between stimuli. The first stimulation was carried out in the absence (S_1) and the second was carried out in the presence (S_2) of the drugs. For the EFS-induced release of ACh, the stimulation was performed in trains of 20 s, and bathing fluid was collected after a further 40 s resting period. EFS was carried out with a pair of platinum electrodes, one at the top and one at the bottom of the strip. The parameters for EFS were as follows: supramaximal

voltage (50 V); 0.5 ms duration; 200 pulses in all, at 10 Hz. All samples collected were kept on ice until assayed (within 3 h). At the end of the experiment, the strips were blotted and weighed to express the release as the amount of ACh per g tissue. The amount of ACh released in response to each stimulation was calculated by subtraction of the output during the immediately preceding resting period of 1 min from the total output during the period of stimulation. All results, in both the figures and in the text, are given in terms of relative amounts of ACh released. Thus, the release of ACh during S_2 is expressed as a percentage of the amount of ACh released during S_1.

Assay of released acetylcholine

The collected samples were assayed for ACh with isolated strips of longitudinal muscle obtained from the most distal region of the guinea-pig ileum, as preveiously described (Takeuchi & Yagasaki, 1987). The medium in the assay bath was kept at 37°C and bubbled with 95% O_2 and 5% CO_2. To eliminate the possibility of interference by DMPP in the collected samples, hexamethonium chloride (0.1 mM) was added to the Tyrode solution for the assays. When substance P was present in the samples, interference was prevented by treating the samples with one-tenth volume of α-chymotrypsin (50 u ml^{-1}) for 20 min at 37°C. When samples containing 5-HT were assayed, 5-HT (2.6 μM) was always present in the solution for the assays. To eliminate the possibility of interference in the assay by the other drugs present in the collected samples, the responses to the bathing fluids were compared with those to a standard solution of ACh to which the tested drugs were added, at the same final concentration.

The active substance in the collected samples was identified as ACh by demonstrating that it was destroyed by boiling in alkali and that its action was antagonized by atropine and potentiated by physostigmine.

Statistical analyses were performed by Student's *t* test.

Drugs

The following drugs were used: acetylcholine chloride (ACh), indomethacin (Ind), physostigmine salicylate, mepacrine dihydrochloride, arachidonic acid, 5-hydroxytryptamine creatinine sulphate (5-HT), α-chymotrypsin (Sigma, U.S.A.), substance P (Peptide institute, Japan), choline chloride, hexamethonium chloride dehydrate (Wako, Japan), morphine hydrochloride (Takeda Chemical Industries, Japan), tetrodotoxin (Sankyo, Japan), and dimethylphenylpiperazinium iodide (DMPP; Aldrich, U.S.A.). Prostaglandin E_2 was a gift from the Ono Pharmaceutical Company (Japan).

All other chemicals were of analytical grade. The drugs were dissolved in distilled water with the exception of indomethacin and prostaglandin E_2 which were dissolved in ethanol as stock solutions. Dilutions were made in Tyrode solution. Ethanol at the concentration used did not affect the release of ACh.

Results

In control experiments, no significant differences were observed between the first (S_1) and the second (S_2) stimulation in terms of the release of ACh induced by each stimulus used in this study. The values for the relative amounts of ACh released (S_2/S_1) were 115.6 ± 8.2, 94.4 ± 3.5, 110.8 ± 10.9 and 115.8 ± 6.7% for DMPP (10 μM), substance P (1 μM), 5-HT (2.6 μM) and EFS (50 V, 10 Hz and 0.5 ms for 20 s), respectively. The actual amounts of ACh released in response to various stimuli were also similar (not shown).

Release of acetylcholine induced by dimethylphenylpiperazinium

DMPP stimulates the release of ACh through the nicotinic receptors in myenteric neurones (Yokoyama *et al.*, 1986). The release of ACh evoked by DMPP (10 μM) was inhibited by Ind at 2.8 μM (to about 39% of control; Figure 1). The release was restored to 86% of control by addition of PGE_2 at 14.3 nM which itself had almost no effect on the release of ACh (Figure 1). $PGF_{2\alpha}$ 14.1 nM was ineffective (not shown). We did not use $PGF_{2\alpha}$ at concentrations higher than 14.1 nM, since we reported previously that the inhibitory effect of Ind on the nicotine-induced contraction was not affected by $PGF_{2\alpha}$ up to 141 nM (Yagasaki *et al.*, 1984). Mepacrine at 5 μM inhibited the DMPP-induced release of ACh to the same extent as Ind, and this inhibition was partially overcome by the addition of arachidonic acid at 10 μM (Figure 1). When Ind at 2.8 μM was used in addition to mepacrine, the restorative effect of arachidonic acid was eliminated (Figure 1). The release of ACh induced by DMPP was inhibited almost completely by 300 nM tetrodotoxin (TTX) (not shown).

Release of acetylcholine induced by substance P

Ind at 2.8 μM caused a significant inhibition of the release of ACh evoked by substance P (1 μM) to about 36% of control (Figure 2). This inhibitory effect of Ind was partially overcome by PGE_2 14.3 nM (Figure 2), but not by $PGF_{2\alpha}$ 14.1 nM (not shown). Mepacrine at 5 μM also depressed the release of ACh in response to substance P to about 40% of control (Figure 2). The inhibitory effect of mepacrine was partially overcome by arachidonic acid and this recovery was also eliminated by 2.8 μM Ind (Figure 2). TTX (300 nM) completely inhibited the stimulatory effect of substance P on the release of ACh (not shown).

Release of acetylcholine induced by 5-hydroxytryptamine

Ind at 2.8 μM failed to inhibit the release of ACh induced by 5-HT at 2.6 μM (Figure 3) but mepacrine at 5 μM was still effective (Figure 3). This inhibitory effect of mepacrine was overcome to a large extent by arachidonic acid at 10 μM (the release of ACh returned to about 88% of control; Figure 3). The recovery with arachidonic acid was not affected by 2.8 μM Ind. TTX (300 nM) inhibited incompletely but significantly, to 36.3 ± 8.7% of control, the release of ACh induced by 5-HT.

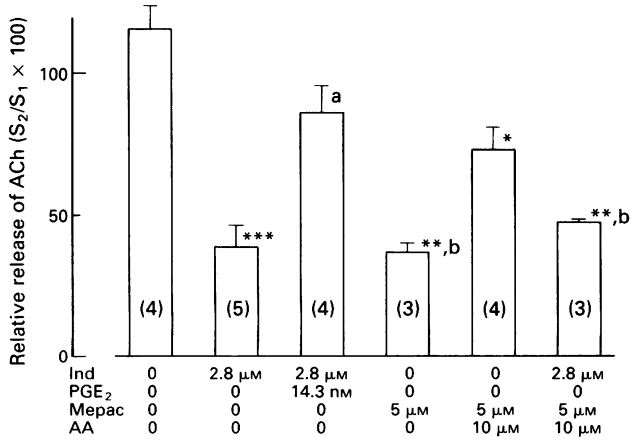


Figure 1 The inhibitory effects of indomethacin (Ind) and mepacrine (Mepac) on dimethylphenylpiperazinium (DMPP)-induced release of acetylcholine (ACh) from the myenteric plexus of guinea-pig ileum and the reversals of these effects by prostaglandin E_2 (PGE_2) and arachidonic acid (AA). DMPP was present at 10 μM. Ind, Mepac and AA were added to the bath 17 min before S_2 and PGE_2 was 2 min before S_2. The columns show the means with s.e. shown by vertical bars. Numbers of experiments are shown in parentheses. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with controls. $^aP < 0.01$ as compared with Ind. $^bP < 0.05$ as compared with Mepac and AA.

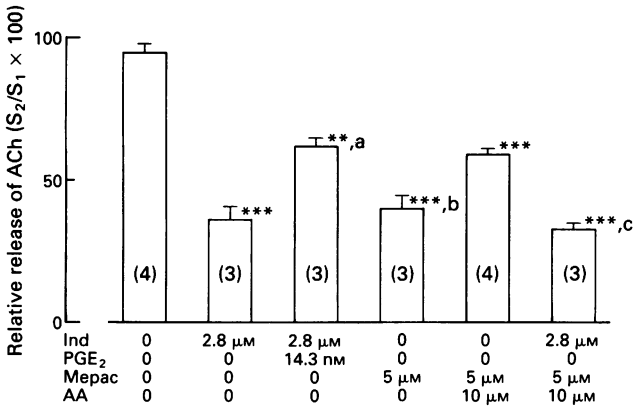


Figure 2 The inhibitory effects of indomethacin (Ind) and mepacrine (Mepac) on substance P-induced release of acetylcholine (ACh) from the myenteric plexus of guinea-pig ileum and the reversals of these effects by prostaglandin E_2 (PGE_2) and arachidonic acid (AA). Substance P was present at 1 μM. Ind, Mepac and AA were added to the bath 17 min before S_2 and PGE_2 was 2 min before S_2. The columns show the means with s.e. shown by vertical bars. Numbers of experiments are shown in parentheses. $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with controls. $^{a}P < 0.05$ as compared with Ind. $^{b}P < 0.05$, $^{c}P < 0.001$ as compared with Mepac and AA.

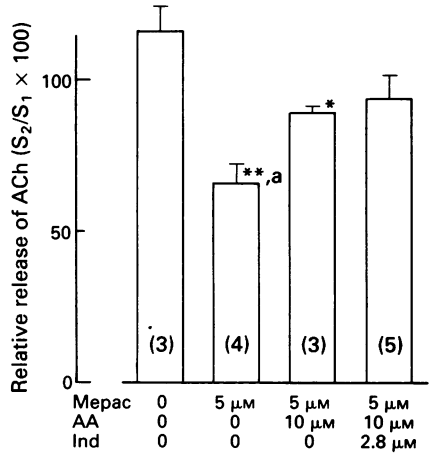


Figure 4 Effects of mepacrine (Mepac) and arachidonic acid (AA) on electrical field stimulation (EFS)-induced release of acetylcholine (ACh) from the myenteric plexus of guinea-pig ileum and the effect of indomethacin (Ind) on the recovery caused by AA. EFS was applied at supramaximal voltage (50 V), 0.5 ms duration and 10 Hz for 20 s. Mepac, AA and Ind were added to the bath 17 min before S_2. The columns show the means with s.e. shown by vertical bars. Numbers of experiments are shown in parentheses. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with controls. $^{a}P < 0.05$, as compared with Mepac and AA.

Electrical field stimulation-induced release of acetylcholine

Previously, we showed that the EFS-induced release of ACh is not affected by Ind (Yagasaki *et al.*, 1984). In the present study, the influence of endogenous arachidonic acid on the release of ACh stimulated by EFS was examined. Mepacrine (5 μM) significantly reduced the EFS-induced release of ACh and this inhibition was overcome by 10 μM arachidonic acid. The effect of arachidonic acid was not altered by 2.8 μM Ind (Figure 4).

Discussion

Both DMPP and nicotine induce the release of ACh by stimulating the nicotinic receptors on the soma of neurones since this release is inhibited by hexamethonium and TTX (Vizi, 1973). Substance P also excites the soma of enteric neurones (Wood, 1987) and causes the TTX-sensitive release of ACh

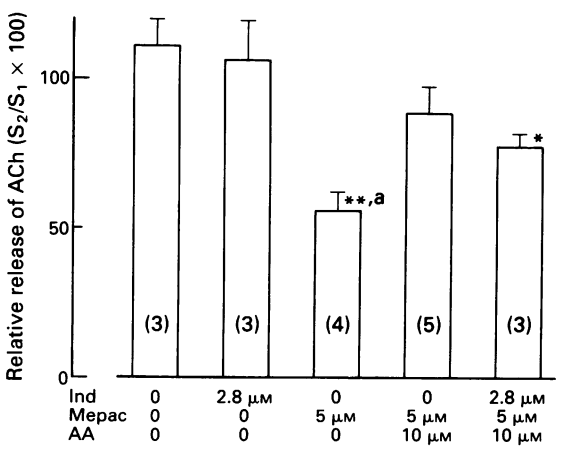


Figure 3 Effects of indomethacin (Ind), mepacrine (Mepac) and arachidonic acid (AA) on 5-hydroxytryptamine (5-HT)-induced release of acetylcholine (ACh) from the myenteric plexus of guinea-pig ileum. 5-HT was present at 2.6 μM. Mepac, AA and Ind were added to the bath 17 min before S_2. The columns show the means with s.e. shown by vertical lines. Numbers of experiments are shown in parentheses. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with controls. $^{a}P < 0.05$, as compared with Mepac and AA.

(Yau & Youther, 1982). In the present study, Ind (2.8 μM) inhibited the release of ACh induced by DMPP and substance P. The concentration of Ind used in this experiment causes a decrease in the production of endogenous prostaglandins by inhibiting the activity of cyclo-oxygenase in this tissue (Flower & Vane, 1974; Botting & Salzmann, 1974). In addition to inhibiting prostaglandin synthesis, Ind has various actions (Northover, 1977), but these effects occur at concentrations more than 10 times higher than that used in the present experiments. Thus, the inhibitory effects of Ind on the release of ACh by these substances may be attributed to decreases in levels of prostaglandins. This hypothesis is supported by the evidence that the addition of PGE_2 overcame the Ind-induced inhibition of the release of ACh evoked by DMPP and substance P. In addition, it has been reported that Ind depresses the release of ACh that is induced by nicotine (Yagasaki *et al.*, 1984), by bradykinin (Yau *et al.*, 1986) and during the atropine-sensitive contraction that is evoked by cholecystokinin (Zseli *et al.*, 1979); the inhibition in each case was overcome by the addition of PGE. These results suggest that endogenous prostaglandins contribute to the maintenance of the excitability of cholinergic neurones by substances that activate the receptors on the soma of myenteric neurones of the guinea-pig ileum. Previously, we showed that the release of ACh induced by the stimulation of axons or terminals was not affected by 2.8 μM Ind (Takeuchi *et al.*, 1989). Thus, the decrease in production of prostaglandins may affect neither the excitability of the axons nor of the terminals of cholinergic neurones.

In contrast to the above results, the release of ACh induced by 5-HT was not inhibited by Ind, although 5-HT has been reported to induce the release of ACh stimulating the 5-HT receptors on the enteric neurones (Taylor & Bywater, 1989). The reason why Ind was ineffective on the release of ACh evoked by 5-HT is not clear at present, but in contrast to the experiments with DMPP and substance P, about 40% of the release of ACh evoked by 5-HT still remained in the presence of TTX. Furthermore, it has been shown that the neuronally-mediated contraction of guinea-pig ileum to 5-HT was resistant to hexamethonium (Sanger & Nelson, 1989), and that the application of 5-HT to the soma of myenteric neurones did not affect the membrane potential in most neurones (Galligan *et al.*, 1988). In our model, therefore, the action of 5-HT may be on the axons and/or terminals. This site of action is consistent with the inability of Ind to affect the release of ACh

induced by the stimulation of axons and terminals (see earlier) and with the evidence that prostaglandins have no effects on the excitability of axons and terminals (Yagasaki *et al.*, 1984).

Mepacrine inhibited the release of ACh induced by all the stimuli used and such inhibition was at least partly overcome by the addition of arachidonic acid. Mepacrine inhibits the activity of phospholipase A_2 (Blackwell *et al.*, 1981) which plays an important role in the release of neurotransmitters (Takeuchi & Yagasaki, 1987). Thus, the inhibitory effect of mepacrine on the release of ACh in the present experiments may be due to the depression of phospholipase A_2 activity. The inhibition of phospholipase A_2 activity would cause a decrease in the liberation of arachidonic acid from phospholipids in membranes and, in turn, it would lower the level of prostaglandins in the tissue (Irvine, 1982; Needleman *et al.*, 1986). As mentioned above, prostaglandins modulated the release of ACh induced by DMPP and substance P. Since Ind cancelled the restorative effects of arachidonic acid on the release of ACh induced by DMPP and substance P, the effect of arachidonic acid may not be attributable to arachidonic acid itself but to its metabolites, the prostaglandins. However, we showed previously that the inhibition by mepacrine was not overcome by PGE_2 alone, and that arachidonic acid is also needed (Takeuchi & Yagasaki, 1987). Thus, these results suggest that the release of ACh induced by DMPP and substance P may also be regulated by both arachidonic acid and prostaglandins.

Ind did not inhibit the restorative effect of arachidonic acid on the EFS-induced release of ACh in the presence of mepacrine. This result is consistent with the result of Yagasaki *et al.* (1984) who found that prostaglandins are not involved in the modulation of the EFS-induced release of ACh. It is known that arachidonic acid is metabolized to lipoxygenase products as well as cyclo-oxygenase products. In the guinea-pig ileum, however, the lipoxygenase products can be considered not to participate in the effect of arachidonic acid on the release of ACh (Takeuchi & Yagasaki, 1987). Thus, arachidonic acid itself may regulate the release of ACh that is stimulated by EFS. EFS excites the axons in the neurones of the myenteric plexus (Yagasaki *et al.*, 1984) and the invasion of the propagated action potential to nerve terminal induces the release of ACh (Paton *et al.*, 1971). Thus, it appears that arachidonic acid may contribute to the modulation of the axonal conductance of neurones or of some aspect of the mechanism of the release of transmitter in the nerve terminals. Arachidonic acid can stimulate the release of hormones by raising intracellular concentrations of Ca^{2+} (Kolesnick *et al.*, 1984) or by activating the turnover of phospatidylinositol (Zeitler & Handwerger, 1985). In the release of neurotransmitter, Ca^{2+} and the turnover of phosphatidylinositol play important roles (Gershon, 1981, Nahorski *et al.*, 1986). From these findings, it seems conceivable that in the process involved in the release of ACh within the nerve terminals, there is a step that is sensitive to arachidonic acid.

In conclusion, endogenous prostaglandins may contribute to the modulation of excitability of the myenteric plexus by DMPP and substance P but not by 5-HT. In addition, endogenous arachidonic acid, a precursor of prostaglandins, also plays an important role in the release of ACh from nerve terminals of cholinergic neurones in the myenteric plexus of the guinea-pig ileum.

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(Received May 15, 1990
Revised July 30, 1990
Accepted October 8, 1990)

Br. J. Pharmacol. (1991), **102**, 386–390 © Macmillan Press Ltd, 1991

5-HT_1-like receptors mediate 5-hydroxytryptamine-induced contraction of guinea-pig isolated iliac artery

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1 The effects of 5-hydroxytryptamine (5-HT) and of the 5-HT_1-like receptor agonists, 5-carboxamidotryptamine (5-CT) and sumatriptan (GR43175) were investigated in isolated ring preparations of guinea-pig common iliac artery.

2 The three agonists induced very weak, if any, contractions of unstimulated preparations, whereas they elicited concentration-dependent contractions in preparations given a moderate tone by a threshold concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

3 Under the latter conditions, E_{max} values for 5-HT and 5-CT reached about 45% of $PGF_{2\alpha}$ maximal effect, whereas the E_{max} value of sumatriptan was significantly lower (about 35%). The rank order of potency (mean EC_{50} value, nM) was 5-CT (6.6) > 5-HT (22.9) > sumatriptan (155). Pargyline, cocaine or deoxycorticosterone were without significant effect on the contractions induced by 5-HT.

4 The 5-HT_3 receptor antagonist, (1αH,3α,5αH-tropan-3-yl) 1-H-indole-3-carboxylic acid ester (ICS 205-930; 1 μM), had no effect on 5-HT-, 5-CT- and sumatriptan-induced contractions.

5 The 5-HT_2 receptor antagonist, ketanserin (1 μM) caused only small rightward shifts (concentration-ratios, about 2) in the concentration-response curves to 5-HT, 5-CT and sumatriptan without significantly depressing the maximum effects.

6 In the presence of ketanserin (1 μM), the non-selective 5-HT receptor antagonist, methiothepin (0.1 μM), shifted the concentration-response curves to 5-HT and 5-CT to the right in a parallel manner and to a similar extent for both agonists (respective mean pK_B values, 8.07 and 8.27). The effect of sumatriptan was also antagonized by methiothepin, but solvent effects precluded quantitative analysis of this antagonism.

7 It is concluded that 5-HT_1-like receptors mediate the contractions induced by 5-HT, 5-CT and sumatriptan in guinea-pig isolated iliac artery. For reasons not yet understood, these receptors are detected only when the tissues are moderately pre-contracted by $PGF_{2\alpha}$.

Introduction

Contractile responses of blood vessels to 5-hydroxytryptamine (5-HT) are mostly mediated by 5-HT_2 receptors (for a review see Saxena, 1989). Notable exceptions are the dog isolated saphenous vein (Apperley *et al.*, 1980; Feniuk *et al.*, 1985; Humphrey *et al.*, 1988), isolated basilar arteries from various species including man (Bradley *et al.*, 1986b; Chang & Owman, 1989; Connor *et al.*, 1989; Parsons & Whalley, 1989; Parsons *et al.*, 1989), cranial arteriovenous anastomoses of pig and cat (Saxena *et al.*, 1986; Feniuk *et al.*, 1987) and dog carotid arterial bed (Feniuk *et al.*, 1989). In all these vessels, 5-HT_1-like receptors have been implicated in the vasoconstrictor response to 5-HT.

5-HT_1-like receptors have been defined as those 5-HT receptors satisfying the following three criteria: 5-carboxamidotryptamine (5-CT) should behave as an agonist and be at least as potent as 5-HT, itself; 5-HT_2 and 5-HT_3 receptor antagonists should be inactive; methiothepin should be a potent antagonist (Bradley *et al.*, 1986a). In addition, sumatriptan (or GR43175) has recently emerged as a selective 5-HT_1-like receptor agonist and as such is potentially a useful tool in the search for 5-HT_1-like receptors (Humphrey *et al.*, 1988).

In the present study, evidence is provided that 5-HT receptors fulfilling these criteria are present in guinea-pig isolated iliac artery. Contraction mediated by these 5-HT_1-like receptors is unmasked when the vessels are moderately precontracted by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). A preliminary account of this work was presented at the 1990 Spring Meeting of the German Pharmacological Society (Schoeffter & Sahin-Erdemli, 1990).

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Methods

Experimental protocol

Male albino guinea-pigs (250–300 g) were killed by a blow to the neck. The abdominal aorta, iliac and femoral arteries were exposed, removed and placed in a Krebs bicarbonate solution (composition in mM: NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 1.25 and glucose 11.5). The right and left common iliac arteries were carefully cleaned of loosely adherent tissue under a microscope. Two ring segments (1.5–2 mm long) were cut in each iliac artery. No attempt was made to remove the endothelium. Rings were mounted between L-shaped stainless steel wires in 10 ml organ baths filled with Krebs bicarbonate solution bubbled with 95% O_2:5% CO_2 at 37°C. An initial tension of 0.5 g was applied and repeatedly readjusted during a 2 h period of washing and equilibration. Isometric changes in tension were recorded. Rings were first contracted by a maximally effective concentration of $PGF_{2\alpha}$ (30 μM). When the contraction had plateaued, carbachol (10 μM) was added and tissues were allowed to relax. $PGF_{2\alpha}$-induced contraction and carbachol-elicited relaxation amounted to 1.00 ± 0.02 g and 54 ± 2% reduction in tone, respectively ($n = 167$). After a further 60–90 min period of washout and resting, during which the tension was readjusted to 0.5 g, tissues were challenged with 5-HT, 5-CT or sumatriptan, either at rest or after the tone had been moderately increased by a threshold concentration of $PGF_{2\alpha}$ (an EC_{10} to EC_{20}, i.e. 0.3 to 1 μM; see Figure 1). 5-HT, 5-CT and sumatriptan were added cumulatively in concentrations increasing by about 3 fold steps. When studied, 5-HT receptor

antagonists, pargyline and uptake inhibitors were present 30 min before $PGF_{2\alpha}$. Agonists and antagonists were tested on only one arterial ring from a given animal; hence n numbers refer to animal numbers. The effects of antagonists and inhibitors were investigated in rings adjacent to those used as controls.

Analysis of data

Concentration-response curves for 5-HT, 5-CT and sumatriptan were analysed using SCTFIT, a non linear regression computerized programme (De Lean *et al.*, 1980). EC_{50} and E_{max} values were derived from this analysis. Antagonist K_B values were calculated according to the formula: $K_B = [B]/(CR - 1)$, where [B] is the concentration of antagonist used and CR (concentration-ratio) is the ratio of agonist EC_{50} measured in the presence of antagonist over that measured in the absence of antagonist (Furchgott, 1972). E_{max} values are expressed as percentages of the amplitude of $PGF_{2\alpha}$ (30 μM)-induced contraction and given as arithmetic means ± s.e.mean. EC_{50} values and concentration-ratios are expressed as geometric means with 95% confidence limits in parentheses. K_B values were given as negative logarithms (pK_B). Statistical comparisons were made by the use of Student's t test. A P level less than 0.05 was considered significant.

Materials

5-HT creatinine sulphate, pargyline hydrochloride, cocaine hydrochloride and carbachol (carbamylcholine chloride) were obtained from Sigma, Saint Louis, MO, U.S.A. $PGF_{2\alpha}$ was purchased from Upjohn in the form of Dinolytic ($PGF_{2\alpha}$ tromethamine salt). 11-Deoxycorticosterone acetate was from Schuchard, Munich, F.R.G. 5-CT hydrogenomaleate, sumatriptan and (1αH,3α,5αH-tropan-3-yl)1-H-indole-3-carboxylic acid ester (ICS 205-930) hydrochloride were synthesized at Sandoz, Basle, Switzerland. Ketanserin tartrate and methiothepin were gifts from Janssen, Beerse, Belgium and from Hoffmann-La Roche, Basle, Switzerland, respectively.

Stock solutions (10 mM) of sumatriptan, ketanserin and methiothepin were prepared daily in a mixture of ethanol:1-methyl- 2-pyrolidone:water (1:1:2) containing ascorbic acid 10 mg ml^{-1}. Subsequent dilutions were made in distilled water. Deoxycorticosterone (10 mM) was dissolved in ethanol. Other substances were dissolved in distilled water.

Results

Effects of 5-hydroxytryptamine, 5-carboxamidotryptamine and sumatriptan

When added to resting rings of guinea-pig iliac arteries, 5-HT (1 nM–0.1 mM), 5-CT (1 nM–0.1 mM) and sumatriptan (10 nM–3 μM) were generally without effect (Figure 1). Occasionally, the highest concentrations of 5-HT and 5-CT ($\geq$10 μM) elicited small contractile responses. By contrast, when tissues had been moderately pre-contracted by a threshold concentration of $PGF_{2\alpha}$ (see Methods), they were further contracted by 5-HT, 5-CT and sumatriptan in a concentration-dependent manner (Figure 1).

The concentration-response curves for 5-HT, 5-CT and sumatriptan obtained under these conditions are shown in Figure 2. 5-HT and 5-CT induced contractions at a concentration as low as 1 nM. With 0.1 μM 5-CT and 0.3 μM 5-HT, the curves reached similar E_{max} values amounting to about 45% the contraction induced by $PGF_{2\alpha}$ (30 μM). At concentrations higher than 1 μM, 5-CT did not induce further contraction, whereas responses to 5-HT were inconsistent: relaxation was frequently observed at 3 μM 5-HT, sometimes followed by re-contraction at higher concentrations; in the other cases, no further increases in tension were produced by 3 μM 5-HT, but 10 μM and higher concentrations produced additional contractions. Sumatriptan (10 nM–3 μM) induced concentration-dependent increases in tension. The E_{max} value was not fully reached at 3 μM, but higher concentrations could not be used because the vehicle began to display relaxing effects. Nevertheless, the computer fitting of the sumatriptan curve yielded an E_{max} of about 35% of that of $PGF_{2\alpha}$ (see Table 1), which is in

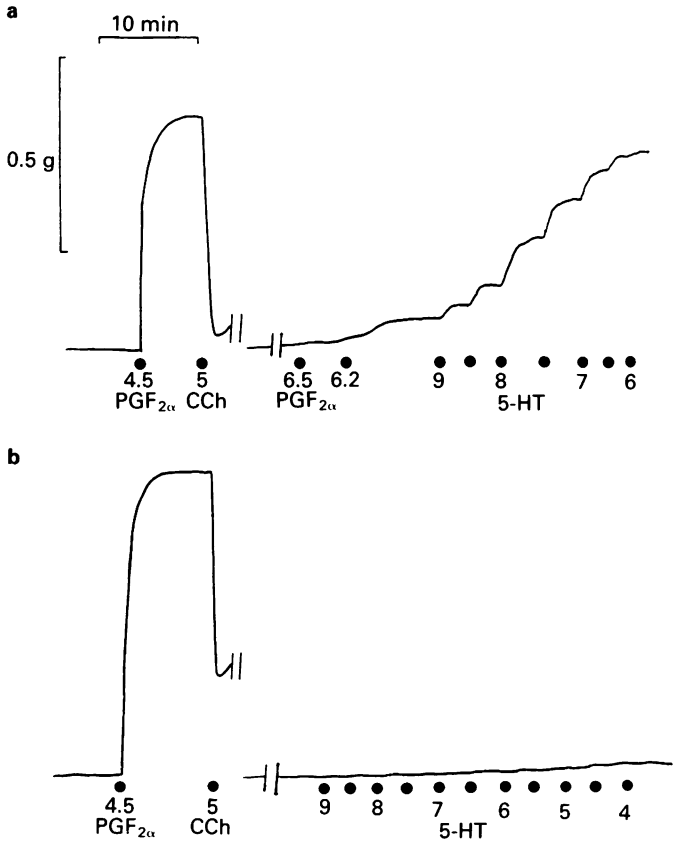


Figure 1 Experimental recordings illustrating the effects of 5-hydroxytryptamine (5-HT) in guinea-pig isolated iliac artery rings, with (a) and without (b) moderate pre-contraction induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The figure also shows the first stimulation by $PGF_{2\alpha}$ (30 μM) followed by the relaxation to carbachol (CCh, 10 μM). Negative logarithms of molar concentrations are indicated. Intermediate concentrations of 5-HT correspond to 3 fold increases.

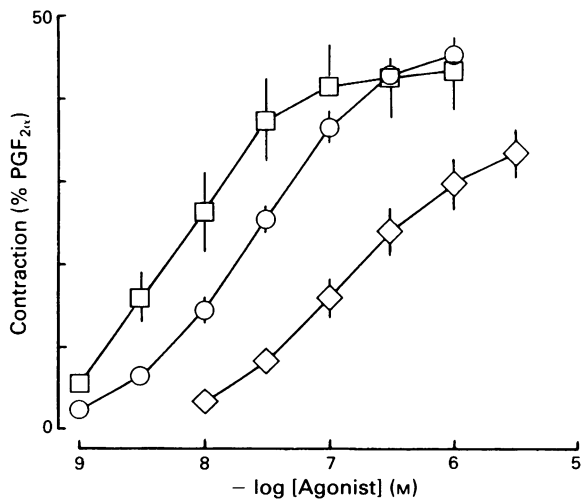


Figure 2 Concentration-effect curves to 5-hydroxytryptamine (○), 5-carboxamidotryptamine (□) and sumatriptan (◇) in guinea-pig isolated iliac artery. Experimental conditions were those described in Figure 1 (a). Points are means with s.e.mean (shown by vertical bars) of 28, 11 and 21 values, respectively.

Table 1 Parameters of 5-hydroxytryptamine, 5-carboxamidotryptamine and sumatriptan agonist activities in guinea-pig isolated iliac arteries

Agonist	EC_{50} (nM)	E_{max} (%)	n
5-Hydroxytryptamine	22.9 (18.2–28.8)	46.3 ± 2.6	28
5-Carboxamidotryptamine	6.6 (4.4–10.0)	44.1 ± 5.1	11
Sumatriptan	155 (100–240)	36.2 ± 3.4	21

EC_{50} values are expressed as geometric means with 95% confidence limits in parentheses; E_{max} values are arithmetic means ± s.e.mean of contractions expressed in percentages of the maximal contraction induced by prostaglandin $F_{2\alpha}$ (30 μM). Experimental conditions were those described in Figure 1a.

line with the overall sigmoidal aspect of the curve (Figure 2). The E_{max} value of sumatriptan was found to be significantly ($P < 0.02$) smaller than that of 5-HT (Table 1). The rank order of potency of the three agonists was as follows (mean EC_{50} values, nM): 5-CT (6.6) > 5-HT (22.9) > sumatriptan (155). Table 1 gives the 95% confidence limits of the EC_{50} values, which do not overlap.

The concentration-response curve to 5-HT was not significantly altered in the presence of either pargyline (50 μM), cocaine (30 μM) or deoxycorticosterone (3 μM), conditions for inhibition of monoamine oxidase, neuronal uptake and extraneuronal uptake, respectively (Figure 3).

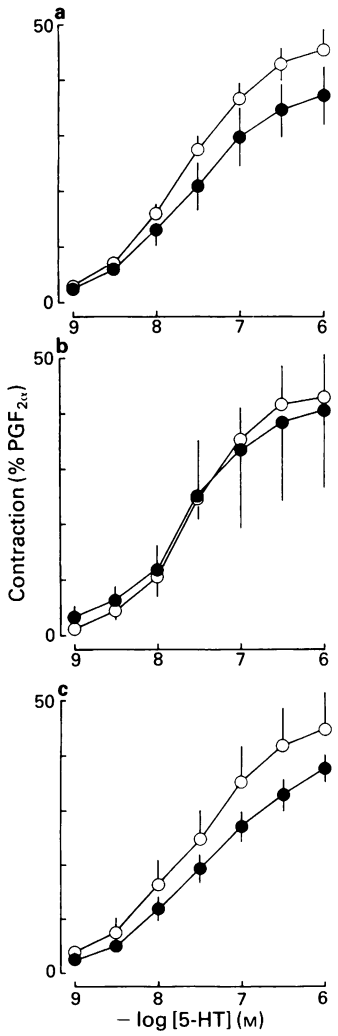


Figure 3 Concentration-effect curves to 5-hydroxytryptamine in guinea-pig isolated iliac artery in the absence (○) and in the presence (●) of (a) pargyline (50 μM); (b) cocaine (30 μM); (c) deoxycorticosterone (3 μM). Experimental conditions were those described in Figure 1 (a), the inhibitors being added 30 min before the second administration of prostaglandin $F_{2\alpha}$. Points are means with s.e.mean (shown by vertical bars) of 5 values.

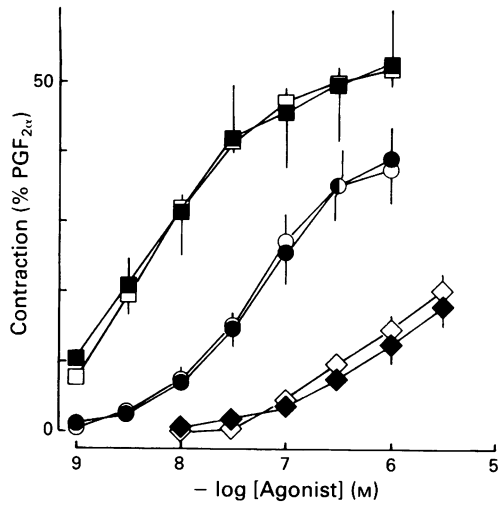


Figure 4 Concentration-effect curves to 5-hydroxytryptamine (circles), 5-carboxamidotryptamine (squares) and sumatriptan (diamonds) in guinea-pig isolated iliac artery in the absence (open symbols) and in the presence (closed symbols) of ICS 205-930 (1 μM). Experimental conditions were those described in Figure 1a, the antagonist being added 30 min before the second administration of prostaglandin $F_{2\alpha}$. Points are means with s.e.mean (shown by vertical bars) of 4 values.

Effects of 5-hydroxytryptamine receptor antagonists

The 5-HT_3 receptor antagonist, ICS 205-930 (1 μM), did not significantly affect concentration-response curves to 5-HT, 5-CT and sumatriptan (Figure 4).

In the presence of the 5-HT_2 receptor antagonist, ketanserin (1 μM) the concentration-response curves to 5-HT, 5-CT and sumatriptan were slightly shifted to the right, without significant depression of the E_{max} values (Figure 5). Concentration-ratio shifts induced by ketanserin (1 μM), as measured at the EC_{50} level, were 2.6 (1.6–4.2), 2.3 (1.5–3.5) and 1.8 (1.1–2.8) against 5-HT ($n = 11$), 5-CT ($n = 9$) and sumatriptan ($n = 13$),

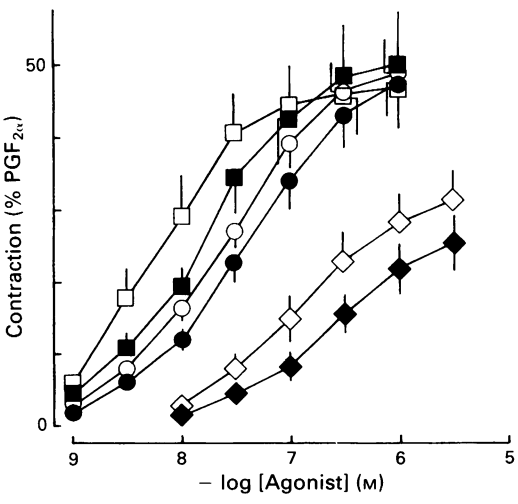


Figure 5 Concentration-effect curves to 5-hydroxytryptamine (circles), 5-carboxamidotryptamine (squares) and sumatriptan (diamonds) in guinea-pig isolated iliac artery in the absence (open symbols) and in the presence (closed symbols) of ketanserin (1 μM). Experimental conditions were those described in Figure 1a, the antagonist being added 30 min before the second administration of prostaglandin $F_{2\alpha}$. Points are means with s.e.mean (shown by vertical bars) of 11 (5-HT), 9 (5-CT) and 13 (sumatriptan) values.

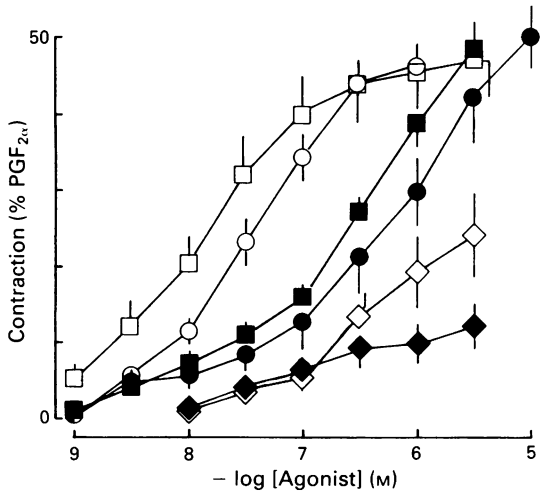


Figure 6 Concentration-effect curves to 5-hydroxytryptamine (circles), 5-carboxamidotryptamine (squares) and sumatriptan (diamonds) in guinea-pig isolated iliac artery in the absence (open symbols) and in the presence (closed symbols) of methiothepin (0.1 μM). Experiments were performed in the presence of ketanserin (1 μM). Experimental conditions were those described in Figure 1a, the antagonists being added 30 min before the second administration of prostaglandin $F_{2\alpha}$. Points are means with s.e.mean (shown by vertical bars) of 5 (5-HT), 6 (5-CT) and 8 (sumatriptan) values.

respectively (geometric means with 95% confidence intervals). That is, the effect of ketanserin (1 μM) was at the borderline of significance.

The effect of the 5-HT receptor antagonist, methiothepin (0.1 μM) was investigated in the presence of ketanserin (1 μM). Control concentration-response curves to 5-HT and to 5-CT (that is, in the presence of ketanserin 1 μM) were displaced to the right by methiothepin 0.1 μM, without loss of E_{max} values (Figure 6). Concentration-ratios were 16 (3–100) and 20 (9–44) for 5-HT ($n = 5$) and 5-CT ($n = 6$), corresponding to pK_B values of 8.07 $\pm$ 0.41 and 8.27 $\pm$ 0.15, respectively. Methiothepin (0.1 μM) also exerted an inhibitory effect on sumatriptan-induced contractions (Figure 6); in this particular case the surmountable nature of the antagonism could not be established, owing to vehicle effects, as mentioned above. In the presence of methiothepin (0.1 μM), nevertheless, a concentration of 3 μM sumatriptan was necessary to reach about the same level of contraction as that produced by 0.3 μM under control conditions (Figure 6). This indicates an approximate concentration-ratio and pK_B value of 10 and 8 respectively.

Discussion

The present study shows that 5-HT and the 5-HT$_1$-like receptor agonists, 5-CT and sumatriptan can elicit concentration-dependent contractions of guinea-pig isolated iliac artery, provided that a minimal tone is previously induced by $PGF_{2\alpha}$. The 5-HT receptor mediating these responses has the characteristics of the 5-HT$_1$-like receptor as proposed by Bradley *et al.* (1986a). Thus, the contractions induced by 5-HT, 5-CT and sumatriptan were relatively resistant to, and unaffected by, the 5-HT$_2$ and 5-HT$_3$ receptor antagonists, ketanserin and ICS 205-930, respectively. The weak antagonist effect displayed by ketanserin in guinea-pig iliac artery is indeed hard to reconcile with its potency in the nanomolar range at 5-HT$_2$ receptors (Van Nueten *et al.*, 1981; Feniuk *et al.*, 1985). In fact, the deduced pK_B value of ketanserin (about 6) fits well with its reported affinity for the various 5-HT$_1$ binding sites (Hoyer, 1988). Second, methiothepin was found to be a potent antagonist of 5-HT, 5-CT and sumatriptan in guinea-pig iliac artery. It was about equiactive and apparently competitive against 5-HT and 5-CT; it also inhibited sumatriptan-induced contractions with an apparently similar potency, although in this case the analysis was hampered by vehicle effects. Third, 5-CT was more potent than 5-HT. Sumatriptan, introduced more recently, was less potent than 5-HT by 6 to 7 fold, an observation in line with its activity at the prototype 5-HT$_1$-like receptor mediating contraction of dog saphenous vein (Humphrey *et al.*, 1988).

As a whole, these results strongly support the view that 5-HT and the 5-HT$_1$-like receptor agonists, 5-CT and sumatriptan evoke contraction of guinea-pig iliac artery through stimulation of a 5-HT$_1$-like receptor. Thus, guinea-pig iliac artery appears to represent another vessel endowed with 5-HT$_1$-like receptors mediating vascular contraction, besides the saphenous vein of the dog and basilar arteries of various species (see Introduction). Removal of agonists by monoamine oxidase, neuronal or extraneuronal uptake are unlikely to bias this interpretation, since pargyline, cocaine and deoxycorticosterone did not significantly affect 5-HT-induced contraction. It is interesting to compare the intrinsic activities of 5-CT and sumatriptan, with respect to that of 5-HT, in the present model and in other models of 5-HT$_1$-like receptor-mediated contraction. In guinea-pig iliac artery, the rank order of intrinsic activities was 5-CT = 5-HT > sumatriptan. In other models, 5-CT usually displays less efficacy than 5-HT (Feniuk *et al.*, 1985; Connor *et al.*, 1989; Parsons & Whalley, 1989; Parsons *et al.*, 1989). In some of these cases, a minor population of 5-HT$_2$ receptors may explain the greater response to 5-HT. This seems not to be the case in guinea-pig iliac artery since 5-CT and 5-HT displayed about equal efficacy. On the other hand, sumatriptan behaved as a full agonist in dog saphenous vein (Humphrey *et al.*, 1988), whereas it behaved as a partial agonist (with about 80% of the intrinsic activity of 5-HT) in guinea-pig iliac artery. Partial agonist activity of sumatriptan has been reported elsewhere (Bond *et al.*, 1989; Parsons *et al.*, 1989; Schoeffter & Hoyer, 1989a; 1990). Such variations in intrinsic activities do not necessarily imply that the receptors involved are heterogeneous, since they may be interpreted in terms of different receptor densities or receptor-coupling efficiencies (see Kenakin, 1984). It is thus quite possible that the 5-HT$_1$-like receptor mediating contraction of guinea-pig iliac artery is the same as that reported in dog saphenous vein (Feniuk *et al.*, 1985; Humphrey *et al.*, 1988).

As regards the identity of the present receptor with one or other of the 5-HT$_1$ receptor subtypes defined by binding studies, it appears difficult to draw any conclusion on the basis of agonist potency ratio and without the use of discriminating 5-HT$_1$ receptor antagonists. It is noteworthy, nevertheless, that sumatriptan is preferentially recognized by 5-HT$_{1D}$ binding sites (Peroutka & McCarthy, 1989; Schoeffter & Hoyer, 1989b; Sumner & Humphrey, 1989; Waeber *et al.*, 1989). In addition, the compound behaves as a full agonist in a proposed 5-HT$_{1D}$ receptor model, inhibition of adenylate cyclase in calf substantia nigra (Schoeffter & Hoyer, 1989b). However, the agonist effect of sumatriptan does not necessarily imply that 5-HT$_{1D}$ binding sites and the 5-HT$_1$-like receptor mediating contraction of guinea-pig iliac artery are identical. Sumatriptan also behaves as a full agonist in 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor models based on second messengers, although with less potency than in the 5-HT$_{1D}$ receptor model (Schoeffter & Hoyer, 1989b). On the other hand, sumatriptan-induced contraction of dog saphenous vein is reportedly unaffected by metergoline (0.1 μM; Humphrey *et al.*, 1988), a drug showing nanomolar affinity to 5-HT$_{1D}$ recognition sites (Heuring & Peroutka, 1987; Waeber *et al.*, 1988).

It is not clear at present why the 5-HT$_1$-like receptor present in guinea-pig iliac artery requires a state of moderate pre-contraction, induced by $PGF_{2\alpha}$, to be functionally 'expressed'. Interestingly, this situation is reminiscent of that described by MacLennan *et al.* (1989) in the human isolated umbilical artery. In that vessel, 5-HT$_1$-like receptors mediating contraction were 'unmasked' under conditions of high

oxygen tension, a mechanism which involved cyclo-oxygenase products. Thus, although 5-HT_2 receptors appear to mediate contraction of most vascular preparations, 5-HT_1-like receptors might be of physiological importance under conditions of increased levels of eicosanoids.

In conclusion, 5-HT and the 5-HT_1-like receptor agonists, 5-CT and sumatriptan induced contraction of isolated rings from guinea-pig iliac arteries, moderately pre-contracted by $PGF_{2\alpha}$. This effect is most probably mediated by a 5-HT_1-like receptor, which could be similar to the 5-HT_{1D} type but further experiments are required to test this possibility.

The expert technical assistance of Mr K. Bruttel is greatly acknowledged, as well as the critical reading of the manuscript by Dr J.R. Fozard. The authors are also indebted to Dr R. Hof for providing laboratory equipment.

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(Received May 9, 1990
Revised July 2, 1990
Accepted October 9, 1990)

Br. J. Pharmacol. (1991), **102**, 391–395 © Macmillan Press Ltd, 1991

Studies of three non-peptide cholecystokinin antagonists (devazepide, lorglumide and loxiglumide) in human isolated alimentary muscle and guinea-pig ileum

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1 Three recently described non-peptide cholecystokinin (CCK) antagonists (devazepide, lorglumide, loxiglumide) have been studied for their antagonism of the contraction to cholecystokinin-octapeptide (CCK-OP) in human alimentary muscle and guinea-pig intestine.

2 Each antagonist caused a concentration-dependent inhibition of the contraction induced by CCK-OP, regardless of regional and species differences.

3 The potencies of each drug, estimated by use of an adaptation of the Cheng & Prusoff equation, were similar in the different regions of human alimentary tract (weighted mean apparent pK_B, ± s.e.mean: devazepide, 5.76 ± 0.08, $n = 20$; lorglumide, 5.82 ± 0.04, $n = 25$; loxiglumide, 5.87 ± 0.07, $n = 24$).

4 In contrast, the potencies differed markedly in the guinea-pig ileum. Apparent pK_B values obtained by the same method as with human tissues were, mean ± s.e.mean: devazepide, 10.61 ± 0.61; lorglumide, 7.43 ± 0.20; loxiglumide, 6.67 ± 0.12. pK_B values obtained from classical competition experiments were: devazepide, 10.09 ± 0.09; lorglumide 7.70 ± 0.12; loxiglumide 6.08 ± 0.22.

5 The CCK receptors in human gut muscle from different regions seem to be similar, but there appear to be species differences.

Introduction

Cholecystokinin (CCK) has long been recognised as the major regulator of gallbladder contraction (Ivy & Goldberg, 1928). The cholecystokinetic activity of different fragments of CCK in man has been established in several studies *in vivo* and *in vitro* (for review see Ryan, 1987).

Our experiments on human stomach, small intestine and colon have shown that CCK octapeptide (CCK-OP) contracts circular muscle strips from the stomach and large intestine, and longitudinal muscle strips from all regions of the gastrointestinal tract, by acting directly on the muscle. In contrast, CCK-OP inhibits the circular layer of the small intestine by stimulating intramural postganglionic inhibitory neurones (D'Amato *et al.*, 1990). Recent studies have shown three non-peptide drugs to be potent and selective CCK antagonists in tissues from laboratory animals (devazepide, formerly MK-329 and L-364,718, Chang & Lotti, 1986; lorglumide, Makovec *et al.*, 1987; loxiglumide, Setnikar *et al.*, 1987). We now show that these drugs are effective in human alimentary muscle. However they, and in particular devazepide, are substantially less potent than in guinea-pig ileum.

Methods

Human alimentary muscle

Macroscopically normal specimens of human gastrointestinal muscle, obtained at operation for benign or malignant conditions, were taken at least 5 cm from any visible lesion. The specimens were placed in Krebs solution bubbled with 95% O_2:5% CO_2 at room temperature and studied either immediately or after storage at 4°C for up to 24 h. Mesentery and fat were removed, and the mucosa/submucosa cut away from the muscle along the submucosal plexus. Depending on the size of the specimen, between 6–16 strips each 3–4 mm wide and 20–30 mm long were cut through the muscle coat parallel to either the circular or longitudinal muscle fibres (taeniae for the colon). The gallbladders were excised because of gallstones; they generally appeared inflamed on macroscopic examination, and histology showed chronic cholecystitis. Gallbladder strips were cut through the whole wall, both parallel and perpendicular to the longitudinal axis.

Guinea-pig ileum

Guinea-pigs of either sex, weighing 250–350 g, were stunned and exsanguinated. Segments of ileum 2–3 cm long were taken at least 15 cm proximal to the caecum.

Experimental procedures

Each strip (human gut) or segment (guinea-pig ileum) was suspended vertically under a load of 1 g in a tissue bath (5–7 ml) at 37°C containing Krebs solution bubbled with 95% O_2:5% CO_2. After equilibration for at least 1 h the responses, magnified 10–15 times in different specimens, were registered by use of isotonic transducers and pen recorders.

An initial non-cumulative concentration-response curve was obtained to acetylcholine (ACh, 0.01–25 μM; contact time 30 s, cycle time 7–10 min which was constant in each preparation). This was followed by reproducible ACh-induced submaximal contractions (40–80% of the maximum response). CCK-OP was then added to the bath for 2 min (human strips) or 1 min (guinea-pig segments; longitudinal muscle activity recorded), and CCK-OP was added again at intervals of 1 h (human strips) or 20 min (guinea-pig segments) until there were reproducible contractions that were 30–70% of maximum; these cycle times for CCK-OP were needed to avoid tachyphylaxis. When at least 2 reproducible control responses to CCK-OP had been obtained, each antagonist was added to a separate preparation from the same specimen either 20 min (devazepide) or 10 min (lorglumide and loxiglumide) before the next dose of CCK-OP, and then washed out at the end of the CCK-OP response. These antagonist contact times were chosen from preliminary studies. One strip in each experiment served as the control. Antagonist concentrations were increased 10 fold sequentially from 0.1–100 μM in the human

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strips, and from 1 pM–10 nM (devazepide), 1 nM to 1 μM (lorglumide) and 1 nM to 10 μM (loxiglumide) in the guinea-pig ileum. The effect of CCK-OP was measured at the end of its contact time and the reduction of the contraction by the antagonist was expressed as the percentage from the control response in the presence of vehicle. IC_{50} values were determined graphically and the variance between and within antagonist treatments calculated.

Pharmacological antagonism is conventionally quantitated by use of the Schild analysis or modifications of this method (e.g. Black *et al.*, 1985). The analysis is a null method based on a rightward displacement of agonist concentration-effect curves to yield a data set of agonist log [EC_{50}] versus log [antagonist]. A parallel displacement of the agonist curves with the same maximum response and a Schild slope not significantly different from unity indicates that the antagonist acts in a simple competitive manner. When the Schild slope is indeed unity, the calculated pA_2 value (the $-\log_{10}$ of the antagonist concentration giving a dose-ratio of 2) is an estimate of pK_B (the $-\log_{10}$ of the equilibrium dissociation constant of a competitive antagonist).

Because of the need to add CCK-OP at hourly intervals to the human tissues, Schild plots could be constructed, and in any case the cost of the peptide would have been prohibitive. For the human data we therefore had to use an alternative analytical method which is less robust because it does not test competitivity. Schild plots, obtained in 4 experiments on guinea-pig ileum with cumulative additions of CCK-OP, produced similar results when analysed as described by Black *et al.* (1985) and validate the alternative method.

The equation of Cheng & Prusoff (1973), typically used to calculate the K_i of a test ligand from its ability to displace a reference radioligand, can be adapted to measure pharmacological antagonism. The K_i is equivalent to the K_B, the latter being the anti-log of the pK_B. If an antagonist is competitive, interacts with a single receptor and produces a concentration-response curve which is a rectangular hyperbola (mid-point slope parameter of unity), it can be shown that $K_B = IC_{50}/(1 + ([A]/[A_{50}]))$, where [A] is a fixed test concentration of an agonist whose concentration-effect curve is a rectangular hyperbola and $[A_{50}]$ is the agonist EC_{50}.

Since the method does not test competitivity of antagonism, the calculated values are referred to in the text as apparent pK_B values. We have analysed the CCK-OP concentration-effect curves for all the tissues, but only those with a mid-point slope not significantly different from unity have been used to calculate the apparent pK_B.

As the amount of agonist used was approximately the EC_{50} (i.e. [A] ~ $[A_{50}]$), then the apparent $K_B \sim IC_{50}/2$. A deviation of ±20% from the EC_{50} for CCK-OP would give an error in the apparent K_B determination of not greater than 0.25 log unit.

The concentration-effect curve results from individual guinea-pig tissues were fitted to a 3-parameter logistic function which calculates the mid-point location (log EC_{50}), mid-point slope, and maximal asymptote (Black *et al.*, 1985). With the human tissues, the small number of CCK-OP concentrations usually possible made it feasible to fit only the mean data for the 3-parameter logistic function. Parallelism of the CCK-OP concentration-effect curves in guinea-pig ileum was tested by one-way ANOVA, comparing the mid-point slope and maximal asymptote between and within antagonist treatment groups. Since no significant differences were observed ($P > 0.05$), the apparent pK_B values were determined from a derivation of the Schild equation (Black *et al.*, 1985).

Although Schild plots are inherently stronger because they examine the criteria of competitivity (i.e. parallel concentration-response curves, the same response maximum, and a Schild slope of unity), the Cheng & Prusoff equation can be used to determine antagonist activity. This is borne out by the similar results we obtained on guinea-pig ileum using the alternative method, compared with our own Schild plots and the results of others.

Drugs

Acetylcholine hydrochloride (Sigma) and cholecystokinin octapeptide (Sigma) were dissolved in 154 mM NaCl. Lorglumide [D,L-4(3,4-dichlorobenzoylamino)-5-(dipentylamino)-5-oxo-pentanoic acid] and loxiglumide [D,L-4-(3,4-dichlorobenzoylamino)-5-(N-3-methoxypropylpentamino)-5-oxo-pentanoic acid; both from Rotta Laboratories] were dissolved in 154 mM NaCl (pH 9.1 with NaOH). Devazepide [3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-1H-indole-2-carboxamide; Merck, Sharp and Dohme] was prepared in 154 mM NaCl containing 0.1% dimethyl sulphoxide. The Krebs solution had the following composition (mM): NaCl 121.5, $CaCl_2 \cdot 6H_2O$ 2.51, KH_2PO_4 1.18, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 25 and dextrose 5.55.

Statistics

The results are presented as means ± s.e.mean. One way analysis of variance was used to compare the potencies of the three antagonists (Snedecor & Cochran, 1976).

Results

Human alimentary tract

There were 58 specimens yielding 116 strips of longitudinal muscle and 131 circular muscle strips from 7 gut regions (Table 1). Gallbladder muscle fibres are irregularly orientated, and since the 'longitudinal' and 'circular' strips from the same specimen responded similarly the results are pooled.

Storage at 4°C overnight (maximum 24 h) did not appear to affect the tissue responses, since on 4 different occasions neighbouring strips from the same preparations responded similarly when used either fresh or the next day. This confirms our other observations (D'Amato *et al.*, 1990). The results on fresh and stored specimens were therefore combined.

During the experiments the muscle tone either decreased somewhat (45% of strips), remained unchanged (40%) or increased (15%). Neighbouring strips from the same specimen showed similar spontaneous activity, responses to ACh and changes in tone. However, only about half of them (140/247; 57%) contracted in response to CCK-OP and were available for analysis, as previously reported (D'Amato *et al.*, 1990).

Mid-point slope parameter for CCK-OP

The mid-point slope parameter for the CCK-OP contraction-response curve did not differ significantly from unity ($P > 0.05$) for the longitudinal muscle of guinea-pig ileum, human stomach, small intestine, ascending and sigmoid colon, or for the circular muscle of human transverse and sigmoid colon. Values significantly greater than unity were obtained with the circular muscle of human ascending colon, and with taenia from the transverse colon (Table 2). The results with the circular muscle of human stomach varied greatly, and should be treated with caution.

Table 1 Region and type of human alimentary muscle studied

Tissue	*No. of specimens*	*Longitudinal muscle strips*	*Circular muscle strips*
Stomach	11	29	18
Jejunum	1	4	6
Ileum	7	6	22
Ascending colon	4	3	14
Transverse colon	4	18	15
Sigmoid colon	21	40	42
Gallbladder	10	16	14
Total	58	116	131

Table 2 Mid-point slope parameter of the cholecystokinin-octapeptide (CCK-OP) curves, as determined from logistic fits

Guinea-pig	
Ileum long	1.14 ± 0.08
Human	
Stomach long	1.09 ± 0.15
Stomach circ	1.98 ± 0.76
Small intestine long	0.92 ± 0.08
Ascending colon long	1.29 ± 0.20
Ascending colon circ	1.82 ± 0.19*
Transverse colon long	1.65 ± 0.27*
Transverse colon circ	1.24 ± 0.19
Sigmoid colon long	1.02 ± 0.18
Sigmoid colon circ	1.22 ± 0.20
Gallbladder	0.98 ± 0.04

The results are means ± s.e.mean for different longitudinal (long) and circular (circ) muscles.
* Significantly different from unity ($P < 0.05$).

Antagonist effects on contractions to CCK-OP in human alimentary muscle

The CCK-OP concentration was chosen from previous concentration-response curves (D'Amato *et al.*, 1990) to give 30–70% of the maximum response for that tissue.

In the control strips, contractions to repeated submaximally effective concentrations of CCK-OP usually varied by no more than 10%, but comparisons of the first and last responses showed >10% decrease in 19% of strips, and >10% increase in 11% of strips.

Each antagonist produced a concentration-dependent inhibition of the contraction to CCK-OP, and the block was always complete with 100 μM. Figure 1 shows the effect of increasing concentrations of devazepide on a human sigmoid taenia coli.

Table 3 Apparent pK_B values for the three cholecystokinin (CCK) antagonists in human alimentary muscles

Specimen	*Strip*	*Drug*	n	*Apparent* pK_B
Stomach	long	Devazepide	3	6.40 ± 0.08
		Lorglumide	4	5.97 ± 0.16
		Loxiglumide	3	6.02 ± 0.20
Ileum	long	Devazepide	1	6.30
		Devazepide	1	5.30
Asc. colon	long	Lorglumide	3	6.06 ± 0.08
		Loxiglumide	3	5.93 ± 0.33
Trans. colon	circ	Devazepide	1	5.18
		Lorglumide	4	5.78 ± 0.36
		Loxiglumide	3	5.16 ± 0.13
Sigmoid colon	long	Devazepide	6	5.73 ± 0.25
		Lorglumide	6	5.55 ± 0.09
		Loxiglumide	5	5.78 ± 0.15
	circ	Devazepide	3	5.57 ± 0.17
		Lorglumide	3	5.93 ± 0.25
		Loxiglumide	4	5.79 ± 0.20
Gallbladder		Devazepide	5	5.64 ± 0.65
		Lorglumide	5	5.85 ± 0.27
		Loxiglumide	6	6.24 ± 0.20
Overall weighted mean		Devazepide	20	5.76 ± 0.08
		Lorglumide	25	5.82 ± 0.04
		Loxiglumide	24	5.87 ± 0.07

The three compounds were about equipotent antagonists of CCK-induced contractions in human alimentary muscle. Results on tissues in which mid-point slope parameter differed significantly ($P < 0.05$) from unity (see Table 2) have been omitted, together with those on the inhibitory responses of the circular muscle from the small intestine which were insufficient for a valid quantitative analysis and those of the stomach circular muscle which varied greatly.

Table 3 compares the three antagonists for those tissues in which the mid-point slope parameter was not significantly different from unity. The mean apparent pK_B values were usually about 6.

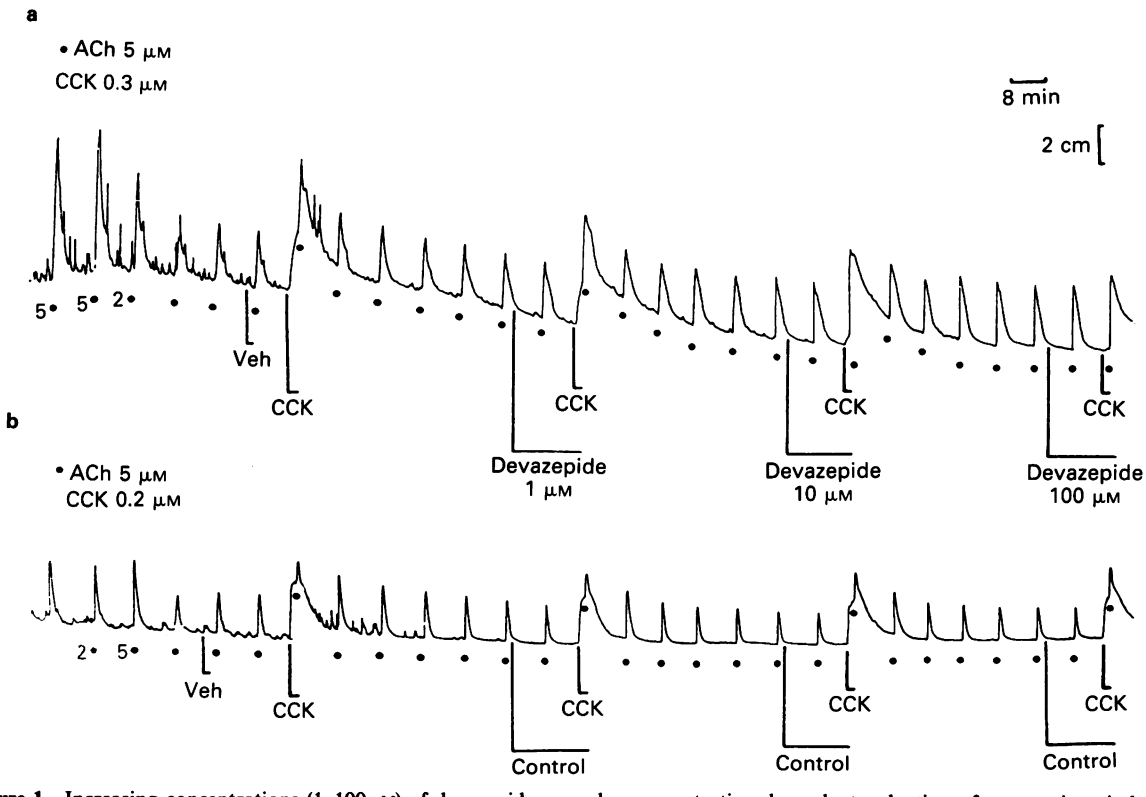


Figure 1 Increasing concentrations (1–100 μM) of devazepide caused a concentration-dependent reduction of contractions induced by cholecystokinin-octapeptide (CCK) (0.3 μM a, 0.2 μM b) in the longitudinal muscle of human sigmoid colon. The numbers 5 and 2 represent respective multiples of ACh concentration used to show that the test responses were submaximal.

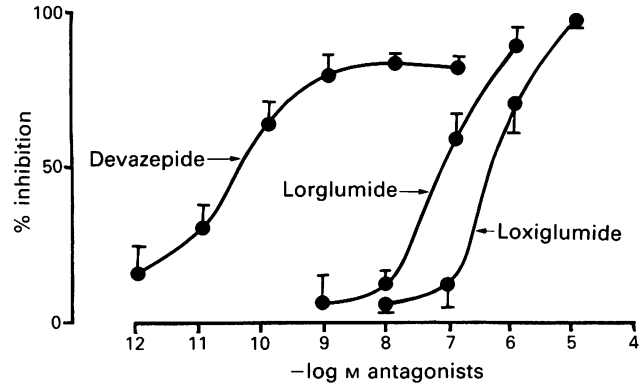


Figure 2 Devazepide, loxiglumide and lorglumide each produced a concentration-dependent block of cholecystokinin-octapeptide (CCK-OP)-induced contractions of guinea-pig ileum. Devazepide was the most potent, but the maximal effect was only 76 ± 6%, whereas the other compounds gave a complete block ($P < 0.05$). $n = 4$. The vertical lines show s.e.mean.

None of the antagonists affected the submaximal contractions to ACh, except for the highest concentration of lorglumide (100 μM) which caused a reversible reduction of 25–80% in 5/12 tissues (the other 7 showed little or no change).

After giving an inhibitory concentration of devazepide and then washing out the bath every 7–10 min, the sensitivity to CCK-OP showed no recovery after 1 h, and usually little or no recovery thereafter. However, on two occasions the response returned to about 50% 4 h after washout. In contrast, 1 h after washout of the other antagonists the CCK-OP response was almost back to normal.

Antagonist effects on the inhibition by CCK-OP in the circular muscle of human small intestine

Inhibition of the circular muscle to CCK-OP (shown as a reduced contraction to ACh) always occurred in circular muscle from the small intestine, and was consistent in 4/7 specimens (3 ileum, 1 jejunum). In the 3 ileum experiments the CCK-induced inhibitions were reduced by 34, 84 and 100% respectively by devazepide 0.1 μM. The block of CCK-OP in the jejunal specimen was almost complete with devazepide 10 μM, lorglumide 30 μM or loxiglumide 40 μM.

Guinea-pig ileum (longitudinal muscle) using the method applied to the human tissues

The concentration of CCK-OP was chosen from preliminary experiments in which the maximum contraction occurred at 100 nM, with an EC_{50} of 2.97 ± 0.05 nM. Each antagonist produced a concentration-dependent reduction of the contraction to CCK-OP but with different potencies. The maximum inhibition achieved with devazepide was only 76 ± 6% of the block by the other antagonists ($P < 0.05$; Figure 2).

Mean apparent pK_B values, derived from the raw data by the adaptation of the Cheng & Prusoff (1973) equation, are about 10.6, 7.4 and 6.7 for devazepide, lorglumide and loxiglumide respectively (Table 4).

pK_B values obtained from classical competition experiments are mainly similar to the apparent pK_B values obtained using the alternative method, and to the pA_2 values reported by others (Table 4).

Table 4 pK_B and pA_2 values of the three cholecystokinin (CCK) antagonists in guinea-pig ileum or (loxiglumide[c]) gallbladder

	Devazepide	*Lorglumide*	*Loxiglumide*
Apparent pK_B	10.61 ± 0.61	7.43 ± 0.20	6.67 ± 0.12
pK_B (our results)	10.09 ± 0.09	7.70 ± 0.12	6.08 ± 0.22
pA_2 (others)	9.89 ± 0.18[a]	7.77 (7.72–7.83)[b]	6.1 (5.7–6.7)[c]

The pK_B and apparent pK_B values for each compound were similar ($P > 0.05$), and agree with the pK_B values obtained by other groups (shown for comparison). The values for devazepide were markedly higher than those for lorglumide and loxiglumide. The values are means ± s.e.mean except where numbers in parentheses show 95% confidence limits. One way analysis of variance of the apparent pK_B values for devazepide vs lorglumide or loxiglumide, $P < 0.001$.

[a] Chang & Lotti (1986); [b] Bartho *et al.* (1987); [c] Setnikar *et al.* (1987).

Discussion

The aims of the present study were to determine the potencies of the three non-peptide CCK-antagonists devazepide, lorglumide and loxiglumide on the response of human isolated alimentary muscle to CCK-OP, and to compare the values with results from guinea-pig ileum. We used both circular and longitudinal muscle strips from the human tissues because some peptides can have opposite effects on gut muscle layers (e.g. vasoactive intestinal peptide in guinea-pig colon, Bennett *et al.*, 1984), and because we previously observed that the response to CCK-OP varied according to the muscle layer and region of the human gut (D'Amato *et al.*, 1990).

Our experimental design was adopted to minimize cost (CCK is very expensive) and to avoid tachyphylaxis which occurs with human gastrointestinal muscles exposed to CCK for > 2.5 min or at intervals of < 1 h between doses (D'Amato *et al.*, 1990). Unfortunately, these factors in human tissues precluded conventional pA_2 determination (Schild, 1957) which provides further information about the antagonism. Nevertheless, by use of the antagonist concentration-response curves against a fixed CCK-OP concentration, and assuming a simple competitive antagonism, the apparent pK_B can be estimated by an adaptation of the Cheng & Prusoff equation (1973).

We compared the results from this alternative procedure with conventional Schild analysis in guinea-pig ileum. The similar values obtained with the two methods and with other investigators' pA_2 values (usually considered a close approximation of the pK_B), validate further our experimental design using a single concentration of agonist.

Devazepide is a benzodiazepine derivative that interacts competitively with rat pancreatic CCK receptors as determined by Scatchard analysis against the specific binding of ^{125}I-labelled CCK. In guinea-pig isolated ileum and colon, devazepide competitively blocked CCK-induced contractions with a potency greatly exceeding that of other non-peptide CCK antagonists such as proglumide, dibutyryl cyclic GMP and benzotript (Chang & Lotti, 1986). We confirm that devazepide is a highly potent CCK-OP antagonist in this tissue.

Lorglumide and loxiglumide are members of a recently discovered class of potent, selective non-peptide CCK_A receptor antagonists (Setnikar *et al.*, 1987). In binding studies on rat pancreatic acini, they gave IC_{50} values of 130 and 330 nM respectively. Lorglumide produced a concentration-dependent inhibition of the contraction to CCK-OP in guinea-pig gallbladder circular muscle (pA_2 7.19, Makovec *et al.*, 1986); loxiglumide was about 10 times less potent (pA_2 6.1, Setnikar *et al.*, 1987). The pA_2 for lorglumide in the longitudinal muscle of guinea-pig ileum was 7.72 (Bartho *et al.*, 1987).

Overall, the published relative potencies of the antagonists on tissues from laboratory animals are devazepide > lorglumide > loxiglumide. Our findings in guinea-pig ileum agree with this, with weighted mean apparent pK_B values of 10.6, 7.43 and 6.67 respectively. In contrast, we obtained similar potencies with all three antagonists in the human tissues (weighted mean apparent pK_B values of 5.76–5.87). Devazepide was therefore roughly 70,000 times less potent than in guinea-pig ileum, compared with about 40 and 6 times less for lorglumide and loxiglumide.

There is strong evidence for two classes of CCK receptors, CCK_A receptors and gastrin/CCK_B receptors, with regional and cellular heterogeneity, on muscle cells and cholinergic neurones in guinea-pig stomach, gallbladder and small intestine (Grider & Makhlouf, 1987). Rodent and human CCK receptors in the central nervous system seem to differ (Cross *et al.*, 1988). The similar potency of each antagonist in all the human alimentary tissues indicates no regional differences, whereas comparison with guinea-pig small intestine (see Table 3) suggests a marked species difference in CCK receptors.

Only in the guinea-pig ileum/devazepide experiments was the CCK-OP block incomplete (76% maximum inhibition). Perhaps the peptide acts partly on CCK_B receptors; if so, the complete inhibition by lorglumide and loxiglumide may indicate partial block of CCK_B receptors by these antagonists.

Gastrin and pentagastrin stimulate human gastric antral and colonic muscle *in vitro* (Bennett *et al.*, 1967; Bennett, 1968). Since CCK and gastrin have the same carboxy-terminal pentapeptide amide sequence, on which the biological activity depends, it might be thought that the CCK antagonists could block responses to gastrin. In human tissues the three antagonists blocked CCK-OP but not ACh, and in guinea-pig ileum devazepide discriminated between CCK, histamine and pentagastrin (Chang & Lotti, 1986), and lorglumide discriminated between CCK, ACh, dimethylphenylpiperazinium, bethanechol, histamine, substance P, substance K and 5-hydroxytryptamine (Bartho *et al.*, 1987). The finding that relatively high concentrations of devazepide are needed to block the responses to CCK-OP in human alimentary muscles might at first sight suggest that the receptors are the gastrin/CCK_B type. However, although gastrin often strongly contracts human isolated stomach muscle, in contrast to CCK-OP it only weakly contracts the colon and usually has no effect on the ileum (Bennett *et al.*, 1967). Thus it seems either that some human gut tissues have CCK_A but not CCK_B receptors, or that there are CCK_B receptors present on which gastrin has a low efficacy compared to CCK-OP. Furthermore, our preliminary unpublished experiments have not detected any effect of the antagonists, in concentrations up to 100 μM, on pentgastrin-stimulated contraction of human gastric antrum ($n = 2$), colon ($n = 1$) or gallbladder ($n = 1$). This apparent paradox in the ability to block CCK-OP in human and guinea-pig alimentary muscle requires further investigation.

CCK antagonists, particularly non-peptides which have the advantage of stability against proteolysis (Morley, 1987), may have value both in research and therapeutics. Since the three non-peptide CCK antagonists studied here block exogenous CCK-OP in human alimentary muscle, they might be of use for investigating the physiology of endogenous CCK, and possibly for treating acute cholecystitis, pancreatitis, anorexia, irritable bowel syndrome and other pathophysiological conditions that may involve this hormone.

We thank The British Council for a grant to M.D'A., Merck, Sharp and Dohme for devazepide, Rotta Research Laboratories for lorglumide and loxiglumide, Mr J.A. Rennie and other surgeons and the pathologists of King's College Hospital who kindly provided the specimens, and in particular Dr R.A.D. Hull for suggesting the alternative method of analysing the data and for making the computations.

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(Received August 3, 1990
Revised October 5, 1990
Accepted October 9, 1990)

Br. J. Pharmacol. (1991), **102**, 396–400 © Macmillan Press Ltd, 1991

Adenosine- and α,β-methylene ATP-induced differential inhibition of cholinergic and non-cholinergic neurogenic responses in rat urinary bladder

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1 The effects of adenosine and α,β-methylene adenosine triphosphate (α,β-Me ATP) on single pulse-induced neurogenic responses and contractions caused by exogenously applied acetylcholine (ACh) and adenosine triphosphate (ATP) were examined in rat urinary bladder.

2 Application of single pulse stimulation (1 ms; 80 V) evoked a biphasic contractile response (abolished by tetrodotoxin, 0.5×10^{-7} M) consisting of a fast (time to peak: 1.02 ± 0.07 s) and a slow component (time to peak: 4.92 ± 1.6 s). The selective inhibition of the slow component by atropine (3×10^{-6} M) suggests the participation of both cholinergic and non-cholinergic neurotransmitters.

3 α,β-Me ATP (5×10^{-6} M) abolished ATP (10^{-4} M)-induced contractions without altering those to ACh (10^{-6} M). Further, the selective inhibition of the fast component of the neurogenic response by α,β-Me ATP is suggestive of the contribution of endogenous ATP to the non-cholinergic component.

4 Adenosine (10^{-8} M to 10^{-4} M) caused dose-related differential inhibition of the fast (IC_{50}, $1.04 \pm 0.25 \times 10^{-5}$ M) and slow (IC_{50}, $2.18 \pm 0.69 \times 10^{-6}$ M) components, thereby further supporting two modes of neurotransmission in bladder.

5 Theophylline (10^{-4} M) antagonized the inhibitory effects of adenosine on the non-cholinergic component, thereby implicating the participation of P_1-purinoceptors in neuromodulation. In contrast, theophylline at this concentration enhanced the adenosine-induced inhibition of the cholinergic component.

6 The magnitude of ATP (10^{-4} M)- and ACh (10^{-8} M)-induced contractions were almost identical to those of the fast and slow components of the neurogenic response, respectively. Comparable reduction of ATP ($30.2 \pm 3.4\%$) and ACh (100%) contractions to those of fast ($44.2 \pm 6.5\%$) and slow ($88.2 \pm 5.5\%$) components suggests the involvement of a postjunctional mechanism in adenosine-induced differential inhibition of neurogenic responses.

7 The lack of effect of erythro-6-amino-9-(2-hydroxy-3-nonyl) adenosine hydrochloride (10^{-6} M) and dipyridamole (10^{-6} M) suggests that endogenous adenosine plays little part in modulation of single pulse-induced neurogenic response.

8 The results of the present study suggest that fast and slow components of neurogenic response are mediated through ATP and ACh, respectively, possibly co-released from the same neurone in the rat bladder.

Introduction

There is considerable evidence implicating acetylcholine (ACh) and adenosine triphosphate (ATP) as the excitatory neurotransmitters mediating cholinergic and non-cholinergic neurogenic responses, respectively in mammalian urinary bladder (Brown *et al.*, 1979; Kasakov & Burnstock, 1983; Hoyle & Burnstock, 1985; Fujii, 1988; Bhat *et al.*, 1989). Further, the role of ATP as a neurotransmitter of the 'purinergic' nerves has been emphasized in guinea-pig urinary bladder by the demonstration of endogenous ATP release on transmural nerve stimulation as well as by the presence of ATP containing nerves shown by fluorescence histochemistry (Burnstock *et al.*, 1978). Purines, particularly adenosine, are known to modulate the release of classical neurotransmitters like ACh, noradrenaline and dopamine in both central and peripheral nervous systems (Snyder, 1985). In view of the release of more than one neurotransmitter (as yet unknown whether from the same or different nerves) on electric field stimulation of intramural nerves in urinary bladder, the neuromodulatory effect of adenosine in respect of both cholinergic and non-cholinergic neurotransmission needs to be examined. Single pulse stimulation parameters that separate the cholinergic and non-cholinergic contractile components distinctly (Bhat *et al.*, 1989) have been used to examine the neuromodulatory role of adenosine in rat urinary bladder. Further, adenosine deaminase inhibitor (erythro-6-amino-9-(2-hydroxy-3-nonyl)adenosine hydrochloride, EHNA) and adenosine uptake inhibitor (dipyridamole) have been employed to elucidate the role of endogenous adenosine in autonomic neurotransmission, if any, in this tissue.

α,β-Methylene ATP (α,β-Me ATP), widely used to desensitize P_2-purinoceptors, has been known to abolish or reduce electrical and contractile responses evoked by nerve stimulation in urinary bladder of several mammalian species (Kasakov & Burnstock, 1983; Fujii, 1988). It is therefore, clearly essential to examine the effects of α,β-Me ATP on both fast and slow components of single pulse-induced neurogenic response so as to establish the contribution of endogenous ATP to the non-cholinergic response in rat bladder.

Methods

Male albino rats, obtained from the Laboratory Animal Resource Section of this Institute, were used in the present study. Animals were stunned with a blow to the head and exsanguinated. The urinary bladder was dissected out and strips of 3×10 mm were cut. The smooth muscle strips were suspended in Tyrode solution of the following composition (mM): NaCl 138, KCl 5.9, $NaHCO_3$ 6.0, glucose 5.5, $NaH_2PO_4 2H_2O$ 0.5, $MgCl_2 6H_2O$ 0.5 and $CaCl_2 2H_2O$ 1.9. The solution was aerated with atmospheric air and maintained at 37 ± 0.5°C (pH 7.4). The tissues were equilibrated for a period of 1 h under a resting tension of 0.5 g.

The responses of the tissue were recorded isometrically with a force displacement transducer connected to a polygraph (M/s Medicare, India). The bladder strip was stimulated with

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a single square wave pulse of 1 ms duration at 80 V, every 15 min. The stimulation caused a biphasic contraction of the urinary bladder, consisting of an early fast phase and a late slow phase, which was abolished by tetrodotoxin (0.5×10^{-7} M), thereby suggesting its neural origin. Atropine (3×10^{-6} M), however, selectively abolished the slow component while the fast component was not altered, thus suggesting both cholinergic and non-cholinergic neurotransmission in the urinary bladder.

In order to achieve P_2-purinoceptor desensitization, the procedure described by Fujii (1988) was followed with slight modification. Briefly, the tissues were superfused with α,β-Me ATP (5×10^{-6} M) for 20 min and successive superfusions were carried out for 30–40 s at 3–4 min intervals.

To examine the effect on single pulse-induced biphasic neurogenic responses, adenosine (10^{-8} M – 3×10^{-4} M) was added cumulatively in the presence of dipyridamole (10^{-6} M) and EHNA (10^{-6} M), agents known to inhibit the inactivation of adenosine. Responses to individual doses (10^{-8} M – 3×10^{-4} M) of adenosine were elicited on some tissues and the concentration-responses relationship obtained with this protocol was the same as that obtained with cumulative doses. In some experiments, the effect of a combination of dipyridamole and EHNA was studied with a view to examining the role of endogenous adenosine, if any, in modifying the neurogenic responses elicited on single pulse field stimulation. Theophylline (10^{-4} M), a P_1-purinoceptor antagonist, was used to study the specificity of adenosine-purinoceptor interaction. Concentration-response relationship with ATP (10^{-6} M – 3×10^{-4} M) and ACh (10^{-9} M – 3×10^{-3} M), added in single doses, was established, maintaining a time interval of 25 min and 5 min, respectively between each addition. Doses of ATP (10^{-4} M) and ACh (10^{-8} M) that induced responses which matched those of fast (non-cholinergic) and slow (cholinergic) components of neurogenic response were selected to evaluate the modulatory effect of adenosine (10^{-5} M) at post-junctional site.

The following drugs were used: acetylcholine chloride (Sigma), adenosine (Sigma), adenosine triphosphate disodium salt (Sigma), α,β-methylene ATP (Sigma), atropine sulphate (Sigma), dipyridamole (Gift: German Remedies), erythro-6-amino-9-(2-hydroxy-3-nonyl) adenosine hydrochloride hydrate (EHNA; gift: Wellcome Research Laboratories), tetrodotoxin (Sigma) and theophylline (Sigma).

Results are presented as mean ± s.e.mean. Student's t test was used for the test of significance. The IC_{50} values were evaluated for each preparation by linear regression analysis.

Results

Rat urinary bladder smooth muscle, normally exhibiting autorhythmicity, when stimulated with single square wave pulses (1 ms pulse duration at 80 V) at an interval of 15 min, produced a biphasic contractile response consisting of a fast component (time to peak: 1.02 ± 0.07 s; $n = 8$) and a slow component (time to peak: 4.92 ± 1.6 s; $n = 8$), the former being always greater in magnitude than the latter. The mean absolute tensions of the fast and slow components were 0.90 ± 0.06 g ($n = 16$) and 0.50 ± 0.05 g ($n = 16$), respectively. At times in tissues exhibiting greater autorhythmicity, a third late contractile component was observed in the descending limb of the second slow phase of contraction. This component, however, was not evident in tissues exhibiting either low or no spontaneity.

Figure 1 depicts the effects of α,β-Me ATP on the responses of rat bladder to ACh, ATP and single pulse stimulation. Contractions caused by α,β-Me ATP (5×10^{-6} M) were partially persistent (about 25% of the initial response) even after five successive superfusions with this agent. However, in the presence of α,β-Me ATP, ATP (10^{-4} M)-induced contractions were abolished while ACh (10^{-6} M)-induced contractions were little affected. Further, α,β-Me ATP caused inhibition of the fast component by 38 ± 3.7% ($n = 4$) while the slow component was not altered. In the tissues pretreated with α,β-Me ATP, atropine (10^{-6} M) selectively abolished the slow component of the neurogenic response without significantly affecting the fast component.

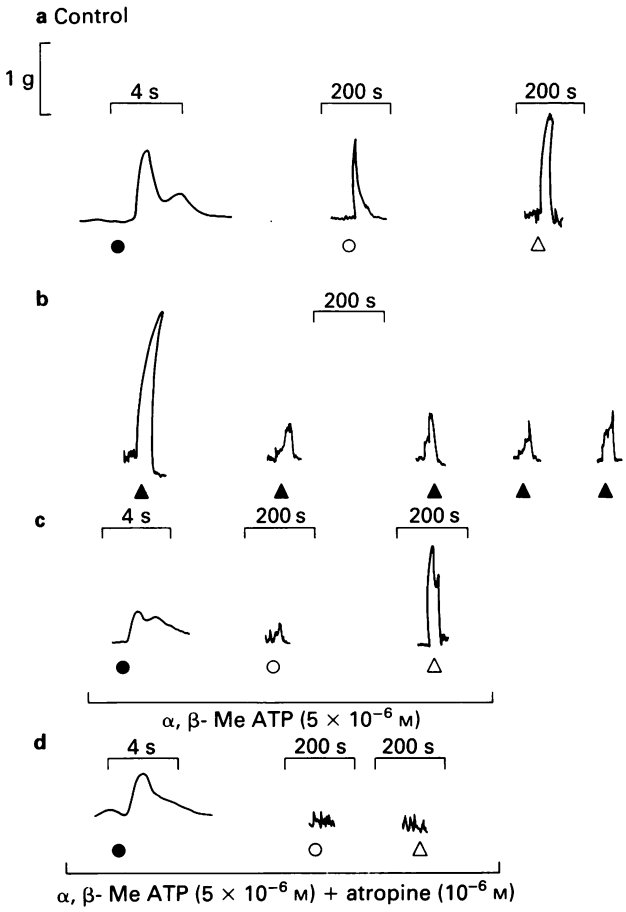


Figure 1 Effects of α,β-methylene ATP (α,β-Me ATP) on the responses to single pulse stimulation (● 1 ms, 80 V) adenosine triphosphate (ATP, ○, 10^{-4} M) and acetylcholine (ACh, △, 10^{-6} M) in rat urinary bladder: (a) control responses; (b) successive superfusions with α,β-Me ATP (5×10^{-6} M); (c) contractile responses in the presence of α,β-Me ATP (5×10^{-6} M) and (d) in the presence of α,β-Me ATP (5×10^{-6} M) and atropine (10^{-6} M).

Incubation of the tissue for 15 min with dipyridamole (10^{-6} M) and EHNA (10^{-6} M) did not significantly modify single pulse-induced biphasic contractions (Figure 2). On the other hand, adenosine (10^{-8} M–3×10^{-4} M) in the presence of dipyridamole and EHNA caused dose-related inhibition of neurogenic responses; the slow component being relatively more susceptible than the fast component (Figures 3, 4 and Table 1). In the tissues treated with dipyridamole and EHNA, theophylline (10^{-4} M) did not affect the fast component of the

Table 1 Inhibitory effect of adenosine (10^{-8}–10^{-4} M) on the fast and slow components of the contractile response to single pulse field-stimulation (1 ms; 80 V) in rat urinary bladder (pretreated with EHNA 10^{-6} M and dipyridamole 10^{-6} M) and its modulation by theophylline (10^{-4} M)

	IC_{50} (M)	
	Fast component	*Slow component*
Adenosine	$1.04 \pm 0.25 \times 10^{-5}$	$2.18 \pm 0.69 \times 10^{-6}$*
Adenosine + theophylline	$2.45 \pm 0.29 \times 10^{-5}$*	$1.32 \pm 0.19 \times 10^{-7}$*

$n = 6$–8; values are mean ± s.e.mean.
*$P < 0.05$.

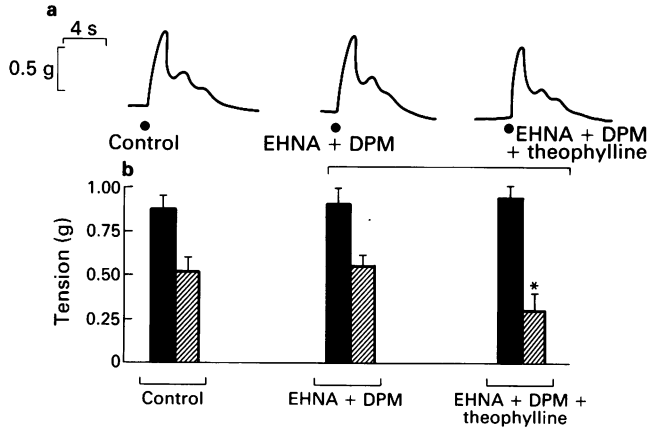


Figure 2 (a) Isometric recordings of rat urinary bladder illustrating the biphasic contractile responses to single pulse stimulation (1 ms; 80 V) and the effect of EHNA (10^{-6} M), dipyridamole (DPM: 10^{-6} M) and theophylline (10^{-4} M) on these responses and (b) mean absolute tension (g) of fast (solid column) and slow (hatched column) components of single pulse stimulus and the effect of erythro-6-amino-9-(2-hydroxy-3-nonyl) adenosine hydrochloride (EHNA, 10^{-6} M), DPM (10^{-6} M), and theophylline (10^{-4} M) on the same. Vertical bars indicate the s.e.mean (*$P < 0.05$; $n = 6$–8).

neurogenic response, while the slow component was significantly ($P < 0.05$) inhibited by about 25% (Figure 2). Further, theophylline antagonized the inhibitory effect of adenosine on the fast component of the neurogenic response (Figure 4a). This is also evident from the significant ($P < 0.05$) rise in the IC_{50} value of adenosine in the presence of theophylline as compared to controls (Table 1). In contrast, adenosine-induced dose-related inhibition of the slow component was markedly potentiated by theophylline (Figure 4b).

ATP (10^{-6} M–3 × 10^{-4} M) and ACh (10^{-9} M–3 × 10^{-3} M) elicited concentration-related contractile responses in rat urinary bladder (Figure 5a). ATP (10^{-4} M) and ACh (10^{-8} M) generated absolute tensions of 1.2 ± 0.12 g and 0.37 ± 0.05 g, respectively which were comparable to those of fast (0.95 ± 0.06 g) and slow (0.42 ± 0.05 g) components of the neurogenic responses. ATP-induced contraction was fast in time course and transient in nature as compared to that of ACh. The mean time to peak values of ATP and ACh was 7.7 ± 0.4 s and 39.3 ± 1.4 s, respectively. Adenosine (10^{-5} M) inhibited ATP- and ACh-induced contractions by about 30 and 100%, respectively as compared to the inhibition of fast (44.2 ± 6.5%; $n = 8$) and slow (88.2 ± 5.5%; $n = 8$) components of the neurogenic response.

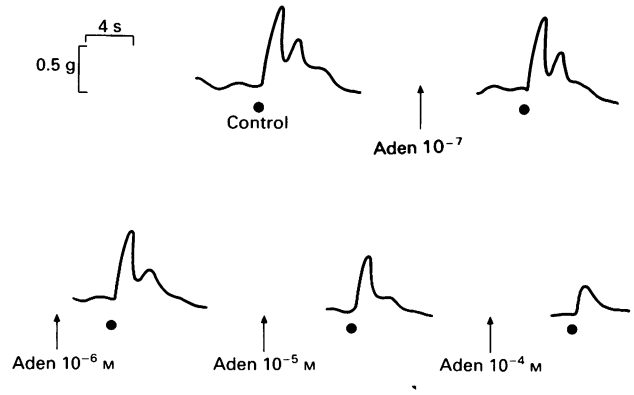


Figure 3 Effect of cumulative concentrations of adenosine (Aden, 10^{-7} M to 10^{-4} M) on the fast and slow components of contractions of single pulse stimulation in rat bladder exposed to erythro-6-amino-9-(2-hydroxy-3-nonyl) adenosine hydrochloride (EHNA, 10^{-6} M) and dipyridamole (10^{-6} M); $n = 8$.

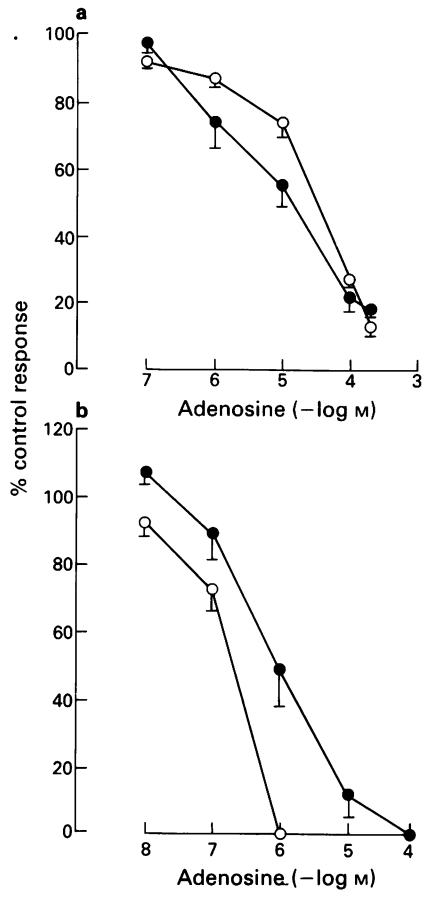


Figure 4 Effect of adenosine (10^{-8} M to 3 × 10^{-4} M) added cumulatively on the fast (a) and slow (b) components of contractions induced by single pulse stimulation in the presence (●) and absence (○) of theophylline (10^{-4} M).

Discussion

The absolute susceptibility of the biphasic contractile response elicited on single pulse stimulation to TTX and the selective inhibition of the slow component by atropine are in conformity with our earlier suggestion that the fast and slow components are mediated by a non-cholinergic neurotransmitter (possibly ATP) and a cholinergic neurotransmitter ACh, respectively (Bhat *et al.*, 1989). In order to substantiate further the role of ATP as a neurotransmitter in rat bladder, desensitization of P_2-purinoceptors by α,β-Me ATP was attempted. Persistence of residual responses to α,β-Me ATP following successive superfusions indicate that this agent partially desensitized P_2-purinoceptors in rat bladder. Similarly, depolarization by α,β-Me ATP has been reported to be partially persistent in the guinea-pig and rabbit mesenteric arteries (Ishikawa, 1985) and in rabbit ear artery (Miyahara & Suzuki, 1987). Nevertheless, in the presence of α,β-Me ATP, ATP-induced contractions were abolished without any effect on those of ACh. Similarly, while the fast component of the neurogenic response was partially inhibited by α,β-Me ATP, the slow component mediated by endogenous ACh was not altered. The selective inhibition of the non-cholinergic component of the nerve-mediated contraction could result from the desensitization of P_2-purinoceptors by α,β-Me ATP and this strongly suggests that the non-cholinergic fast component to single pulse stimulation is partly mediated by ATP. Although the role of α,β-Me ATP in inhibiting neurotransmitter release cannot be excluded in the present study, this possibility may be of little significance in view of previous reports where α,β-Me ATP has been shown not to affect neurotransmitter release in other smooth muscle preparations (rabbit

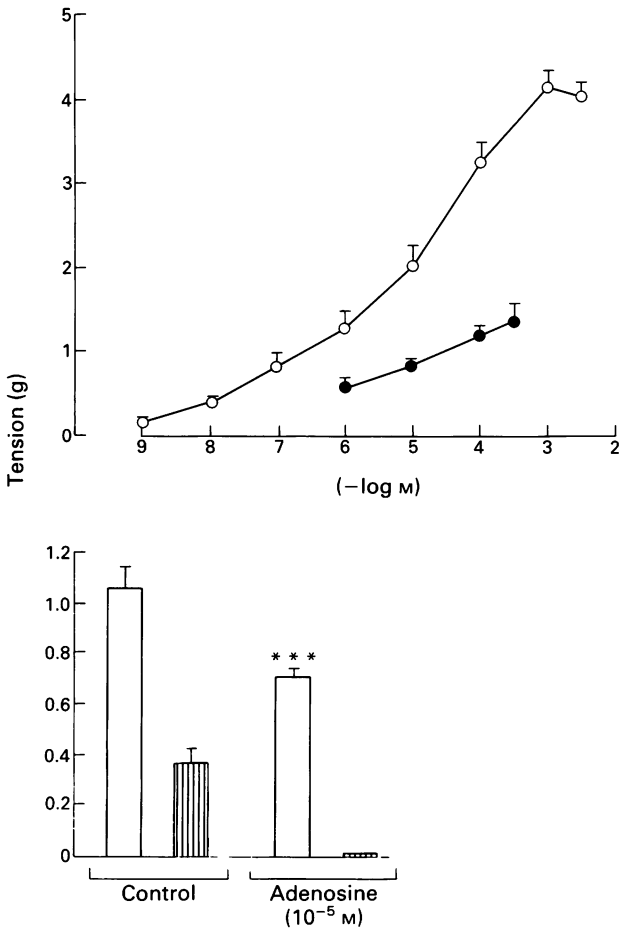


Figure 5 (a) Concentration-response curves to acetylcholine (ACh, ○) and adenosine triphosphate (ATP, ●) in rat urinary bladder and (b) effect of adenosine on the absolute tension of ATP (10^{-4} M) (open columns) and ACh (10^{-8} M) (lined columns). Vertical bars indicate the s.e.mean (***$P < 0.001$; $n = 6$).

mesenteric artery: Ishikawa, 1985; Kugelgen & Starke, 1985; guinea-pig vas deferens: Allcorn *et al.*, 1986). As observed in the present study the resistance of a significant level of the fast component to α,β-Me ATP could possibly be due to partial desensitization of P_2-purinoceptors in rat bladder. Alternatively the contribution of some neurotransmitter other than ATP to the fast component cannot be ruled out.

Since ATP and ACh are the possible neurotransmitters in rat urinary bladder, responses which matched those of fast and slow components of neurogenic responses were elicited by exogenously added ATP (10^{-4} M) and ACh (10^{-8} M), respectively. The effect of adenosine on these two agonist-induced contractile responses was evaluated with a view to (1) elucidating the postjunctional actions and (2) to examining the involvement of presynaptic/postjunctional mechanisms in adenosine-induced varied inhibition of the neurogenic responses. In rat urinary bladder, the contractions produced by ATP were fast in time course (time to peak: 7.7 ± 0.4 s) as compared to those of ACh (time to peak: 39.3 ± 1.4 s) at an equimolar concentration (10^{-4} M). This observation is in agreement with the electrophysiological observations made on the guinea-pig bladder (Fujii, 1988). Further, the characteristics of contractions to exogenously applied ATP and ACh mimic the fast and slow components of contraction originating from single pulse stimulation, in as far as the time course of action is concerned.

The abolition of the ACh response and a significant inhibition of the ATP response by adenosine is in conformity with its postjunctional inhibitory action in bladder muscle (Husted *et al.*, 1983). A similar mechanism might be involved in adenosine-induced differential inhibition of neurogenic responses as is evident from a 44% reduction of the fast component (compared to 30% with exogenous ATP) and an 88% inhibition of the slow component (compared to 100% with exogenous ACh), respectively. However, an inhibitory effect of adenosine on presynaptic release of neurotransmitter(s) in rat bladder cannot be ruled out. Assuming that ATP and ACh come from the same nerve, a prejunctional action of adenosine which might reduce the release of ATP and ACh in parallel would not result in a parallel decrease in the amplitude of neurogenic responses. Thus the results obtained with adenosine together with those of α,β-Me ATP suggest that possibly ATP and ACh are co-released from a single neurone in rat bladder and is consistent with the hypothesis of 'co-transmission' as reported earlier in several other tissues (rabbit ear artery: Saville & Burnstock, 1988; dog mesenteric artery: Machaly *et al.*, 1988; vas deferens: Sneddon *et al.*, 1984). However, the involvement of separate populations of nerves releasing ACh and ATP independently cannot be ruled out.

The specificity of antagonism by theophylline of adenosine-induced inhibition of the fast component is consistent with its P_1-purinoceptor antagonism. However, the potentiation of the inhibitory effect of adenosine on the slow component by theophylline is difficult to explain in terms of its purinoceptor antagonism. The inhibitory effect on the slow component and the potentiation of the adenosine effect by theophylline could possibly be related to its phosphodiesterase inhibitory effect as observed in other smooth muscles (Gustafsson *et al.*, 1981).

In several tissues including rat bladder, the adenosine uptake inhibitor, dipyridamole, and adenosine deaminase inhibitor, EHNA, are known to inhibit the inactivation of endogenous adenosine (Fredholm *et al.*, 1978; Dahlen & Hedqvist, 1980). However, in the present study EHNA and dipyridamole did not modify the single pulse-induced neurogenic response. This is in contrast to the inhibitory effect of dipyridamole on field-stimulated rat urinary bladder linked to accumulation of endogenous adenosine (Dahlen & Hedqvist, 1980). The discrepancy observed could probably be due to higher frequency of stimulation (e.g. 5 Hz) used in earlier studies as compared to single pulse field stimulation in the present study. Although the lack of inhibitory effect of EHNA and dipyridamole on single pulse-induced response suggests that endogenous adenosine plays little part, we cannot exclude its role altogether in neuromodulation. This is because, in the absence of a sufficient concentration of spontaneously released adenosine, any accumulation of breakdown products of purines during single pulse stimulation of intramural nerves might not have any appreciable effect before the peak contractions were reached.

In conclusion, it is suggested that (1) the varied susceptibility of cholinergic and non-cholinergic neurogenic responses in rat bladder to adenosine may involve postjunctional mechanisms, (2) the excitatory neurotransmitters possibly, ATP and ACh mediate the fast and slow components, respectively and (3) endogenous adenosine appears to play little part in the modulation of the neurogenic response elicited by single-pulse field stimulation.

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(Received June 13, 1990
Revised September 10, 1990
Accepted September 24, 1990)

Br. J. Pharmacol. (1991), **102**, 401–407 © Macmillan Press Ltd, 1991

An amplifying effect of exogenous and neurally stored 5-hydroxytryptamine on the neurogenic contraction in rat tail artery

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1 The interactions between sympathetic neuroeffector transmission and 5-hydroxytryptamine (5-HT) were investigated in segments of rat isolated tail artery.

2 Contractile responses to field stimulation of the artery segments were abolished by tetrodotoxin (3×10^{-7} M). A subthreshold concentration of acutely applied exogenous 5-HT (10^{-8} M) markedly enhanced the contractions induced by sympathetic nerve stimulation, through an action on postjunctional 5-HT_2-receptors.

3 The amplifying effect of 5-HT involved an enhanced influx of extracellular calcium into the smooth muscle cells. In contrast, the neurogenic contractions in vessels not exposed to 5-HT were not dependent on extracellular calcium.

4 The adrenergic component of the amplified response involved postjunctional α_1- but not α_2- adrenoceptor activation.

5 Exposure of the vessels to 5-HT (5×10^{-7} M) for 30 min resulted in uptake of the amine into the perivascular sympathetic nerves, as demonstrated by immunohistochemistry. After chemical sympathectomy with 6-hydroxydopamine *in vitro* or *in vivo*, or surgical sympathectomy, there was little or no uptake.

6 Exposure to 5-HT followed by repeated washing resulted in an enhancement of the neurogenic contraction, which was still fully tetrodotoxin-sensitive. The enhanced response was blocked by ketanserin (10^{-8} M) and prevented by the presence of the 5-HT uptake blocker, paroxetine (3×10^{-8} M), during the period of exposure to 5-HT.

Introduction

An amplifying effect of 5-hydroxytryptamine (5-HT) on the contractions induced by sympathetic nerve stimulation or exogenous noradrenaline (NA) has been reported in many blood vessels (for review see Van Nueten *et al.*, 1988; de la Lande, 1989). This effect of 5-HT usually, but not always (Chang & Owman, 1989), involves activation of 5-HT_2-receptors on the vascular smooth muscle. It has been suggested that the potentiating effect of 5-HT on the NA-induced contractions plays an important role in various diseases including essential hypertension (Vanhoutte, 1982) and cerebral and coronary vasospasm (Owman, 1988; Cohen, 1988), where vascular smooth muscle adrenoceptors may become exposed to enhanced circulating or local concentrations of 5-HT, perhaps even released from the perivascular sympathetic nerves after prior uptake.

The underlying mechanism of the amplifying effect of 5-HT is not fully understood. Recent studies, investigating the interactions between NA and 5-HT (Meehan *et al.*, 1988), or phenylephrine and 5-HT (Marwood, 1988), where all agents were exogenously applied, indicate that the 5-HT-induced enhancement of extracellular calcium influx to the vascular smooth muscle cells may play an important role. In an attempt to elucidate further the role of calcium in the amplifying effect, the interaction between exogenously applied 5-HT and endogenous NA released from the sympathetic nerves by transmural field stimulation was studied in the rat tail artery.

In various vascular preparations, including cerebral (Levitt & Duckles, 1986; Saito & Lee, 1987; Owman, 1988), coronary (Cohen, 1985; 1988), mesenteric (Kawasaki & Takasaki, 1984) and ear arteries (Meehan *et al.*, 1986), neuronal uptake of 5-HT may play a role in the regulation of vascular tone. 5-HT that accumulates in perivascular sympathetic nerves can be released by transmural field stimulation, and causes contraction of the smooth muscle, as demonstrated in canine coronary (Cohen, 1985; 1988), rabbit basilar (Saito & Lee, 1987) and rat mesenteric arteries (Kawasaki & Takasaki, 1984). In the present study we investigated whether such uptake also occurs in perivascular nerves of the rat tail artery and, if so, whether such accumulated 5-HT, upon release, could amplify the effect of neuronally released NA in a manner similar to exogenously applied 5-HT.

Methods

Male Sprague-Dawley rats weighing 300–400 g were killed by diethyl ether anaesthesia. A segment (1 cm in length) of the intermediate part of the tail artery (located from 6 to 7 cm from the proximal end of the tail) was dissected out and immediately placed in an ice-cold Krebs-Ringer buffer solution. Within 30 min of dissection, segments (2 mm in length) of the vessels were mounted on two L-shaped metal holders between platinum electrodes (0.3 mm in diameter, 4 mm apart, placed parallel to the vessel) in an organ bath for measurement of isometric circular muscle contractions following exposure to various agents and electrical field stimulation (Hardebo *et al.*, 1986). The organ bath contained a Krebs-Ringer buffer solution of the following composition (in mM): NaCl 118, KCl 4.5, $CaCl_2$ 1.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.0 and glucose 6.0. The bath temperature was kept at 37°C and the pH at 7.35 by continuous slow aeration with 88.5% O_2 + 11.5% CO_2.

After mounting, the segments were subjected to a passive load of 4–5 mN (depending on vessel size) and then allowed to stabilize for 40–60 min. The passive load applied was of a magnitude that allowed a maximum contractile response of the vessels as tested in separate control experiments. Agents used to enhance or block the responses to electrical stimulation were added to the bath 15 min beforehand, except for 5-HT 10^{-8} M which was added 2 min beforehand. Incubations

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for 30 min in the presence of 5-HT 5×10^{-7} M (in some experiments together with paroxetine 3×10^{-8} M) and of prostaglandin $F_{2\alpha}$ 5×10^{-6} M were also performed. Several segments of each tail artery from the individual animals were used.

Electrical field stimulation

Electrical stimulation with 10 s trains of 9 V monophasic square wave impulses were delivered from a Grass S44 stimulator. The current during the pulses was 72 mA. The frequency was set at 6 Hz, and the duration of individual impulses was 0.1 ms. These stimulus parameters were optimal to produce a fully tetrodotoxin (TTX, 3×10^{-7} M)-sensitive and thus purely neurogenic response (see Szabó & Hardebo, 1990).

Denervations

In some experiments the vessels were acutely denervated *in vitro* with 6-hydroxydopamine (6-OHDA) according to the method of Aprigliano & Hermsmeyer (1976). The solution in the organ bath was replaced for 10 min by a bicarbonate-free solution containing 6-OHDA 30 μg ml^{-1} and reduced glutathione 20 μM. The O_2/CO_2 mixture aerating the bath was turned off during this treatment. Following this denervation procedure, the segments were allowed to recover in normal aerated solution for 150 min. *In vivo* denervation was achieved by i.p. injection of 6-OHDA (50 mg kg^{-1}, dissolved in saline containing 0.1% ascorbic acid) two days before and 100 mg kg^{-1} one day before the rats were killed. Surgical denervation was performed by transection of both ventral collector nerves according to Sittiracha *et al.* (1987) 2 cm from the proximal end of the tail. The animals were killed 4 days later and a segment 5 cm long, starting 3 cm distal to the section level, was dissected out.

Immunohistochemistry

After incubation for 30 min in 37°C Krebs-Ringer solution with 5×10^{-7} M 5-HT, or without 5-HT (control), the tail arteries were fixed for 2 h in a fixative solution containing 2% formaldehyde and 15% of a saturated aqueous picric acid solution in 0.2 M phosphate buffer (pH 7.2). After fixation, they were thoroughly rinsed for 24 h in a Tyrode solution containing 10% sucrose at 4°C, followed by sectioning in a cryostat at 12 μm thickness and mounted on chromalum-coated slides. The indirect immunohistochemical procedure of Coons *et al.* (1955) was used. The specimens were incubated in a humid chamber of 4°C for 24 h with antiserum against 5-HT raised in rabbit (generous gift from H.W.M. Steinbusch, Department of Pharmacology, Free University, Amsterdam, The Netherlands) at a dilution of 1:250 or with antiserum against tyrosine hydroxylase raised in a rabbit (lot no. 1012, Eugene Tech, Allendale, U.S.A.) at a dilution of 1:320. After repeated rinsing in 0.1 M phosphate buffer solution containing 0.3% Triton X-100 for 30 min the vessels were incubated with porcine anti-rabbit IgG conjugated with fluorescein isothiocyanate (DAKOPATTS, Denmark) at a dilution of 1:20 for 45 min at room temperature. The slides were then rinsed twice in phosphate buffer solution containing 0.3% Triton X-100 before mounting in a mixture of the buffer and glycerin (1:1). They were examined in a Zeiss fluorescence microscope equipped with incident illumination.

Drugs

The following drugs were used: ascorbic acid (Sigma), Bay K 8644 (Bayer), D-600 (methoxyverapamil, gift from Knoll AG), ketanserin (Janssen), α,β-methylene-ATP (Sigma), neuropeptide Y (NPY; Peninsula), reduced glutathione (Sigma), paroxetine hydrochloride (Ferrosan), phenylephrine hydrochloride (Winthrop), prazosin hydrochloride (Pfizer), prostaglandin $F_{2\alpha}$ (Astra), rauwolscine hydrochloride (Roth), 5-HT creatinine sulphate (Sigma), tetrodotoxin (TTX, Sigma). Prazosin was dissolved in a few drops of methanol, followed by 10% lactic acid, in a stock solution that was further diluted with 0.9% NaCl solution. All other agents were dissolved in 0.9% NaCl solution.

Statistics

For statistical analyses, Student's t test was applied for paired and unpaired data. The indicated values are means $\pm$ s.e.mean.

Results

Characterization of the neurogenic response

The electrical field-induced contractions were totally abolished by TTX (3×10^{-7} M). The effect on the neurogenic responses of the α-adrenoceptor antagonists prazosin and rauwolscine, the 5-HT_2 receptor antagonist ketanserin, and D-600 (a blocker of potential-sensitive calcium channels) in untreated vessels are summarized in Figure 1a. Prazosin (5×10^{-8} M) and rauwolscine (10^{-6} M) significantly attenuated neurogenic responses in untreated vessels by 63 and 39%, respectively. A marked (88%) inhibition of neurogenic responses was produced by the combination of prazosin and rauwolscine and further addition of a high concentration of NPY (10^{-7} M) to pre-occupy junctional NPY receptors almost eliminated the response. Ketanserin (10^{-8} M) and D-600 (10^{-6} and 10^{-5} M) had no effect on the neurogenic responses in untreated vessels.

Neurogenic response in the acute presence of 5-hydroxytryptamine

The presence of 5-HT (10^{-8} M), added to the vessel chamber 2 min beforehand, enhanced the contractions induced by sympathetic nerve stimulation, while this concentration of 5-HT did not, on its own, alter the vascular tone (Table 1, Figure 2a). The neurogenic response was enhanced by 92% in the presence of 5-HT.

Ketanserin (10^{-8} M) or D-600 (10^{-5} M) eliminated the enhancement by 5-HT of the neurogenic contraction. A similar inhibition was observed after application of 5×10^{-8} M prazosin or 10^{-6} M rauwolscine (Figure 1b). The combined application of prazosin and rauwolscine did not totally inhibit the response, in contrast to the effect in untreated vessels. The combined presence of prazosin and rauwolscine did not cause a greater inhibition than prazosin alone, again contrasting to the effect in untreated vessels. However, a further inhibition of the response was obtained when D-600 was applied in combination with prazosin and rauwolscine (Figure 1b). This inhibition by the calcium antagonist was concentration-dependent, in contrast to the absence of effect in untreated vessels. Furthermore, the combined presence of the α-antagonists and D-600 caused a more complete inhibition of the response than D-600 alone. Thus, in the presence of 5-HT, all individual inhibitors or combinations of them caused significant reductions of the enhanced neurogenic response, whereas in untreated vessels only α-adrenoceptor antagonists caused significant reductions of the response.

Neurogenic response in the acute presence of other constrictors

To elucidate whether this enhancement was specific to 5-HT, the possible amplifying effect of various other constrictor agents on the neurogenic response was studied. The results are given in Table 1. A minor elevation of the extracellular potassium (from 5.5 mM to 10 mM) caused a significant enhancement of the contraction, without changing the resting tone.

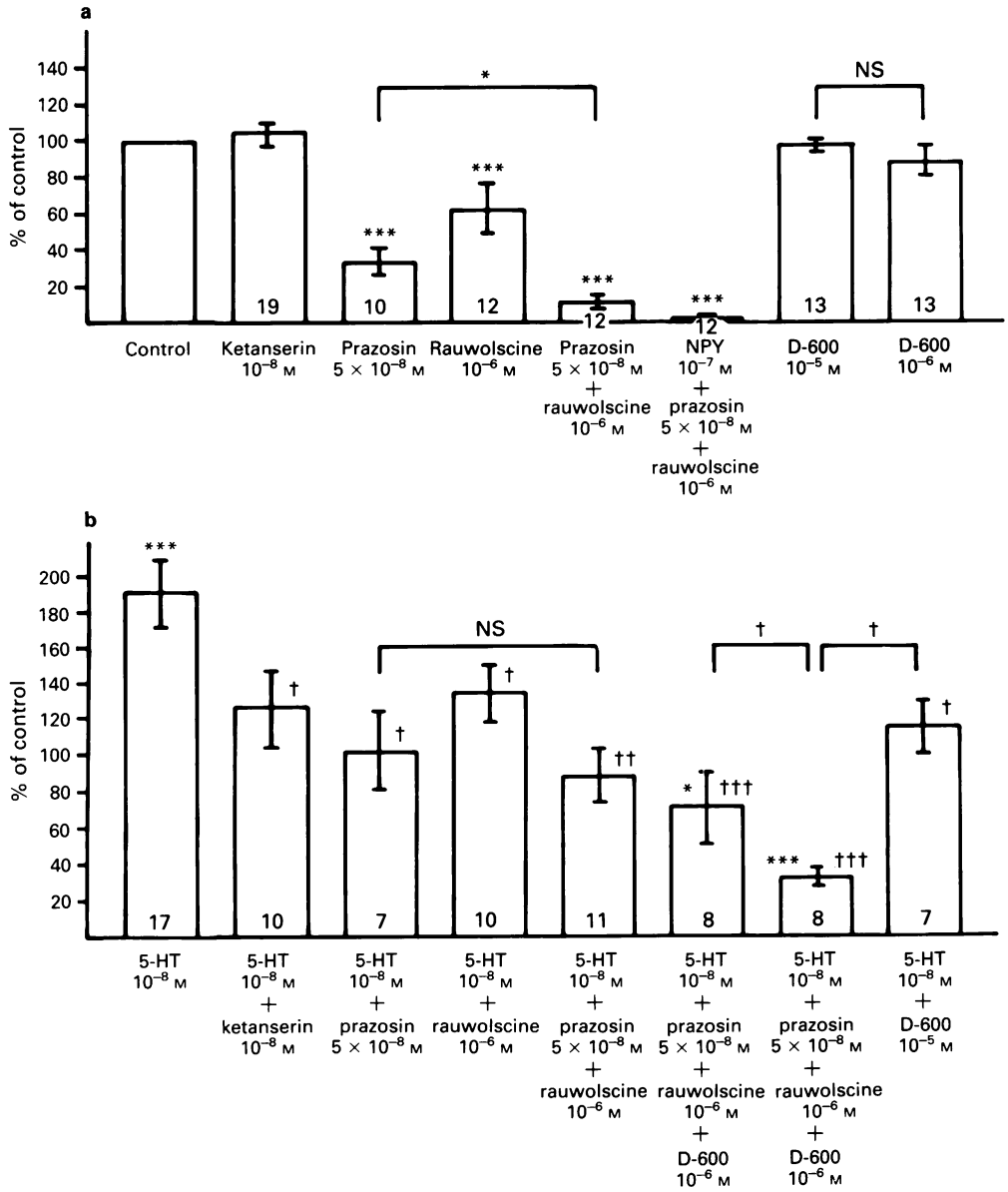


Figure 1 Effects of α_1- and α_2-adrenoceptor antagonists, a 5-HT_2-receptor antagonist and a calcium channel-blocker, and the presence of a high concentration of neuropeptide Y (NPY), on the neurogenic contractions induced by transmural nerve stimulation in the rat tail artery. (a) Effects of the blockers and NPY on the response in untreated vessels. (b) Effects of the blockers on the response in the presence of 5-hydroxytryptamine (5-HT) 10^{-8} M. Significant differences from the control value, i.e. in untreated (control) vessels, and also between different treatments in (a), * $P < 0.05$ and ** $P < 0.001$, respectively. Significant differences between the contractile response in the presence of 5-HT (10^{-8} M) alone and in the combined presence of 5-HT and the blocking agents, and also between different treatments in (b): † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$ respectively. NS = nonsignificant. All reported values represent % of the response in untreated (control) vessels, which is set as 100%.

Table 1 Effects of various agents on the contractions (in mN) induced by transmural nerve stimulation on the resting tone in rat tail artery

Agent	n	*Contraction before the application of agent* (mN)	*Contraction after the application of agent* (mN)	*Direct effect of the contractile agent on tone* (mN)
5-HT 10^{-8}	17	3.50 ± 0.36	5.93 ± 0.49***	none
K^+ 10 mM	20	3.35 ± 0.38	4.83 ± 0.72***	none
α,β-methylene-ATP 10^{-6} M	20	3.66 ± 0.44	5.24 ± 0.66***	1.43 ± 0.32**
Phenylephrine 10^{-8} M	6	3.22 ± 0.53	3.08 ± 0.44	none
Phenylephrine 10^{-7} M	6	3.22 ± 0.53	3.31 ± 0.53	none
Phenylephrine 10^{-6} M	12	3.08 ± 0.40	3.50 ± 0.30	2.65 ± 0.38***
Prostaglandin $F_{2\alpha}$ 5×10^{-7} M	17	3.50 ± 0.41	3.73 ± 0.51	0.09 ± 0.05
Prostaglandin $F_{2\alpha}$ 5×10^{-6} M	14	3.73 ± 0.53	6.66 ± 0.89***	2.04 ± 0.25***
Bay K 8644 10^{-8} M	8	3.22 ± 0.73	5.78 ± 1.32**	none

Significant differences ** $P < 0.01$ and *** 0.001, respectively from control values or from the resting tone. n = number of vessels.

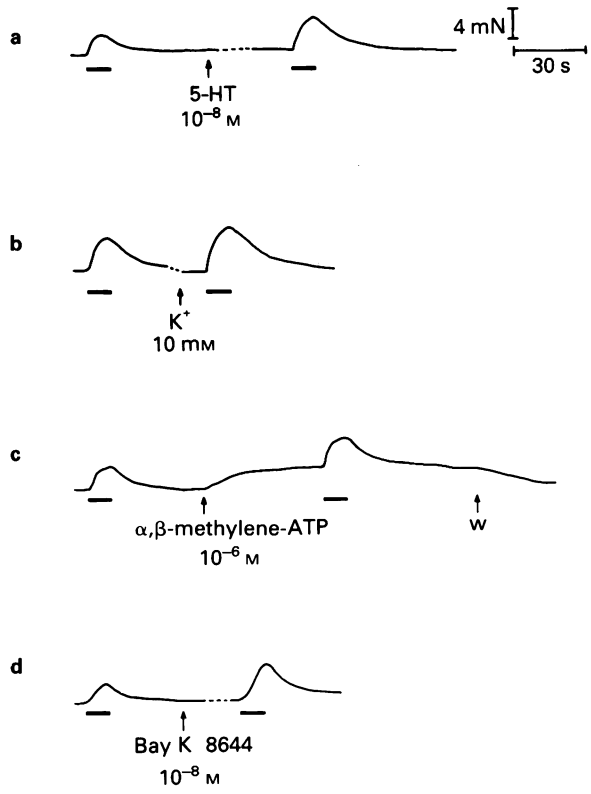


Figure 2 Representative individual experiments on neurogenic contractions induced by transmural nerve stimulation (9 V, 72 mA, 0.1 ms pulses at 6 Hz for 10 s) in the rat tail artery. The effect on the responses of: (a) acute exposure to 5-hydroxytryptamine (5-HT) 10^{-8} M; (b) elevation of the extracellular potassium concentration from 5.5 to 10 mM; (c) exposure to α,β-methylene-ATP 10^{-6} M; (d) Bay K 8644 10^{-8} M.

The depolarizing purinoceptor agonist α,β-methylene-ATP (10^{-6} M) significantly elevated the resting tone and significantly enhanced the neurogenic contractions. Phenylephrine did not affect the neurogenic response, either at low concentrations (10^{-8}–10^{-7} M), which did not affect the resting tone, or at a higher concentration (10^{-6} M) which caused contraction. Prostaglandin $F_{2\alpha}$, at a concentration (5×10^{-7} M) that caused a slight contraction had no effect on the neurogenic response, whereas a concentration of 5×10^{-6} M, that caused a more marked contraction, strongly enhanced the neurogenic response. Activation of potential-sensitive calcium channels by Bay K 8644, at a concentration of 10^{-8} M, markedly enhanced the neurogenic contraction (77%), without affecting the resting tone. Typical tracings of some of these responses are shown in Figure 2.

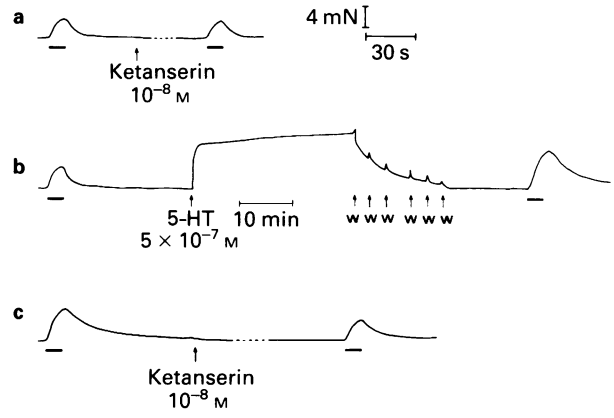


Figure 3 Individual experiment on a rat tail artery showing: (a) the absence of an effect of ketanserin, 10^{-8} M, on the neurogenic response to transmural nerve stimulation, (b) the stable contraction during exposure to 5-HT 5×10^{-7} M for 30 min, its reversal after repeated washing (W), and the enhanced neurogenic response after the incubation; (c) the partial blockade by ketanserin 10^{-8} M of the enhanced neurogenic response. Note the change in time scale in the intermediate part of the tracing in (b).

Neurogenic response after preincubation with 5-hydroxytryptamine

In the second part of the study the vessels were incubated for 30 min in 5×10^{-7} M 5-HT, followed by repeated washouts and a return of tone to the baseline. Following this treatment the neurogenic response was enhanced (Table 2, Figure 3b). Ketanserin (10^{-8} M) markedly attenuated the enhancement and now the response was not significantly different from that before incubation with 5-HT (Table 2). When the 5-HT uptake blocker paroxetine (3×10^{-8} M) was present during the incubation with 5-HT, no significant enhancement of the subsequent neurogenic response was observed. Further, the difference in enhancement between 5-HT incubation with and without uptake blocker was significant.

In the presence of prostaglandin $F_{2\alpha}$ (5×10^{-6} M) a significant enhancement of the neurogenic response was observed (Table 1), which was abolished after washout (Table 2).

Neurogenic response in the acute presence of 5-HT or after preincubation with 5-HT following sympathetic denervation

Immunohistochemical staining for 5-HT showed no or very sparse specific fluorescence in untreated vessels (Figure 4b),

Table 2 Effect of incubation of the vessels with 5-hydroxytryptamine (5-HT) 5×10^{-7} M for 30 min, incubation with 5-HT in the presence of paroxetine 3×10^{-8} M, the effect of application of ketanserin 10^{-8} M or tetrodotoxin (TTX) 3×10^{-7} M after incubation with 5-HT, or incubation with prostaglandin $F_{2\alpha}$ 5×10^{-6} M for 30 min on the electrically induced neurogenic contractions in rat tail artery

Incubation for 30 min	n	*Contraction before incubation* (mN)	*Contraction after incubation* (mN)	*Contraction after incubation as % of contraction before incubation*
5-HT 5×10^{-7} M	15	3.00 ± 0.32	5.86 ± 0.72***	212 ± 23%***
5-HT 5×10^{-7} M + paroxetine 3×10^{-8} M	7	3.77 ± 0.47	4.37 ± 0.70	123 ± 20%†
5-HT 5×10^{-7} M followed by ketanserin 10^{-8} M	10	3.17 ± 0.29	3.46 ± 0.33	115 ± 12%††
5-HT 5×10^{-7} M followed by TTX 3×10^{-7} M	8	3.79 ± 0.63	0***	0***,†††
Prostaglandin $F_{2\alpha}$ 5×10^{-6} M	6	3.27 ± 1.04	3.42 ± 1.09	102 ± 2%††

Significant differences between contractions before and after incubation with 5-HT: *** $P < 0.001$. Significant differences (in %) between the enhanced response after incubation with 5-HT alone and the other treatments. † $P < 0.05$, †† 0.01 and ††† 0.001, respectively. n = number of vessels.

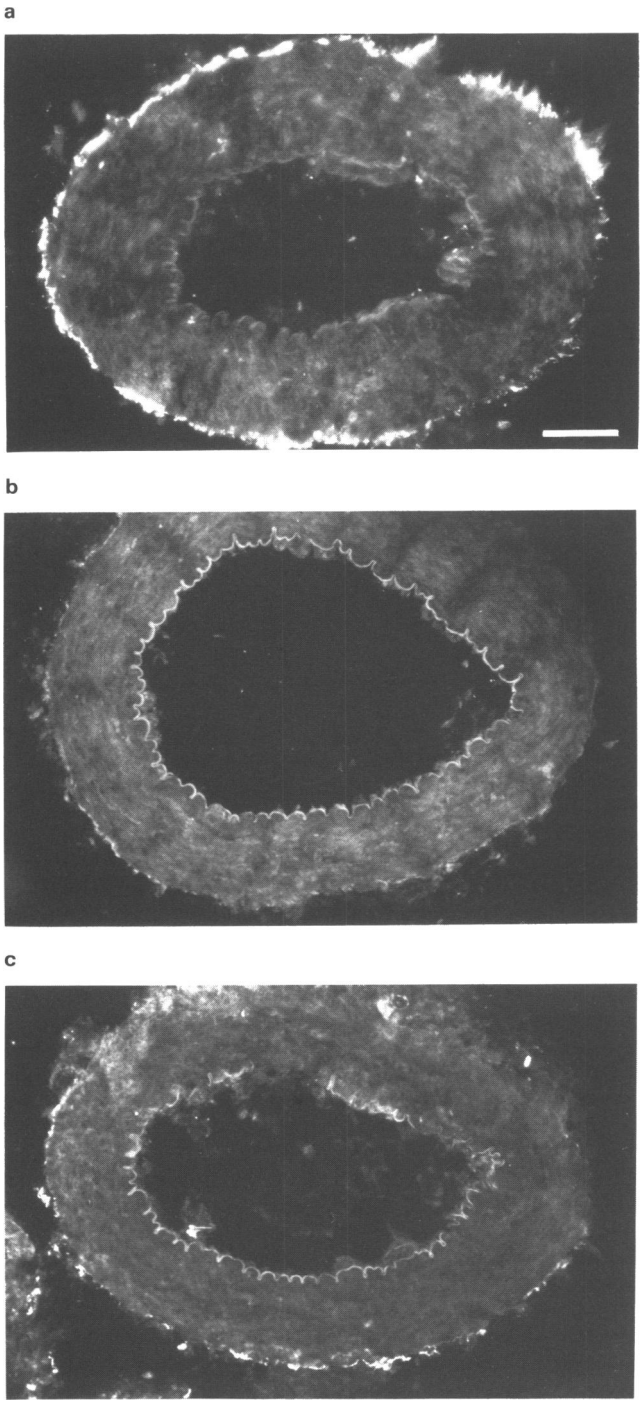


Figure 4 (a) 5-Hydroxytryptamine (5-HT)-immunofluorescent nerve fibres in the adventitial-medial border in a cross-section of a rat tail artery following incubation for 30 min with 5-HT 5×10^{-7} M. (b) Control vessel with no or very few visible 5-HT-containing fibres. (c) Vessel from *in vitro* 6-hydroxydopamine-treated rat following incubation in the presence of 5-HT, few 5-HT-containing fibres are visible. Bar = 100 μm.

whereas after 30 min incubation in 5-HT (5×10^{-7} M), dense 5-HT-immunofluorescence was observed in the adventitial-medial border (Figure 4a). After *in vivo* treatment with 6-OHDA or section of both ventral collector nerves, the majority of sympathetic nerves, as demonstrated by tyrosine hydroxylase immunoreactivity, had disappeared, and only few 5-HT-immunofluorescent fibres were observed after prior incubation (Figure 4c).

The effects of the various denervation procedures on the neurogenic response are summarized in Table 3. In vessels not exposed to 5-HT, only a weak, TTX-sensitive response remained after 6-OHDA treatment *in vivo*, and there was no response at all after *in vitro* treatment. After treatment, no facilitation of the weak response was seen in the presence of 5-HT (Table 3a) or after incubation with 5-HT for 30 min (Table 3b). However, after section of both ventral collector nerves, a procedure which reduces the sympathetic nerve density by about 80% (Sittiracha *et al.*, 1987), a marked potentiation of the remaining weak response was seen both in the presence of 5-HT and after incubation. The seemingly larger relative enhancement of the response after 5-HT treatment compared to that in untreated vessels is, in all probability, due to a smaller response prior to 5-HT exposure in denervated than in intact vessels.

Discussion

Presence of 5-hydroxytryptamine extracellularly

The results of the present study support previous findings in rat tail artery that 5-HT in subthreshold concentrations enhances contractions induced by sympathetic nerve stimulation, and that this effect of 5-HT involves activation of postjunctional 5-HT_2 receptors (Medgett *et al.*, 1984; Xiao & Rand, 1989). In this vessel, 5-HT causes depolarization of the smooth muscle cell membrane (Neild & Kotecha, 1987) and influx of extracellular calcium through potential-sensitive calcium channels (Chang & Owman, 1987). The present finding of a marked inhibition of the 5-HT-induced potentiation by the potential-sensitive calcium channel blocker D-600 indicates that such a mechanism is also operating for the 5-HT-induced enhancement of neurogenic responses in this artery. Only intracellular calcium mobilization appears to be involved in the neurogenic response in untreated vessels, since the calcium channel blocker did not inhibit the response, in accordance with findings by Medgett & Rajanyagam (1984). It has been shown that activation of postjunctional α_1-adrenoceptors, but not α_2-adrenoceptors in this vascular smooth muscle causes mobilization of intracellular calcium (see Timmermans & Thoolen, 1987). Accordingly, the α_1-adrenoceptor antagonist prazosin inhibited the response to a greater extent than the α_2-adrenoceptor antagonist rauwolscine in untreated vessels.

The facilitating effect on neurogenic responses by vasoactive agents other than 5-HT may also be due to depolarization and calcium influx through potential-sensitive channels into the smooth muscle cells. It has been shown in rat tail artery and rabbit ear artery that a modest elevation of the potassium concentration (Neild & Kotecha, 1987) or exposure to Bay K 8644 (Cheung & Mackay, 1986) or α,β-methylene-ATP (Neild & Kotecha, 1986) causes a small depolarization of the smooth muscle membrane. Recent investigations have shown that even a small depolarization of the cell membrane substantially alters the availability of extracellular calcium to the smooth muscle cells by altering the opening-closing state of the calcium channels in the membrane. In this way, contractions to exogenously applied NA are facilitated (Nelson *et al.*, 1988). Prostaglandin $F_{2\alpha}$ and the α_1-adrenoceptor agonist phenylephrine, in concentrations that did not contract the vessels, did not facilitate the neurogenic response, probably because the receptors on which they act are not linked to enhanced calcium influx (Uski & Andersson, 1984; van Zwieten & Timmermans, 1987).

Recent studies on the potentiating effect of 5-HT on the contractions caused by exogenously applied adrenoceptor agonists have suggested that the effect is dependent upon extracellular calcium, since it is counteracted by diltiazem or lowered extracellular calcium (Meehan *et al.*, 1988; Xiao & Rand, 1989). These results are in agreement with our findings, that D-600 abolished the enhanced part of the neurogenic response, similarly to the effect of ketanserin. Since the present combined application of the calcium channel blocker and α-

Table 3 Effect in rat tail artery of *in vitro* 6-hydroxydopamine (6-OHDA) denervation, *in vivo* 6-OHDA denervation and section of both ventral collector nerves, on the enhanced neurogenic contractile response normally seen (a) in the presence of 5-hydroxytryptamine (5-HT) 10^{-8} M and (b) after incubation with 5-HT 5×10^{-7} M for 30 min

a

Denervation	n	*Contraction* (mN) *untreated vessels*	*Contraction* (mN) *in the presence of 5-HT* (10^{-8} M)
In vitro 6-OHDA denervation	4	0	0
In vivo 6-OHDA denervation	8	0.31 ± 0.35	0.34 ± 0.23
Surgical denervation	5	1.10 ± 0.39	3.04 ± 1.08*

b

Denervation		*Contraction* (mN) *before incubation*	*Contraction* (mN) *after incubation for 30 min with 5-HT* (5×10^{-7} M)
In vitro 6-OHDA denervation	4	0	0
In vivo 6-OHDA denervation	8	0.25 ± 0.18	0.28 ± 0.26
Surgical denervation	10	0.84 ± 0.29	2.38 ± 0.48**

Significant differences from control values. * $P < 0.05$ and ** 0.01, respectively. n = number of vessels.

adrenoceptor antagonists caused a more pronounced inhibition of the contraction than either blocker alone, our results support the proposal that the binding sites on the calcium channels for blockers like verapamil and D-600 represent the overlapping area (or common binding site) for 5-HT_2-receptors and α_1-adrenoceptors involved in the amplifying effect (Marwood, 1988). Thus our results support an interaction between 5-HT and NA on the postjunctional receptors, which involves an enhanced calcium influx through potential-sensitive channels when NA is of endogenous origin. However, since there was a significant (30%) remaining contraction in the presence of 5-HT, even if both α-blockers and D-600 were applied, additional amplifying mechanisms may be present.

While the neurogenic response in untreated vessels was markedly blocked after combined α_1- and α_2-adrenoceptor blockade, a less pronounced blockade by these two antagonists was obtained in the presence of 5-HT 10^{-8} M. To explain this, an additional amplifying mechanism involving postjunctional α_2-adrenoceptors and 5-HT_2-receptors might be present. However, this is made unlikely by the finding that combined blockade by α_1- and α_2-adrenoceptor antagonists in vessels exposed to 5-HT was not more effective than α_1-blockade alone, in contrast to the situation in untreated vessels. A possible interaction between postjunctional 5-HT_2-receptors and γ-receptors, which have been proposed to exist in several vascular beds including the rat tail artery (for review see Hirst & Edwards, 1989), may be considered. If they exist, they play only a very minor role in sympathetic neuroeffector mechanisms in the rat tail artery, since the small component of the neurogenic contractile response remaining in untreated vessels after combined α_1- and α_2-adrenoceptor blockade was almost fully attributable to activation of NPY-receptors. Therefore, the additional amplifying mechanism by 5-HT might possibly consist of an interaction between exogenous 5-HT and NPY, released upon nerve stimulation. Previous studies on the rat tail artery has excluded the possibility of endogenous cholinergic, purinergic and prostanoic mechanisms in the tonic response to field stimulation at the present stimulation parameters (Szabó & Hardebo, 1990).

Presence of 5-hydroxytryptamine in perivascular sympathetic nerves

The second part of the study demonstrates that incubation of the vessels with 5-HT for 30 min followed by washout enhances the neurogenic contractions. The enhanced response was still fully TTX-sensitive. The effect is due to release of 5-HT from the perivascular sympathetic nerves after prior uptake, since (1) 5-HT could be clearly demonstrated by immunohistochemistry in perivascular nerves after prior exposure to 5-HT (but not or to a very minor extent in untreated vessels); (2) the perivascular sympathetic nerves, together with the 5-HT-containing fibres in vessels that had been exposed to 5-HT, were markedly reduced in number after *in vivo* 6-OHDA treatment; (3) no enhanced contractile response was seen following 6-OHDA treatment; (4) the presence of the 5-HT uptake blocker, paroxetine, counteracted this enhancement; and (5) ketanserin significantly reduced the response. Upon incubation, 5-HT may also accumulate extraneuronally in arteries (Fukuda *et al.*, 1986). Release from such stores is not responsible for the presently observed facilitation of the neurogenic response, since the enhanced contraction was fully TTX-blockable.

The surgical denervation procedure that was used eliminates only about 80% of the perivascular sympathetic fibres (Sittiracha *et al.*, 1987). There was uptake of 5-HT into the remaining sympathetic nerves, and it enhanced the small contractile response to electrical field stimulation. Furthermore, the denervation causes a supersensitivity of postjunctional receptors to 5-HT (see Szabó & Hardebo, 1990). The lack of enhancement of the neurogenic contractions after incubation with prostaglandin $F_{2\alpha}$ clearly shows that the enhancement is not due to an altered calcium influx (or rearrangement of intracellular calcium) in the smooth muscle cells secondary to a prolonged contraction.

In conclusion, the study demonstrates a capacity of perivascular sympathetic nerves in the rat tail artery to accumulate 5-HT. Upon release, this neuronal 5-HT is able to enhance the contractions caused by stimulation of the adrenergic nerves to an extent similar to exogenously applied 5-HT in a concentration of 10^{-8} M. The enhanced response results from an opening by 5-HT of potential-sensitive calcium channels. Upon exposure of the vessel wall to 5-HT such a mechanism may contribute to an enhanced contractile response when the perivascular sympathetic nerves become activated. This may occur when platelets disrupt in conjunction with thrombosis formation, especially at sites with endothelial damage (Cohen, 1988). In hypertension, where the vascular postsynaptic 5-HT receptors are supersensitive (Van Nueten & Janssens, 1986), such a neurogenic contractile response may become even larger.

This work was supported by a grant from the Swedish Medical Research Council (14X-732/5680). Technical assistance by Ms Joanna Daszkiewicz-Nilsson and excellent typing of the manuscript by Ms Maud Mårtensson is highly appreciated.

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(Received March 27, 1990
Revised August 13, 1990
Accepted August 31, 1990)

Br. J. Pharmacol. (1991), **102**, 408–414 © Macmillan Press Ltd, 1991

Evidence for regulatory control of iron uptake from ferric maltol across the small intestine of the rat

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1 ^{59}Fe absorption from the novel iron compound, ferric maltol, was studied in rats pretreated twice daily for two weeks with non-radioactive ferric maltol in oral doses containing 7 mg elemental iron. Tissue accumulation of ^{59}Fe 2 h after administration of radioactive ferric maltol into the stomach was significantly lower in iron pretreated animals than in saline-treated controls.

2 ^{59}Fe uptake from ferric maltol into isolated fragments of ileum and of duodenum was of similar magnitude in control animals but in iron-treated animals, duodenal uptake was significantly lower than that of the ileum.

3 Absorption of ^{59}Fe was also investigated in anaesthetized rats after intestinal perfusion with saline (controls) or with 5 mM chenodeoxycholate to render the intestines more permeable.

4 Changes in permeability of the small intestine were monitored by estimating the amount of [^{14}C]-mannitol absorbed and fluid secreted with reference to the non-absorbable [^{3}H]-inulin in the perfusate effluents.

5 Despite the increased permeability of the intestines after bile salt treatment, there was little difference from control in the tissue accumulation of ^{59}Fe from ferric maltol 2 h after intraduodenal administration. However ^{59}Fe absorption from ferrous sulphate was significantly increased after bile salt treatment.

6 Gel filtration profiles of plasma made 5 and 60 min after intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol demonstrated that metal and ligand do not enter the circulation as the complex even when intestinal permeability is increased.

7 Uptake of ^{59}Fe was investigated in isolated fragments of rat small intestine after saline or bile salt perfusion. Although ^{59}Fe uptake from ferric maltol was somewhat greater in the bile salt-treated intestinal fragments, saturable kinetics were still observed. By contrast, ^{59}Fe uptake from ferrous sulphate: ascorbate was greatly enhanced by bile salt pretreatment and a very large diffusional component of uptake was evident.

8 It is concluded that iron uptake from ferric maltol may well be under endogenous regulatory control even in damaged intestines, so it is unlikely that this novel iron compound can bring about iron overload when administered orally.

Introduction

Since few regulatory mechanisms exist for eliminating body iron, normal control of iron status is exerted primarily at the point of intake i.e., at the intestinal mucosa. However, it is still not clearly understood how intestinal absorption of non-haem iron, which constitutes the bulk of dietary iron, is controlled. Three key steps i.e., uptake from the gut lumen, retention in the mucosal storage pool and transfer to the plasma appear to be involved in intestinal iron absorption. Should any one of these steps be by-passed or be abnormal, then changes in iron status, particularly iron overload, may result, as for example in haemochromatosis, where altered serosal iron transfer has been identified (McClaren *et al.*, 1988).

A novel iron compound, ferric maltol (3-hydroxy-2-methyl-4-pyrone), has been developed as a more palatable and less irritant alternative to ferrous compounds in the treatment of iron deficiency (Hider *et al.*, 1984). Since the ferric iron is bound with high affinity to maltol forming an uncharged complex with an oil:water partition coefficient sufficiently high (0.5) to allow diffusion across lipid membranes, it was originally supposed that ferric maltol might diffuse across the intestinal wall and donate its ferric iron directly to transferrin in the plasma. If this were the case, then danger of iron overload would be possible. However, saturable uptake kinetics for iron entry from ferric maltol have been observed in intact tissues both *in vivo* and *in vitro* (Barrand *et al.*, 1987; Levey *et al.*, 1988). Furthermore, reduction of the ferric iron to the ferrous state with consequent dissociation of metal and ligand probably take place immediately prior to iron absorption (Barrand *et al.*, 1990). The iron must thus be donated to the endogenous uptake carrier and thereby remains under regulatory control.

The present work provides further evidence that iron from ferric maltol may enter the normal regulatory uptake pathways in the intestine so that under iron replete conditions or when the intestine is damaged, excessive uptake of metal will not occur.

Methods

Absorption and distribution of ^{59}Fe in whole animals

Male Wistar rats, 150–200 g in weight, were given, by gavage twice daily for two weeks, ferric maltol in 500 μl of saline. Control animals received saline alone. In initial experiments, a further control group of animals received a saturated solution of maltol alone. Iron solutions for oral dosing were prepared just before use by mixing solid $FeCl_3$ and maltol powder at a molar ratio of 1:4 in sufficient saline to provide 7 mg elemental iron in each 500 μl dose. The final dose given contained 5 μCi of ^{59}Fe. The animals were killed by cervical dislocation 2 h later. Samples were taken of blood and liver together with urine, kidneys, femurs, spleen and the unabsorbed contents and washed segments of small intestine and their content of ^{59}Fe measured in a Packard Auto Gamma 500 counter. It has already been established that the ^{59}Fe content of the femurs provides a consistent index i.e. 1/12th of ^{59}Fe present in the total bone marrow (Barrand *et al.*, 1987).

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In initial experiments, the small intestines from animals after dosing for two weeks with ferric maltol or with saline were examined for overt signs of structural damage and biochemical abnormalities. Portions of duodenum were fixed overnight in 4% glutaraldehyde and processed for electron microscopy. Samples of small intestine were homogenised in 1 mM potassium phosphate buffer at pH 7.8 and assayed for monoamine oxidase activity by a radiochemical method (Callingham & Laverty, 1973) using [^{3}H]-5-hydroxytryptamine as substrate. Other samples of small intestine were homogenized in 2 mM HEPES buffer at pH 7.4 containing 50 mM mannitol and assayed for sucrase activity by a spectrophotometric method (Dahlqvist, 1968). Glucose uptake in the small intestine was assessed by the method described below for ^{59}Fe uptake into isolated fragments with 2-deoxy-D-[2,6-^{3}H]-glucose as substrate and [^{14}C]-mannitol as a marker of extracellular fluid volume.

For ^{59}Fe uptake studies in anaesthetized animals following intestinal perfusion (see below), the intestines were first washed through with saline alone (0.9% NaCl w/v), tied at each end and 500 μl volumes of the iron solutions (either ferric maltol prepared as above or $FeSO_4$ containing 5 μCi ^{59}Fe and 7 mg element iron) injected into the duodenum close to the pyloric end. The animals were maintained under anaesthesia for a further 2 h and then killed by anaesthetic overdose before being dissected as outlined above.

Perfusion of rat small intestine

Male Wistar rats, 150–350 g body weight, fasted for 18 h before use were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). The abdomen was opened by a midline incision and two cannulae (i.d. 3 mm) inserted into the small intestine, with inflow near the pylorus and outflow approximately 30 cm distal to this. The cannulae were tied in place avoiding obstruction of blood flow and the inflow connected to a peristaltic pump delivering warmed perfusion fluid at a rate of 0.6 ml min^{-1}. The intestines were flushed out initially with saline alone and then perfused for 30–45 min with saline containing 10 mM glucose, [^{14}C]-mannitol at 0.002 μCi ml^{-1} and [^{3}H]-inulin at 0.01 μCi ml^{-1} to obtain baseline levels and then for a further 45 min with the above perfusion mixture with or without 5 mM chenodeoxycholate. Each 5 min effluent was collected and measured both for volume and also for ^{14}C and ^{3}H content by liquid scintillation counting in a Packard 2000CA spectrometer with automatic external standardization. Fluid secretion and mannitol absorption relative to inulin was calculated per cm of small intestine using the amount of [^{3}H]-inulin as the nonabsorbable marker. For morphological evidence of tissue damage, samples of intestine were taken after perfusion, fixed overnight in 4% glutaraldehyde and processed for examination by electron microscopy.

Sampling and gel filtration of plasma

Blood samples were obtained via carotid cannulae from anaesthetized rats 5 and 60 min after intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol centrifuged at 6000 *g* for 2 min at 20°C and the plasma passed down 10 ml columns (PD-10) of Sephadex G-25 (Pharmacia, Uppsala, Sweden) previously equilibrated with 20 mM HEPES, pH 7.4 in 154 mM NaCl and eluted in 1 ml fractions. Each fraction was analysed for ^{59}Fe and for tritium content by liquid scintillation spectrometry.

^{59}Fe uptake into isolated fragments of small intestine

This was carried out as described previously (Levey *et al.*, 1988) with tissue obtained both from rats dosed for two weeks with saline or with ferric maltol and from rats after intestinal perfusion. Briefly, intestinal fragments 30–50 mg in weight were immersed in buffer (composition, in mM, HEPES 16, NaCl 125, KCl 3.5, $CaCl_2$ 1, $MgSO_4$ 10, D-glucose 10, and pH 7.4) and incubated for 10 min at 37°C with the appropriate ^{59}Fe solutions containing also [^{3}H]-inulin (0.01 μC ml^{-1}) as extracellular fluid marker. Iron solutions for these *in vitro* studies were prepared by mixing $FeCl_3$ and maltol in 16 mM HEPES buffer at pH 7.4 at a ratio of 1:4 or by mixing $FeSO_4$ with ascorbic acid at a ratio of 1:5, $^{59}FeCl_3$ being added so as to achieve a final concentration of ^{59}Fe in the incubation media of 0.1–0.25 μCi ml^{-1}. Fragments, removed at intervals and digested in Soluene-350 (Packard, Reading, UK), were analysed for their ^{59}Fe and tritium content.

The amount of ^{59}Fe accumulated was calculated, after correction for extracellular fluid volume and wet weight of each tissue fragment, as a distribution ratio (tissue/medium) and subsequently converted to pmol min^{-1} mg^{-1} wet weight of tissue. Since ^{59}Fe uptake appears to be linear over 10 min (Levey *et al.*, 1988), initial rates of iron uptake may be estimated from the data.

Materials

D-[1-^{14}C]mannitol, 2-deoxy-D-[2,6-^{3}H]glucose, 5-hydroxy-[G-^{3}H] tryptamine creatinine sulphate, [^{3}H]inulin and $^{59}FeCl_3$ were purchased from Amersham International (Bucks, UK) and the pyrone, maltol (Veltol) obtained from Pfizer Ltd (Sandwich, Kent, UK). [^{3}H]-maltol was synthesized as described in Barrand *et al.* (1991). All other chemicals either were bought from Sigma Chemical Co Ltd (Poole, Dorset, UK) or were standard laboratory reagents of analytical grade.

Statistical methods

Statistical significance was determined by Student's *t* test (unpaired) comparing each group with its respective control.

Results

Ultrastructure, enzyme activities and glucose uptake capability of rat small intestine following two weeks treatment with oral ferric maltol

No gross differences in morphology of intestinal epithelial cells were observed between rats treated for two weeks with saline and with ferric maltol. There were no obvious signs of damage to the mitochondria or to the microvilli along the brush border of the epithelium, nor were there any gaps between the cells suggesting loss of intercellular contact (Figure 1). Activities of the outer mitochondrial membrane enzyme, monoamine oxidase, and of the brush border enzyme, sucrase, were similar in homogenates taken from the two groups of animals (monoamine oxidase activities of 68.2 $\pm$ 4.14 in the ferric maltol treated group compared with 72.8 $\pm$ 4.5 nmol product mg^{-1} protein h^{-1} in control tissue, $n = 6$ and sucrase activities of 36.2 $\pm$ 0.8 in the ferric maltol treated group compared with 34.0 $\pm$ 3.2 μg product mg^{-1} protein h^{-1} in control tissue, $n = 6$). Rates of initial uptake of [^{3}H]-deoxyglucose into isolated fragments of small intestine taken from each of the two groups of rats were similar (8.4 $\pm$ 1.1 in the ferric maltol treated group compared with 6.8 $\pm$ 1.1 pmol min^{-1} mg^{-1} wet tissue in the control group, $n = 12$).

^{59}Fe absorption following two weeks of oral ferric maltol

The amount of ^{59}Fe present in the blood and the accumulation of ^{59}Fe in liver, bone marrow, kidneys, spleen and urine was measured 2 h after stomach administration of [^{59}Fe]-ferric maltol at a dose containing 7 mg elemental iron. Previous studies (Bothwell *et al.*, 1979; Barrand *et al.*, 1987) have shown that the liver and bone marrow constitute the major sites of ^{59}Fe accumulation. Levels of ^{59}Fe significantly lower than control were found in the animals that had been

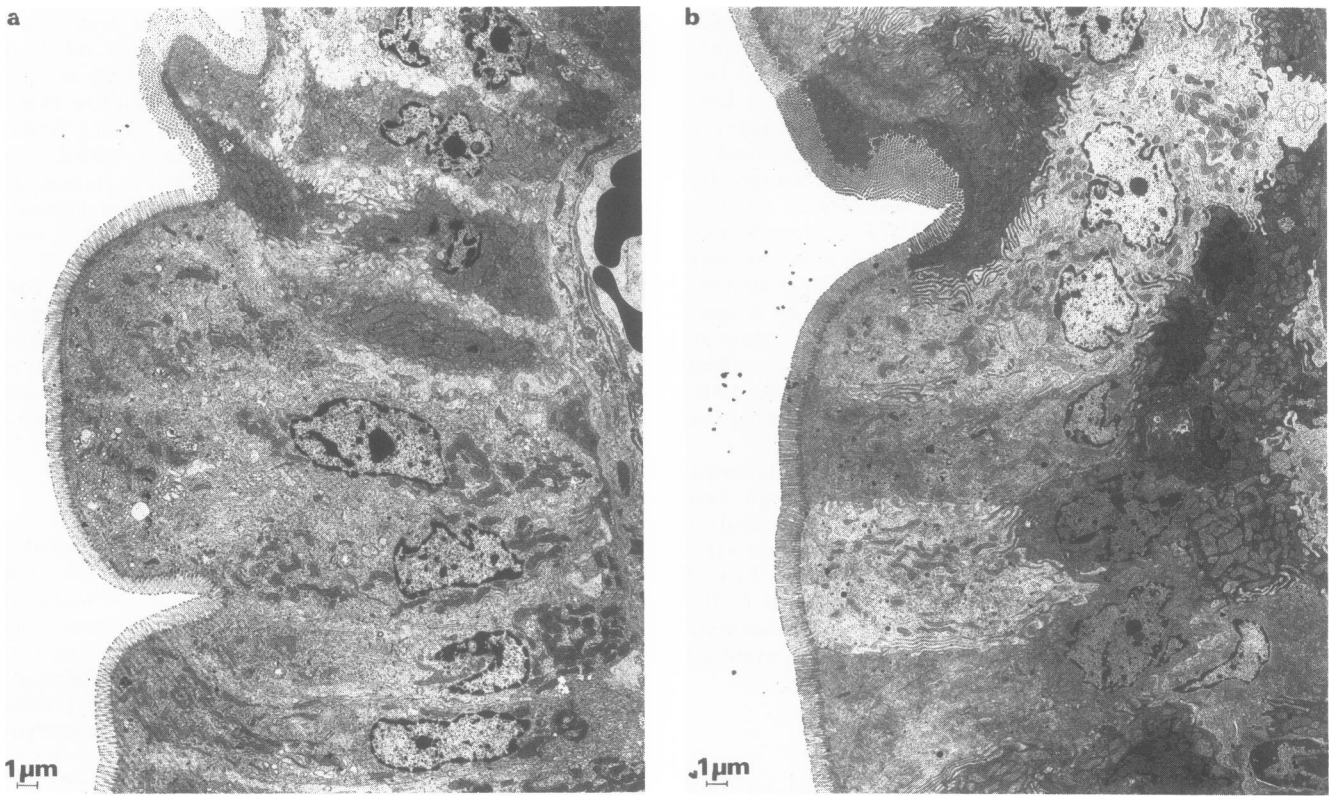


Figure 1 Electron micrographs of mucosal epithelium from the proximal part of the small intestine taken from rats treated orally for two weeks with twice daily doses of (a) ferric maltol (each dose containing 7 mg elemental iron) or (b) saline.

pretreated twice daily for two weeks with oral doses of ferric maltol containing 7 mg elemental iron before administration of the ^{59}Fe maltol (Table 1). In these experiments, the control animals received saline alone.

In initial experiments, control animals received either saline alone or maltol alone. It was found to be impossible, in the absence of iron, to dissolve maltol in amounts equivalent to those present in the ferric maltol doses. Animals therefore received oral doses of a saturated solution of maltol. This resulted in looseness of the stools, a feature not observed when ferric maltol was used for oral dosing. Free maltol is unlikely to be present within the gut lumen in any significant amounts when ferric maltol is administered. Both iron and maltol appear to be absorbed rapidly after dissociation in the gut lumen (Barrand *et al.*, 1991) and if not absorbed, ferric maltol remains as the complex which can be visualised by its deep red colour. Control experiments tracing the passage of the orally administered ferric maltol showed that it remained in the undissociated form throughout the small intestine. Since maltol alone has very different biochemical and physical properties from the ferric maltol complex, it did not appear to provide a very satisfactory control. In all subsequent experiments, only controls receiving saline alone were used.

Table 1 Tissue accumulation of ^{59}Fe 2 h after gavage with [^{59}Fe]-ferric maltol dose

	Ferric maltol treated	*Controls*
Marrow	23.1 ± 1.4**	30.1 ± 1.5
Liver	53.2 ± 1.5*	156.1 ± 35.0
Blood	27.09 ± 2.8*	33.46 ± 2.1
Spleen	2.73 ± 0.63	3.78 ± 0.56
Kidney	1.4 ± 0.14*	3.15 ± 0.7
Urine	0.42 ± 0.14	0.42 ± 0.07

[^{59}Fe]-ferric maltol, containing 7 mg of elemental iron was administered to rats, previously treated, twice daily, for two weeks with oral doses of either unlabelled ferric maltol containing 7 mg of elemental iron or saline (controls). Values shown are the mean ± s.e.mean of data from 6 rats in each group and are given as μg of administered ^{59}Fe recovered in each tissue. Significant differences between the groups are shown by use of Student's *t* test (** $P < 0.01$ and * $P < 0.05$).

^{59}Fe uptake into isolated fragments of ileum and duodenum from rats treated for two weeks with oral doses of ferric maltol

In control, saline-treated animals, rates of ^{59}Fe uptake from ferric maltol into isolated fragments of ileum and of duodenum were of similar magnitude (Figure 2b). In the iron-treated animals however, rates of ^{59}Fe uptake were significantly lower in duodenal fragments ($P < 0.01$, Figure 2a). In a previous study on iron deficient animals (Callingham & Barrand, 1987) the converse was observed i.e., rates of iron uptake were significantly higher into duodenal fragments than into ileal fragments. Data from these experiments are shown in Figure 2c for comparison.

Permeability changes in perfused small intestines

Virtually no [^{14}C]-mannitol was absorbed from the intestines during perfusion with saline/glucose alone. However, 5 to 10 min after changing the perfusion medium to one containing 5 mM chenodeoxycholate, [^{14}C]-mannitol absorption increased, levelling off 20 to 30 min later (Figure 3a). Changes in fluid secretion showed a similar time course (Figure 3b). Extensive damage to the intestines was observed histologically (Figure 4) after perfusion with 5 mM chenodeoxycholate. The goblet cells were ruptured with their content of mucus extruded and epithelial cells had been stripped away leaving the villus tips denuded of epithelium. These permeability and histology changes are similar in pattern to those reported by others (Erickson *et al.*, 1987).

^{59}Fe absorption from ferric maltol and ferrous sulphate following intestinal perfusion

Despite the increased permeability of the intestines perfused with the bile salt, there appeared to be little difference between

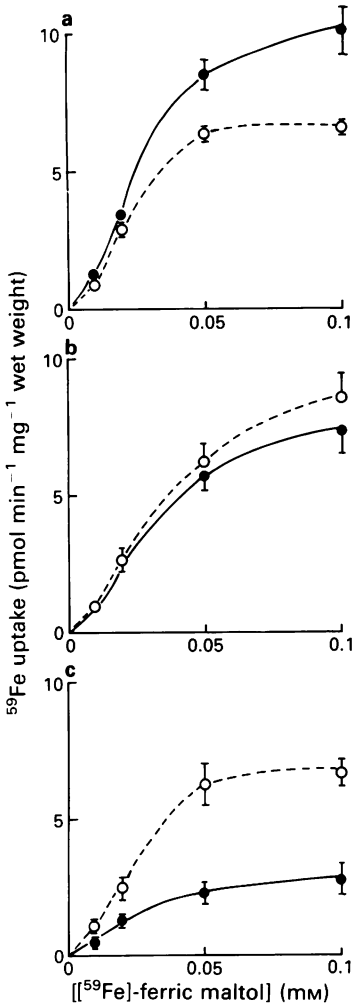


Figure 2 The effect of ferric maltol concentration on the initial rates of uptake of ^{59}Fe into isolated fragments of ileum (●) and of duodenum (○) taken from (a) rats given twice daily oral doses of ferric maltol containing 7 mg of elemental iron for two weeks, (b) saline-treated control rats and (c) rats made iron-deficient by feeding from weaning on a low iron diet. Each value represents the mean of data from 4–6 separate experiments, quadruplicate determinations being obtained from each experiment: vertical bars show s.e.mean. Values are given as pmol of iron min^{-1} mg^{-1} wet weight of tissue. The rates of uptake were significantly different in (a) and (c) ($P < 0.01$) at [^{59}Fe]-maltol concentrations above 0.05 mM.

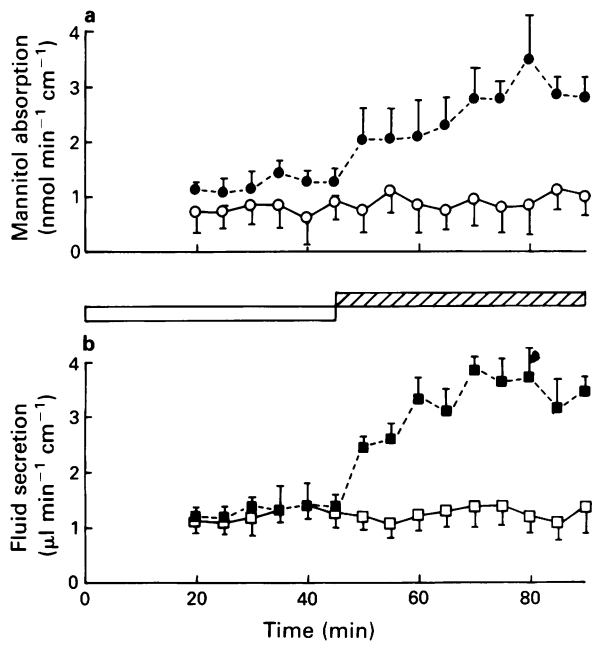


Figure 3 The effects of bile salt perfusion through the intestine of anaesthetized rats on mannitol absorption (a) and fluid secretion (b) in the intestines. Rats were perfused in pairs. For the first 45 min (indicated by the open bar), both rats received saline containing 10 mM glucose. During the second 45 min (indicated by the hatched bar), the experimental rat (closed symbols) was perfused with 5 mM chenodeoxycholate whilst the control rat continued to receive saline/glucose. Each value represents the mean of data taken from 4 separate experiments, i.e., 8 rats; vertical bars show s.e.mean. Duplicate determinations of the effluent composition were taken at each time point for calculation of mannitol absorbed in nmol min^{-1} cm^{-1} lengths of intestine and of fluid secreted in µl min^{-1} cm^{-1} lengths of intestine.

experimental and control animals in the amount of ^{59}Fe accumulated in the tissues 2 h after intraduodenal administration of ferric maltol at a dose containing 7 mg elemental iron (Table 2). When equivalent doses of ferrous sulphate were administered, significant increases in tissue ^{59}Fe accumulation were seen in the bile salt-treated animals. ^{59}Fe absorption from ferrous sulphate was however very much lower than that from ferric maltol. This may be explained on the basis of the lower bioavailability of the ferrous iron. Precipitates of iron could be seen adhering to the mucosal lining at the end of the 2 h exposure. Since the intestines were tied off, movement of iron down the gut was prevented, thus accentuating the differences between ferric maltol which can hold iron in soluble form and ferrous sulphate which rapidly precipitates at neutral pHs.

Disposition of ^{59}Fe and ^{3}H in plasma samples after [^{59}Fe]-ferric [^{3}H]-maltol administration into perfused intestines

Gel filtration studies were carried out on plasma samples taken 5 and 60 min after intraduodenal administration of ^{59}Fe complexed to [^{3}H]-maltol. ^{59}Fe eluted from the gel column at the void volume, a position corresponding to the high molecular weight proteins, whereas ^{3}H was detectable only in the fractions containing smaller molecular weight material (Figure 5). This pattern was the same in plasma taken at 5 and at 60 min, whether or not the intestines had been rendered leaky by bile salt perfusion.

Table 2 Tissue accumulation of ^{59}Fe 2 h after intraduodenal administration of [^{59}Fe]-ferric maltol and [^{59}Fe]-ferrous sulphate

	Ferric maltol		*Ferrous sulphate*	
	Control	*Treated*	*Control*	*Treated*
Marrow	48.3 ± 9.1	65.8 ± 11.2	18.9 ± 2.8	**30.1 ± 3.5
Liver	626 ± 142	702 ± 63.0	78.4 ± 16.1	***274 ± 28.0
Kidney	37.1 ± 10.5	55.3 ± 11.2	2.1 ± 0.7	***14.0 ± 7.0

Either [^{59}Fe]-ferric maltol or [^{59}Fe]-ferrous sulphate containing 7 mg of elemental iron was administered into the duodenum of anaesthetized rats previously perfused for 45 min through the intestines with saline alone (controls) or with 5 mM chenodeoxycholate in saline. Values shown are the mean ± s.e.mean of data from 6 rats in each group and are given as µg of administered ^{59}Fe recovered in each tissue. Statistical analysis by Student's *t* test shows significance compared with control values as *** $P < 0.001$ and ** $P < 0.01$.

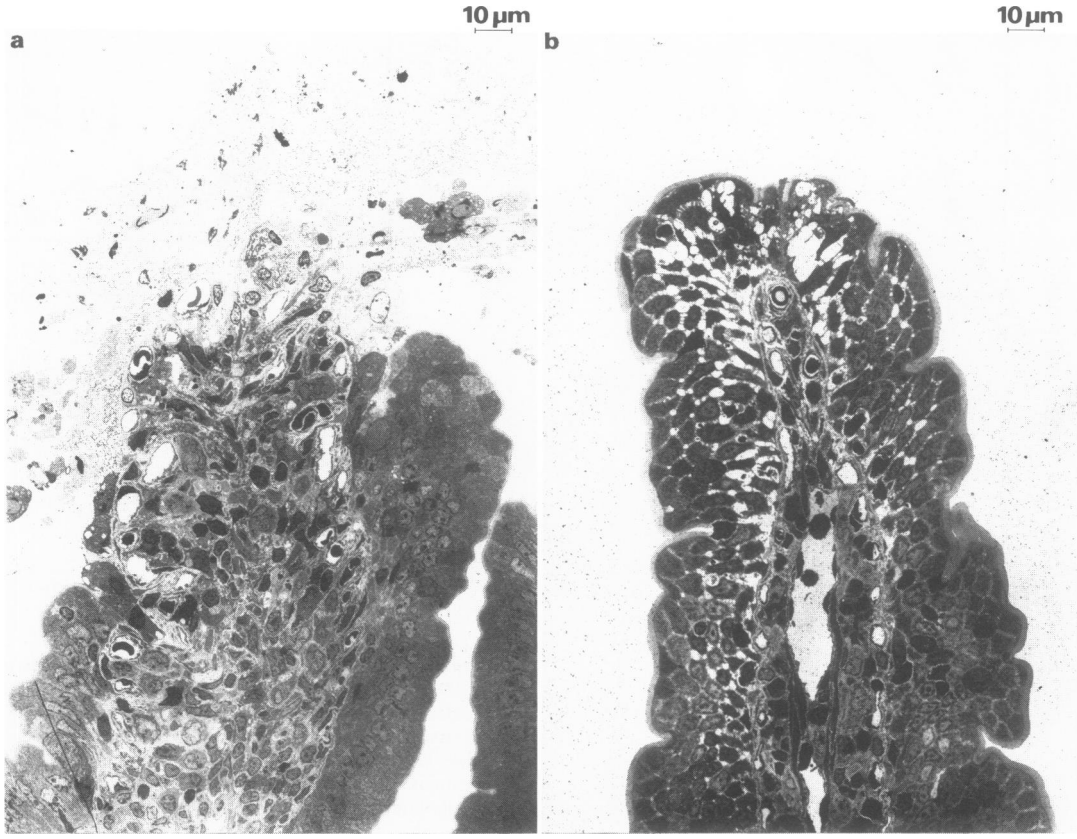


Figure 4 Electron micrographs of mucosal tissue taken from the proximal part of the rat small intestine following perfusion for 45 min with (a) 5 mM chenodeoxycholate in saline or (b) saline alone to demonstrate the extent of damage to the epithelial cells at the tips of the villi caused by the bile salt.

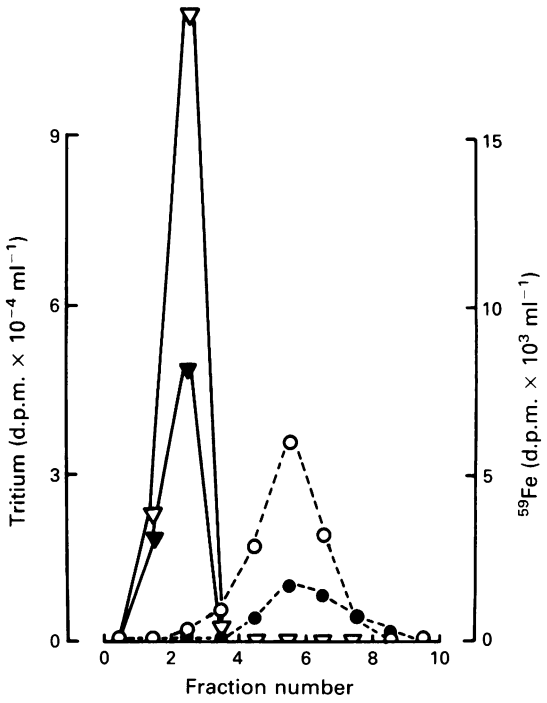


Figure 5 Gel filtration profiles of ^{59}Fe (▼, ▽) and of tritium (●, ○) in plasma samples taken 5 min (closed symbols) and 60 min (open symbols) after intraduodenal administration [^{59}Fe]-ferric [^{3}H]-maltol at a dose of 7 mg elemental iron into anaesthetized rats previously perfused for 45 min with 5 mM chenodeoxycholate in saline/10 mM glucose; 100 μl volumes of plasma were applied to 10 ml columns of Sephadex G-25 and 1 ml fractions eluted. Values are shown as d.p.m. of radioactive material in each fraction. Data presented are from one of the three separate experiments.

^{59}Fe uptake from ferric maltol and from ferrous sulphate/ascorbate into isolated intestinal fragments following perfusion

Rates of ^{59}Fe uptake were increased significantly at all concentrations of iron both from ferric maltol and from ferrous sulphate/ascorbate in fragments taken from bile salt perfused intestines (Figure 6). However, ^{59}Fe uptake from ferric maltol still remained largely saturable with only a small diffusional component being evident. By contrast, ^{59}Fe uptake from ferrous sulphate/ascorbate showed a large diffusional component and this was dramatically enhanced by bile salt treatment.

Discussion

Twice daily oral administration of high doses of ferric maltol to rats over the course of two weeks was sufficient to bring about reduced iron uptake from a single dose of ^{59}Fe but produced no obvious signs, either morphological, biochemical or physiological, of intestinal damage. Each dose used i.e., 7 mg elemental iron would be equivalent to 2 g in a 70 kg human subject. It is well known that iron absorption decreases when body iron stores are raised and increases during periods of iron deficiency and that these alterations are effected by mechanisms within the duodenum (Forth & Rummel, 1973). Indeed even single doses of iron insufficient to alter body iron stores may bring about decreased absorption of subsequent doses (O'Neil-Cutting & Crosby, 1987). The results with ferric maltol are thus entirely in keeping with the idea that ferric maltol donates its iron to normal regulatory uptake pathways in the intestine.

The mechanisms concerned with this regulation are not entirely elucidated but are most likely multifactorial, not only

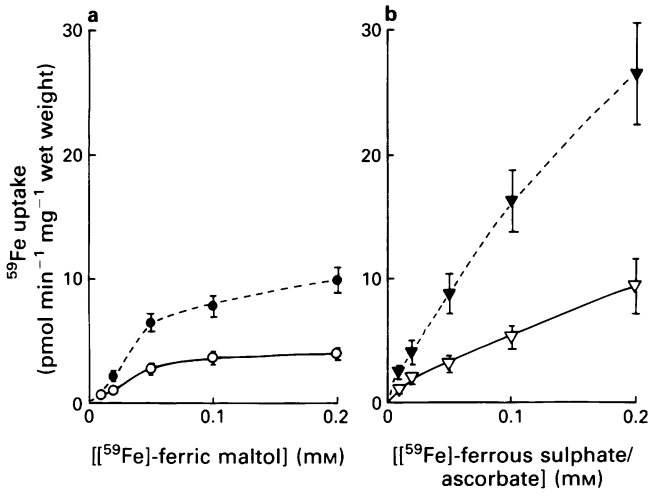


Figure 6 The effect of concentration on the initial rates of uptake of ^{59}Fe from [^{59}Fe]-ferric maltol (a, ●, ○) and from [^{59}Fe]-ferrous sulphate/ascorbate (b, ▼, ▽) into fragments isolated from the proximal part of the small intestine from rats following 45 min perfusion of the intestines with 5 mM chenodeoxycholate in saline (closed symbols) or with saline alone as control (open symbols). Each value represents the mean of data from four separate experiments, quadruplicate determinations being made in each experiment; s.e.mean shown by vertical bars. Values are given as pmol of iron min^{-1} mg^{-1} wet weight of tissue.

alterations in demand e.g. via erythropoeisis modifying iron transfer from serosal surface to plasma but also changes taking place at the luminal surface of the duodenum modifying initial uptake (Muir & Hopfer, 1985; Raja *et al.*, 1988). In the present study, significantly lower rates of iron uptake from ferric maltol were observed in duodenal fragments compared with ileal fragments isolated from animals pretreated for two weeks with ferric maltol. This contrasts with the similar rates of ^{59}Fe uptake observed in duodenal and ileal fragments taken from control animals and with the higher rates of uptake seen in the duodenal fragments isolated from iron-deficient animals. It is probably not valid to compare directly the absolute uptake rates between animals of different iron status since values are given with reference to the wet weight of the tissue and structural changes in the shape and dimensions of the epithelial cells are known to occur with altered dietary regimens (Callingham & Barrand, 1987; Powers *et al.*, 1988).

Differences in intracellular processing of iron within the intestinal mucosa may provide further regulatory control of iron absorption (Topham *et al.*, 1985). Mucosal ferritin levels are known to vary with body iron status and defects here have been identified in hereditary haemochromatosis where iron absorption is inappropriately high (Cook & Skikne, 1987). Previous studies have shown that ^{59}Fe taken up from ferric maltol into intestinal fragments isolated from normal iron-replete animals is largely sequestered by ferritin in the duodenal mucosa but that in fragments isolated from iron-deficient animals the ^{59}Fe from ferric maltol is passed on to mucosal transferrin (Callingham & Barrand, 1987).

From the results obtained so far there is no evidence to suggest that iron taken up from ferric maltol can in any way by-pass the normal regulatory mechanisms of intestinal iron absorption. Even under conditions where the intestines had been rendered more permeable by treatment with chenodeoxycholate, ^{59}Fe absorption from ferric maltol was not greatly enhanced, tissue accumulation of ^{59}Fe being not significantly higher than in control animals. As seen from the ^{59}Fe uptake studies into isolated intestinal fragments, initial rates of ^{59}Fe uptake from ferric maltol were increased after bile salt treatment. Thus factors involved in subsequent transfer of iron to the plasma were still able to control overall iron absorption from ferric maltol. Undissociated ferric maltol was not found in the plasma either 5 min or 60 min after administration even with the more permeable intestines.

Saturable uptake kinetics were still evident with ferric maltol in the intestinal fragments treated with the bile salt. What seemed to account for the increased iron uptake was the appearance of a small diffusional component. This component was present even in normal intestinal fragments during iron uptake from ferrous sulphate/ascorbate and was predominant during uptake into the bile salt-treated intestinal fragments. Several pathways have been suggested for transfer of iron from the intestinal lumen into the mucosa (Dorey *et al.*, 1988; Peters *et al.*, 1988) including a saturable carrier-mediated transfer and an intercellular permeability pathway. It is possible that the diffusional component seen in the present studies represents iron passing between cells. This is more noticeable with ferrous sulphate than with ferric maltol and increases with damage to the intestinal mucosa.

It is concluded that iron uptake and absorption from ferric maltol in the rat is probably under regulatory control. Even where there is intestinal cell damage, and increased intestinal permeability, ferric maltol does not cross the intestinal barrier as the complex. Diffusional entry of iron into the intestinal epithelial cells still appears to be limited and subsequent steps in iron transfer to the plasma still control the overall absorption. It is therefore possible that the use of ferric maltol in human subjects as an oral agent in the treatment of iron deficiency will not provide any greater potential danger of iron overload than with iron preparations currently used in clinical practice.

We wish to thank Roussel Laboratories Ltd for their generous support for this work and for the salary of M.A.B. We are particularly grateful to Mr M.A. Pratt and Dr P. Miller for their many helpful discussions. We are indebted to Angela Rudolph for her skill with the electron microscopy

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(Received March 2, 1990
Revised June 26, 1990
Accepted August 15, 1990)

Br. J. Pharmacol. (1991), **102**, 415–421 © Macmillan Press Ltd, 1991

5-Hydroxytryptamine-stimulated accumulation of 1,2-diacylglycerol in the rabbit basilar artery: a role for protein kinase C in smooth muscle contraction

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1 5-Hydroxytryptamine (5-HT) produced a concentration-dependent increase in the membrane concentration of 1,2-diacylglycerol (DG) in the rabbit isolated basilar artery, but did not stimulate the hydrolysis of membrane phosphoinositide.

2 The 5-HT-induced accumulation of DG could be blocked with the putative phospholipase C inhibitor 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC; 70 μM), but not with the protein kinase C inhibitor, 1-(5-isoquinolinesulphonyl)- 2-methyl piperazine (H7; 50 μM).

3 Direct stimulation of protein kinase C with phorbol 12,13-dibutyrate (PDBu) produced sustained smooth muscle contraction which was fairly rapid in onset and could be reversed by H7 but not by NCDC. The inactive phorbol, 4α phorbol 12,13-didecenoate, did not produce contraction in the basilar artery.

4 5-HT-induced contractions (1 nM–100 μM) were blocked or greatly reduced in the presence of the protein kinase inhibitor H7 or polymyxin B, and with the phospholipase C inhibitor, NCDC. The concentrations of these inhibitors which abolished contraction to 5-HT, did not alter smooth muscle contraction produced in response to 30 mM K^+-physiological salt solution (PSS).

5 These data suggest that DG production and the subsequent activation of PKC forms an important component of the cerebrovascular contractile response to 5-HT. As the DG does not appear to arise from membrane phosphatidylinositol, it appears that 5-HT can stimulate the production of this second messenger in cerebral arteries by a mechanism which is different from peripheral arteries.

Introduction

Agonist-induced contraction in vascular smooth muscle involves both voltage-dependent and voltage-independent membrane mechanisms, the relative contribution from each varying with different agonists (Bolton, 1979; 1986). 5-Hydroxytryptamine (5-HT) generally contracts vascular smooth muscle. In the rabbit basilar artery, 5-HT produces both smooth muscle depolarization and contraction, but the contraction is largely independent of the change in smooth muscle membrane potential (Garland, 1987). The contraction of this artery in response to both 5-HT and noradrenaline can be rapidly abolished by the removal of extracellular calcium, indicating that if intracellular calcium stores are present they are depleted more rapidly than those in peripheral arteries (Towart, 1981; McCalden & Bevan, 1981). Alternatively, for some agonists the intracellular calcium stores in this cerebral artery may not be linked to receptor activation. Overall, these observations suggest that 5-HT-induced contraction in the basilar artery may follow a receptor-operated influx of calcium, which could result from the production of secondary messengers such as D-myo-inositol 1,4,5-trisphosphate (IP_3) and sn-1,2 diacylglycerol (DG). 5-HT-induced contraction in vascular smooth muscle cells from peripheral arteries occurs, at least in part, as a consequence of the breakdown of membrane polyphosphoinositide, indicating a possible secondary messenger role for both IP_3 and DG (Nakaki *et al.*, 1985; Roth *et al.*, 1986). IP_3 has been shown to release stored intracellular calcium in vascular smooth muscle cells, as it does in other cell types, although there is no evidence to suggest that IP_3 can directly increase the movement of calcium into smooth muscle (Berridge, 1984; Somlyo *et al.*, 1985; Hashimoto *et al.*, 1986).

A role for DG in excitation-contraction coupling in vascular smooth muscle is suggested by the observation that protein kinase C (PKC) activation, usually with phorbol esters, is followed by contraction (Rasmussen *et al.*, 1984; Baraban *et al.*, 1985; Forder *et al.*, 1985; Chatterjee & Tejada, 1986; Gleason & Flaim, 1986). Smooth muscle contraction induced in this way can be reversed with PKC inhibitors such as 1-(5-isoquinolinesulphonyl)-2-methyl piperazine (H7), which can also reverse contraction to both 5-HT and phenylephrine (Khalil & Van Breemen, 1988; Clark & Garland, 1989). PKC activation has been shown to increase the influx of calcium into vascular smooth muscle (Gleason & Flaim, 1986; Sperti & Colucci, 1987; Khalil & Van Breemen, 1988), although whether this influx is sufficient to induce contraction is not clear. An alternative is that small changes in cytoplasmic calcium concentration have a permissive action at an early stage in the contractile process. This explanation would then fit with the observation that cytoplasmic calcium is not significantly raised by PKC activation in vascular smooth muscle (Sperti & Colucci, 1987; Jiang & Morgan, 1989; Ruzycky & Morgan, 1989; Sharma & Bhalla, 1989), which may reflect the increased rate of calcium efflux which follows the activation of PKC (Sperti & Colucci, 1987).

In the present study, we have investigated the possibility that both inositol phosphates and protein kinase C contribute to the contraction induced in the rabbit basilar artery with 5-HT. Some of the results have been presented in preliminary form (Clark & Garland, 1989; 1990).

Methods

Measurement of smooth muscle contraction

New Zealand white rabbits of either sex (2–4 kg) were anaesthetized by an intravenous injection of sodium pentobarbitone (60 mg kg^{-1}), and killed by exsanguination. The brain was removed, placed in physiological salt solution (PSS) at room temperature, and the basilar artery carefully removed. Endothelial cells were destroyed by either perfusing the lumen of the basilar artery with a 0.1% solution of Triton-X 100 in

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PSS, or gentle rubbing of the luminal surface. A 2 mm segment of artery was then cut and mounted in a temperature-controlled organ bath (approximate volume 1.5 ml) by passing two tungsten wires (each of 25 μm diameter) through the segment's lumen. Each wire was attached to a small plastic foot, one of which was connected to an isometric force transducer (Harvard Biosciences, 52-9529) and the other to a micrometer (Prior, code 71). The segment was superfused (3 ml min^{-1}) with PSS which had been gassed with a mixture of 95% O_2 and 5% CO_2, and warmed to 36.5 $\pm$ 0.5°C. The artery was then equilibrated at a predetermined optimum resting tension of 500 mg for 60–90 min. The absence of functional endothelial cells was demonstrated by a failure to produce relaxation with 1 μM–10 μM acetylcholine, in segments precontracted with histamine. Agonists and enzyme inhibitors were equilibrated with the superfusate before entering the tissue bath. Cumulative and non-cumulative concentration-response curves were then constructed to 5-HT. Segments were equilibrated with enzyme inhibitors H7, NCDC, and polymyxin B for 20 min before the application of 5-HT. Cocaine (3×10^{-7} M) was present in the PSS to inhibit uptake of 5-HT by nerves.

Measurement of diacylglycerol concentration

DG was assayed after conversion to phosphatidic acid with DG kinase in the presence of [^{32}P]-γ-ATP. Segments of basilar artery (2 mm in length) were gassed (95% O_2 + 5% CO_2) in PSS at 37°C for 30 min, transferred to plastic microtubes and 400 μl PSS containing 5-HT (10 nM–100 μM) added. H7 or NCDC were incubated with segments 20 min before the addition of 5-HT. The reaction was terminated at between 30 s and 5 min with 3 ml chloroform:methanol (1:2 v:v); 400 μl 1 M NaCl was added, the tubes vortexed, 1 ml chloroform plus 1 ml 1 M NaCl added, the tubes vortexed again and then centrifuged at 5000 *g* for 2 min. The upper aqueous layer was discarded and the chloroform layer evaporated under vacuum. Separate tubes were prepared containing 100 μl of standard DG concentrations (31–1000 pmol). Standards were also evaporated and 20 μl of detergent solution (n-octyl-β-glucopyranoside 5 mM, cardiolipin 7.5% w:v, in 1 mM dithylenetriaminepentaacetic acid (DETAPAC)) which had been sonicated for 5 min, was added to both standards and samples. Tubes were vortexed, sonicated for 5 min and 70 μl of a solution containing 10 μl *E. coli* diacylglycerol kinase (Amersham International); 50 μl 0.1 M imidazole/HCl buffer pH 6.6 containing 0.1 M NaCl, 25 mM $MgCl_2$ and 2 mM EGTA; 10 μl 0.02 M dithithreitol added to each tube. The reaction was initiated by adding 10 μl of [^{32}P]-γ-ATP in a solution of 5 mM ATP in 100 mM imidazole/HCl containing 1 mM DETAPAC, to give 1 μCi [^{32}P]-γ-ATP in each tube. Tubes were vortexed and incubated at 25°C for 30 min. The reaction was terminated with 20 μl of 1% perchloric acid and 450 μl chloroform:methanol (1:2 v:v); the tubes vortexed, left at room temperature for 10 min and centrifuged (2000 *g*) for 1 min. This procedure was repeated after adding 150 μl chloroform and 150 μl perchloric acid.

The aqueous layer was discarded, 1 ml of perchloric acid added followed by further vortexing and centrifugation. This was repeated twice, with the aqueous layer discarded each time.

In the standard tubes, 150 μl of the lower phase was added to 10 ml of water in scintillation vials. Further purification was not necessary as ^{32}P was predominantly present in phosphatidic acid. The lower phase from the experimental tubes was then added to Amprep columns (Amersham International) which had been preconditioned with hexane, and washed with 2 ml chloroform followed by 2 ml 15% ethyl acetate in hexane. [^{32}P]-phosphatidic acid was eluted from the columns with 2 ml of chloroform:methanol:acetic acid (65:10:10) and made up to a final volume of 10 ml in water. Samples were then counted for 4 min on a Beckman LKB 1219 counter. The DG produced was calculated from a standard curve of log d.p.m. against log DG standard pmol/tube. Results were expressed as the amount of DG formed (pmol) per mg dry tissue weight, and changes expressed relative to control values from tissue not exposed to 5-HT. The assay was validated by running eluted samples (basal and plus 1 μM 5-HT) and a standard (formed by the conversion of 1 nmol DG with DG kinase) on silica gel plates with chloroform:methanol:acetic acid as solvent. The plates were then dried and visualized by autoradiography. Each track (apart from basal) ran to a single peak with an R_F value between 0.3 and 0.4 (Figure 1). This corresponds to the R_F for phosphatidic acid which is 0.35 with this solvent.



Figure 1 Autoradiograph of a thin-layer chromatography plate (silica-gel) run with chloroform:methanol:acetic acid as the solvent. Tracks ran to a single peak with an R_F value between 0.3 and 0.4, which corresponds to the known value for phosphatidic acid (0.35) with this solvent. (a) Standard 1,2-diacylglycerol (DG) sample (0.12 nmol) which was not run through an Amprep column. Note contamination at the origin. (b) Sample stimulated with 1 μM 5-hydroxytryptamine showing a single peak of radioactivity. (c–e) Standard DG samples (0.12, 0.5 and 1 nmol) after conversion to phosphatidic acid with DG kinase. In (b–e) samples were passed down Amprep columns and do not show any contamination at the origin.

Measurement of inositol phosphates

The method employed to measure the hydrolysis of polyphosphoinositide was essentially that described by Donaldson & Hill (1985). Segments (2 mm in length) of rabbit basilar artery or thoracic aorta were incubated in PSS for 30 min at 37°C, under an atmosphere of 95% O_2/5% CO_2. The segments were then transferred to PSS (basilar segments, 1 ml; aorta, 5 ml) containing 50 μCi [^{3}H]-myo-inositol, incubated at 37°C and bubbled every 20 min (95% O_2/5% CO_2) for a total of 3 h. Segments were washed in normal PSS (20 ml) for 1 h at 37°C, under an atmosphere of 95% O_2/5% CO_2, and then transferred to plastic insert tubes containing PSS (190 μl) and lithium chloride (10 mM final concentration). The tubes were gassed with 95% O_2/5% CO_2, capped and incubated for a further 30 min at 37°C. 5-HT (1 μM–1 mM) or sodium fluoride (15 mM) was added in PSS (10 μl), the tubes gassed, recapped and subsequently gassed every 20 min. The reaction was terminated after 1 h by the addition of 0.94 ml chloro-

form:methanol:HCl (100:200:1), and the tubes vortexed. Water (0.31 ml) and chloroform (0.31 ml) were added and the vortexing repeated; 0.8 ml of the upper aqueous phase was removed, made up to 3 ml in water and added to columns containing 0.5 ml Dowex-1 in the formate form (0.25 ml Dowex-1: 0.25 ml water). Ten ml of 5 mM myo-inositol was then passed through the columns to remove free [^{3}H]-myo-inositol. Water-soluble phosphates were then eluted into glass vials with 1.5 ml ammonium formate (1 M): formic acid (0.1 M), added to Optiphase scintillant (12 ml) and counted. The amount of [^{3}H]-myo-inositol which remained incorporated was measured after evaporating the lower organic phase, and adding scintillant (4 ml).

To allow comparison of data from the basilar artery and aorta, and to correct for variation in [^{3}H]-inositol accumulation and segment weight between experiments, [^{3}H]-inositol phosphate formation was calculated as a percentage of the total amount of [^{3}H]-inositol incorporated into each individual artery segment.

$$\frac{\text{d.p.ms in aqueous fraction} \times 100}{\text{d.p.ms in organic fraction} + \text{d.p.ms in aqueous fraction}}$$

The mean total d.p.m. of the organic phase was 10,133 ± 929 in the basilar artery ($n = 34$), 86,804 ± 10,433 in the rabbit aorta ($n = 12$) and 3,084 ± 552 ($n = 23$) in the rat aorta.

Chemicals and solutions

The chemicals used were: acetylcholine chloride; 5-hydroxytryptamine creatinine sulphate; histamine acid phosphate; 1-(5-isoquinolinesulphonyl)-2-methyl piperazine; 2-nitro-4-carboxyphenyl *N,N*-diphenyl carbamate; phorbol 12,13-dibutyrate and sodium fluoride, all Sigma. Stock solutions were made up in deionised water, except for H7, NCDC and PDBu which were initially dissolved in dimethylsulphoxide (final concentration <0.4%). Subsequent dilutions were made in PSS. Chemicals used in the DG assay, including isotope, were purchased from Amersham International U.K. [^{3}H]-myo-inositol (20 Ci mmol^{-1}) was obtained from New England Nuclear and passed through a column of Dowex (Dowex-1:formate form) before use, to remove polar contaminants. Dowex-1 (×8, chloride form 100–200 mesh) was purchased from Sigma, and formated with sodium formate (1 M).

Displacement of chloride from the anion-exchange column was monitored by adding aliquots of the elutant to acidified silver nitrate.

Physiological salt solution (PSS) used in the experiments had the following composition (mM): NaCl 121.8, $NaHCO_3$ 25.5, KCl 5.2, $MgSO_4$ 1.2, $CaCl_2$ 1.6, disodium EDTA 0.027, ascorbate 0.114 and glucose 9.4. Potassium-rich solutions were prepared by direct replacement of NaCl with KCl.

Results

5-Hydroxytryptamine-stimulated 1,2-diacylglycerol production

5-HT produced significant, concentration-dependent increases in the membrane concentration of DG in the basilar artery. The basal accumulation of DG (720 ± 165 pmol mg^{-1} tissue dry weight) varied somewhat between individual experiments. However, the percentage increase in DG which was produced in response to any particular concentration of 5-HT was very comparable between all the experiments. Figure 2a shows the effect that different concentrations of 5-HT had on basal DG production in the basilar artery. The changes shown were all produced after 30 s of exposure to the various concentrations of 5-HT tested. With longer periods of exposure to 5-HT (2 and 5 min), the increases in DG concentration were not as marked.

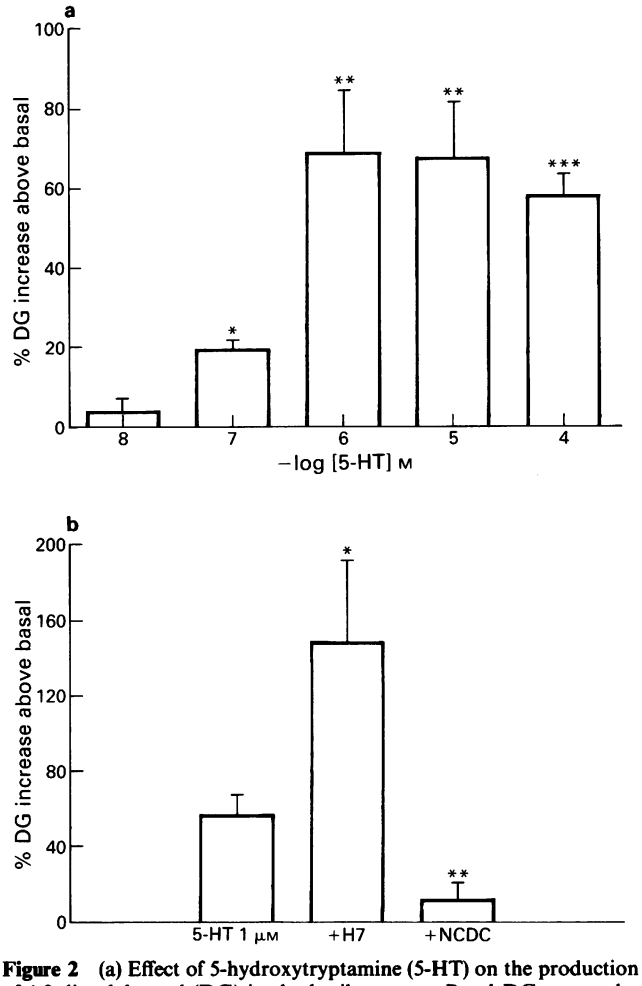


Figure 2 (a) Effect of 5-hydroxytryptamine (5-HT) on the production of 1,2-diacylglycerol (DG) in the basilar artery. Basal DG accumulation was 720 pmol mg^{-1} tissue. DG was determined by the conversion to and subsequent assay of [^{32}P]-phosphatidic acid, as described in the methods. Values are the mean ± s.e.mean of $n = 6$–16 duplicate observations from a total of 25 rabbits; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (b) Modification of 5-HT (1 μM)-stimulated increases in DG production by 1-(5-isoquinolinesulphonyl)-2-methyl piperazine (H7, 50 μM) and 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate (NCDC, 70 μM). Each column is the mean of 9 observations on tissue from 4 rabbits, and is expressed as the percentage change from basal DG production; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$.

Incubation of artery segments with 70 μM NCDC significantly reduced the 5-HT-stimulated increase in DG accumulation, whereas in the presence of H7 (50 μM) accumulation was increased Figure 2b.

Effect of 5-hydroxytryptamine on inositol phosphate accumulation

5-HT (1–100 μM) failed to stimulate any significant increase in the intracellular formation of inositol phosphates in the basilar artery, although significant increases to 5-HT could be produced in both the rabbit and rat aorta (Figure 3). All the experiments were performed in the presence of 10 mM lithium, utilized as an uncompetitive inhibitor of inositol 1-phosphatase.

This concentration of lithium did not inhibit either the increase in DG ($n = 3$) or the smooth muscle contraction ($n = 2$) produced in response to 5-HT.

The basal formation of inositol phosphates was 1370 ± 309 d.p.m. in the basilar artery ($n = 8$), 175 ± 39 d.p.m.

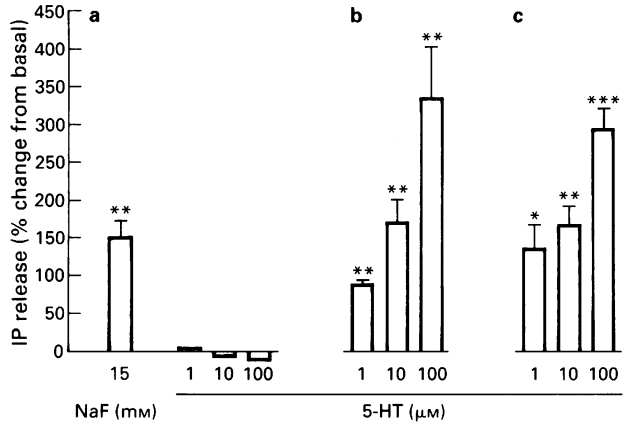


Figure 3 Effect of 5-hydroxytryptamine (5-HT) on the intracellular formation of inositol phosphates in (a) rabbit basilar artery (b) rat aorta (c) rabbit aorta. Points are the mean of duplicate observations in (a) 8, (b) 3 and (c) 4 animals; vertical bars show s.e.mean. The amounts of tissue used in (a) and (b) were not significantly different. Data are expressed as percentage change from the basal IP release from each artery, values for which are listed in the results section. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

in the rat aorta ($n = 6$) and 150 ± 58 d.p.m. in the rabbit aorta ($n = 7$). The mean dry weights of the tissue used in each series were 0.25 ± 0.02 mg for the basilar artery, 0.24 ± 0.02 mg for the rat aorta and 10.6 ± 1.7 mg for the rabbit aorta. The value for basal formation of inositol phosphates in the rabbit aorta has been standardized for a tissue weight of 0.25 mg, allowing a direct comparison with the other two arteries.

One possible explanation for the observations with the basilar artery, is that the technique used to measure inositol phosphate accumulation is not of sufficient sensitivity to detect changes in the small amount of basilar artery tissue. This explanation is unlikely, however, as significant stimulation of inositol phosphate production could be detected with 5-HT in the same amount of tissue from the rat aorta. The 5-HT-stimulated intracellular formation of inositol phosphates in the rat aorta was similar in magnitude to the formation obtained in this vessel by Turla & Webb (1990).

An alternative possibility is that rabbit vascular smooth muscle cells do not hydrolyse membrane polyphosphoinositides in response to 5-HT. Again, this is clearly not the case, as 5-HT induced significant accumulation of total inositol phosphates in the rabbit aorta, although these increases were somewhat less than those obtained in response to other agonists in non-vascular smooth muscle (Donaldson & Hill, 1985; Chilvers *et al.*, 1989). In addition, the relatively high basal formation of inositol phosphates in basilar artery, and the significant increase in release which was produced in the presence of NaF, shows that cells in this artery can turn over membrane phosphoinositide.

Direct activation of protein kinase C with phorbol 12,13-dibutyrate (PDBu)

Segments of basilar artery contracted in response to PDBu. The contraction was fairly rapid in onset (68–100 s; $n = 9$) and sustained, continuing throughout the duration of an experiment (maximum around 240 min), even if PDBu was removed from the superfusate after contraction had developed. The threshold concentration of PDBu required for contraction varied between 0.1 nM and 10 nM. The contractions were concentration-dependent, with 10 μM PDBu producing a maximum contraction of 873 ± 154 mg ($n = 11$). Maximal contractions with 10 μM PDBu were completely reversed in the presence of 50 μM H7 ($n = 5$), over a period of about 80 min. The relaxation could be reversed and contraction re-established by the removal of H7 during this period. Contraction did not decline during the course of these experiments in time-control segments which were stimulated with PDBu but not exposed to H7. The inactive phorbol, 4α phorbol 12,13-didecenoate (10 μM, $n = 3$) did not elicit a response from the basilar artery.

Effect of H7, polymyxin B and NCDC on 5-HT-induced contraction

5-HT produced concentration-dependent contraction in the basilar artery, the overall size of contraction varying between individual arteries. The shape of the concentration-response curves were, however, consistent between each series of experiments. Figure 4 shows the mean responses obtained in the presence of different concentrations of the protein kinase C inhibitor, H7. The curve could be split into two significantly separate components and was similar to the responses to 5-HT reported in cerebral arteries by other workers (Young *et al.*, 1986; Cain & Nicholson, 1989).

The first phase of contraction peaked with between 10 and 100 μM 5-HT, and the second phase with concentrations above 1 mM. The maximum contraction obtained with 5-HT was 66 ± 5% of the contraction produced in response to 100 mM K-PSS in this series of experiments.

Both H7 and polymyxin-B blocked contraction of the basilar artery to 5-HT. The extent of this inhibition increased with increasing concentrations of each of the inhibitors. In the presence of the highest concentration of H7 (50 μM) used against 5-HT, contraction could only be produced with concentrations of 5-HT above 100 μM and the responses to these high concentrations were greatly reduced from control levels (Figure 4). H7 in concentrations up to 50 μM did not reduce the contraction produced in response to 30 mM K^+-PSS, which indicates that H7 is not blocking contraction by acting on for example, myosin light chain kinase which can be activated by the sustained rise in intracellular calcium which is produced by potassium (Jiang & Morgan, 1989).

The mean contraction produced in response to 30 mM K-PSS, 1960 ± 81 mg, was not significantly altered (2056 ± 158 mg) in the presence of 50 μM H7 ($n = 8$). However, higher concentrations of H7 did depress contraction to potassium, 30 mM K-PSS producing a response of only 803 ± 114 mg ($n = 5$) in the presence of 100 μM H7.

A similar pattern of inhibition was produced in the presence of polymyxin B, although overall this inhibitor was not as potent as H7. Contraction to 5-HT persisted, although it was

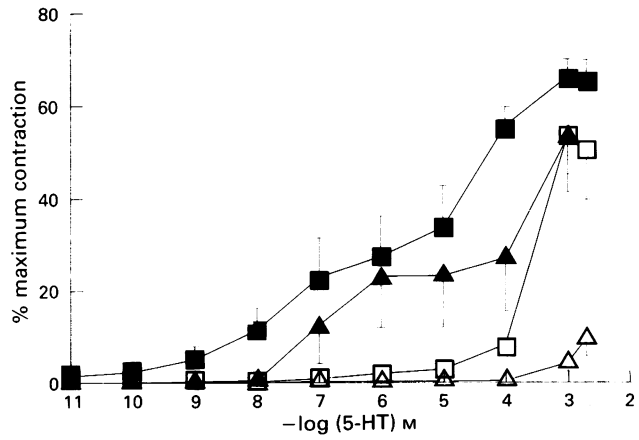


Figure 4 Depression of the contractile response to 5-hydroxytryptamine (5-HT) in the rabbit basilar artery in the presence of different concentrations of the protein kinase C inhibitor 1-(5-isoquinolinesulphonyl)-2-methyl piperazine (H7). Responses are expressed as a % of the artery response to 100 mM K^+-PSS; control (■); 1 μM H7 (▲); 10 μM H7 (□); 50 μM H7 (△). Points are the mean with s.e.mean shown by vertical bars, from 7 experiments.

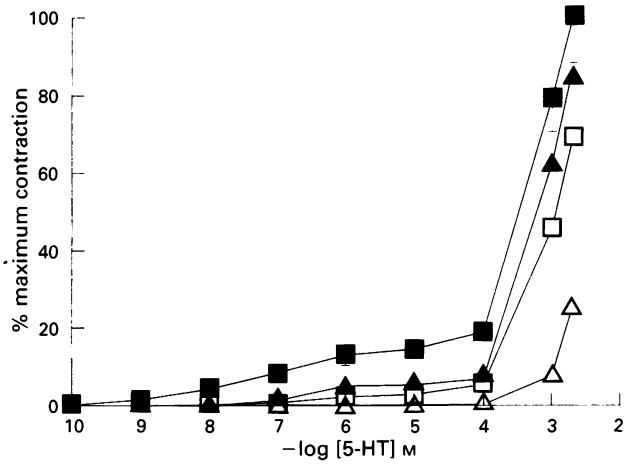


Figure 5 Depression of the contractile response to 5-hydroxytryptamine (5-HT) in the rabbit basilar artery in the presence of different concentrations of 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC). Responses are expressed as a % of the artery response to 100 mM K^+-PSS: control (■); 1 μM NCDC (▲); 10 μM NCDC (□); 70 μM NCDC (△). Points are the mean from 5 experiments.

markedly reduced. 5-HT (100 μM) produced a mean response of 611 ± 221 mg in the presence of 10 μM polymyxin B (n = 7), and 146 ± 35 mg (n = 5) with 100 μM, compared to a control response of 1348 ± 153 mg (n = 7). Higher concentrations of polymyxin B did not further reduce the 5-HT-induced contraction and polymyxin B did not alter the contraction produced in response to raised extracellular potassium. In these experiments 30 mM K-PSS produced a mean contraction of 2771 ± 273 mg in the absence, and 2750 ± 340 mg in the presence of 100 μM polymyxin B (difference NS; n = 5).

2-Nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC), a putative inhibitor of phospholipase C (Walenga *et al.*, 1980), blocked contraction to 5-HT (Figure 5). The inhibition increased with increasing concentrations of NCDC, and as with the PKC inhibitors, contraction persisted, although in a reduced form, with the highest concentrations of 5-HT. Contractions produced in response to 30 mM K^+-PSS were not altered in the presence of NCDC.

Discussion

These data indicate that protein kinase C plays a major role in the contractile response of the basilar artery to 5-HT. The most direct evidence for this is based along two lines. First, the major physiological activator of PKC, DG, is produced rapidly and over the same 5-HT concentration-range as smooth muscle contraction. The fact that the production of DG reaches a maximum with 1 μM 5-HT, suggests that PKC activation is involved at an early stage in the initiation of smooth muscle contraction. Even low concentrations of 5-HT will stimulate significant DG production and the subsequent PKC activation will make a major contribution to the ensuing contraction. This idea is supported by the susceptibility of the contractions to low concentrations of 5-HT, to the inhibitors H7 and polymyxin B. Contraction to higher concentrations of 5-HT will receive a proportionally greater contribution from other mechanisms, such as the opening of voltage-dependent calcium channels caused by the significant membrane depolarization produced by high concentrations of 5-HT (Garland, 1987). Contraction induced by other mechanisms will be superimposed on the PKC response. Second, the fact that the phorbol ester PDBu was able to contract the basilar artery shows that protein kinase C activation is linked to smooth muscle contraction in this vessel. Phorbol esters activate protein kinase C, both *in vitro* and *in vivo*, by interacting with the same membrane site – or one very closely related – as DG (Nishizuka, 1984). PKC represents a large family of proteins, with variation in enzymatic characteristics, tissue expression and intracellular location between the multiple sub-species (Nishizuka, 1988; 1989). Therefore, the possibility that different sub-species are linked to different stimulus-responses in cells cannot be discounted. Together with the fact that phorbol esters produce a widespread activation of PKC, possibly in a heterogeneous fashion (Beh *et al.*, 1989), means that apart from implicating PKC activation in smooth muscle contraction, a quantitative comparison cannot reasonably be made between the PDBu- and 5-HT-induced contractions.

Further evidence supporting a role for PKC in the contractile responses to 5-HT is provided by the experiments with H7, polymyxin B and NCDC. NCDC completely blocked the 5-HT-stimulated accumulation of DG, presumably reflecting the inhibition of phospholipase C. This compound blocks the phospholipase C isolated from blood platelets, and the 5-HT-induced hydrolysis of phosphoinositide in the rat aorta (Walenga *et al.*, 1980; Nakaki *et al.*, 1985). The same concentration of NCDC produced almost total block of contraction to 5-HT in the present study, but did not depress responses of the basilar artery to potassium, ruling out a non-specific action of NCDC on the contractile process. In contrast, H7 did not reduce the production of DG, but rather increased the amount of DG produced in response to 5-HT. This indicates a negative feedback action of PKC on phospholipase C activity, an interaction which has been described in other cell types (Berridge, 1987). Although H7 did not reduce the production of DG it did block contraction to both 5-HT and PDBu, indicating a direct inhibitory action on PKC. As with NCDC, both H7 and polymyxin B failed to block the contraction to potassium, which results from the activation of myosin light chain kinase by persistent raised intracellular Ca^{2+} (Jiang & Morgan, 1989). Some caution is required, however, as neither of the protein kinase C inhibitors are totally selective, although both H7 and polymyxin B are more active against protein kinase C than the Ca^{2+}-calmodulin-dependent kinases such as myosin light chain kinase (MLCK), and both have varying actions on adenosine 3′:5′-cyclic monophosphate (cyclic AMP)- and guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-dependent kinases. The K_i value for H7 with protein kinase C and MLCK is 6 μM and 97 μM, respectively, whereas the K_i values with cyclic AMP- and cyclic GMP-dependent kinases are 3 μM and 5.8 μM (Hidaka *et al.*, 1984). Polymyxin B shows a similar relative selectivity for protein kinase C over Ca^{2+}-calmodulin-dependent kinases, with K_i values of 1.8 μM and 7 μM against protein kinase C extracted from heart and pancreas, and with respective values of 17 μM and 950 μM against Ca^{2+}-calmodulin-dependent kinases. In addition, polymyxin B has no effect on cyclic AMP- and cyclic GMP-dependent kinases (Mazzei *et al.*, 1982; Wrenn & Wooten, 1984). In view of these variations in K_i values between different tissues, it is impossible to predict what effect H7 and polymyxin B will have on different kinases in the basilar artery. However, the fact that H7 totally reversed the effect of protein kinase C stimulation with PDBu in the basilar artery and at the same concentration inhibited the contraction to 5-HT, does suggest that these agents act via a common mechanism. This idea is supported by the reduction in 5-HT-induced contraction which was obtained in the presence of polymyxin B. This agent inhibits protein kinase C by a different mechanism from H7, which competes with ATP for the enzymes co-substrate site (Hidaka *et al.*, 1984). The amphipathic antibiotic polymyxin B, by its ability to bind phospholipids, is thought to interfere with the interaction of the enzyme with membrane phospholipids such as phosphatidylserine, which are necessary for the activation of PKC (Mazzei *et al.*, 1982; Nishizuka, 1984). That the highest concentrations of H7 and polymyxin B used against 5-HT did not depress the contraction to raised extracellular potassium, argues against any significant inhibition of Ca^{2+}-calmodulin-

dependent kinases. An effect due to the inhibition of cyclic AMP- or cyclic GMP-dependent kinases is also unlikely for two reasons.

First, raised smooth muscle levels of both cyclic AMP and cyclic GMP are usually associated with smooth muscle relaxation not contraction. Second, if cyclic AMP- and cyclic GMP-dependent kinases were involved in the responses to 5-HT, H7 and polymyxin B might be expected to show a marked difference in their inhibitory effects, as polymyxin B is thought not to inhibit either group of enzymes (Mazzei *et al.*, 1982).

As these results suggest an important role for protein kinase C in responses of the basilar artery smooth muscle to 5-HT, the failure of 5-HT to stimulate the hydrolysis of membrane phosphatidylinositol was surprising, particularly as available evidence clearly shows that 5-HT can hydrolyse smooth muscle phosphoinositide in smooth muscle from peripheral arteries. One explanation may be, that although vascular 5-HT receptors are linked to polyphosphoinositide hydrolysis in the rat (Nakaki *et al.*, 1985; Roth *et al.*, 1986), this is not the case in the rabbit. This appeared not to be the case as 5-HT stimulated significant, concentration-dependent intracellular formation of inositol phosphates in the rabbit aorta. This increase, was less than the approximate 6 fold accumulation of inositol phosphates produced by 5-HT in the rat aorta by Nakaki *et al.* (1985), but similar to the increase reported by Turla & Webb (1990) in the aorta of WKY rats. This discrepancy may reflect failure to approach or achieve steady-state labelling with [^{3}H]-inositol in the rat aorta by Nakaki *et al.* Chilvers *et al.* (1989), have shown that variable labelling of the agonist-sensitive inositide pool in tracheal smooth muscle can alter the amount of labelled inositol phosphate accumulation. Additional evidence that phospholipase C is linked to the hydrolysis of phosphatidylinositol in the rabbit aorta and basilar artery is provided by the NaF-stimulated accumulation of inositol phosphates in these arteries. NaF stimulates phospholipase C and the subsequent production of IP_3 and DG, by activating a GTP-binding protein (Blackmore *et al.*, 1985). In addition, the basal formation of inositol phosphates was much higher in the basilar artery compared to both the rabbit and rat aorta. This suggests that the turnover of phosphoinositides may in fact be more marked in cerebral arteries.

Overall, these data provide evidence that the 5-HT-induced formation of DG in the basilar artery is from a membrane phospholipid(s) other than phosphatidylinositol. Recent evidence indicates that DG can be formed from either phosphatidylcholine or phosphatidylethanolamine. Bocckino *et al.* (1965) stimulated DG production in isolated hepatocytes with various agonists, and by subsequent analysis of the fatty acid side chains showed that the DG was partially derived from sources other than the inositol phospholipids. Subsequently, it has been shown that a number of different agents, including platelet-derived growth factor, bombesin and vasopressin can stimulate the hydrolysis of phosphatidylcholine in different cell types, including a smooth muscle cell line, by activating phospholipase C (Besterman *et al.*, 1986; Irving & Exton, 1987; Muir & Murray, 1987; Grillone *et al.*, 1988). In isolated hepatocytes, breakdown of phosphatidylcholine by a phospholipase C appears to be linked to the activation of a GTP-binding protein (Irving & Exton, 1987). This is of interest, as HA-*ras* transformed fibroblasts show increased levels of DG with no detectable change in the levels of inositol phosphates. However, the cells do have increased steady-state levels of phosphorylcholine and phosphorylethanolamine, suggesting that the DG is produced by phospholipase C-mediated breakdown of phosphatidylcholine and phosphatidylethanolamine (Lacal *et al.*, 1987). The *ras* p21 proteins bind guanine nucleotides and hydrolyse GTP, and may therefore activate a phospholipase C for phosphatidylcholine in these cells.

If 5-HT does release DG from phospatidylcholine and phosphatidylethanolamine by activating a phospholipase C in the basilar artery, this would then explain the susceptibility of the contractions to the phospholipase C inhibitor NCDC.

Whatever the source for DG is in the basilar artery, these results show that the mechanism of contraction to 5-HT is very different from that in peripheral arteries, where 5-HT will stimulate the hydrolysis of phosphatidylinositol. This difference is associated with different receptor types in the respective arteries. 5-HT-induced contractions in peripheral arteries are generally mediated by 5-HT_2 receptors, whereas the 5-HT receptors present in cerebral arteries are different and fit the general criteria for '5-HT_1-like' receptors (Bradley *et al.*, 1986a,b).

In the rabbit basilar artery, the receptor apparently does not correspond to the various sub-types in this group viz. 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} (Parsons & Whalley, 1989). Whether any of these differences from smooth muscle in peripheral arteries can explain the complex form of the 5-HT response-curve in the basilar artery is not clear. Responses of cerebral arteries to 5-HT may reflect the presence of more than one receptor sub-type or multiple mechanisms for inducing smooth muscle contraction, and is currently under investigation. Unique receptor mechanisms for 5-HT-induced contractions in cerebral arteries offer the obvious possibility of developing selective antagonists and inhibitors for 5-HT, which would be of potential application in the numerous cerebrovascular disorders where responses to 5-HT have been implicated.

We are grateful to the Wellcome Trust for financial support. C.J.G. is a Nuffield Foundation Science Research Fellow.

We would like to thank Dr R.B. Barlow, University of Bristol and Dr B.J. Chapman for advice on aspects of data analysis.

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(Received June 6, 1990
Revised October 11, 1990
Accepted October 19, 1990)

Br. J. Pharmacol. (1991), **102**, 422–428 © Macmillan Press Ltd, 1991

The mechanism of action of endothelin in human lung

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1 The peptides endothelin-1 (ET-1) and endothelin-2 (ET-2) elicited potent and sustained contractions of human isolated bronchus and pulmonary artery.

2 ET-1 is one of the most potent contractile agonists investigated in these tissues with an EC_{50} value of 18.3 nM (95% confidence interval: 12.9, 25.9 nM; $n = 26$) in bronchus and 3.2 nM (95% confidence interval: 0.4, 23.9 nM; $n = 5$) in the arterial preparation.

3 ET-1 is 2.5 times more potent than ET-2 in both the airway and vascular tissues, and both forms of the peptide have geometric mean EC_{50} values 5 times greater in the isolated bronchial tissue than in the pulmonary artery.

4 Neither pretreatment with the voltage-dependent calcium (VDC) channel antagonist verapamil (10 μM) nor with indomethacin (25 μM) significantly altered the response curve to ET-1 in human isolated bronchus. Removal of calcium from the Krebs-Henseleit solution did not affect ET-1-induced responses.

5 Specific binding on the smooth muscle of human airway and pulmonary arterial tissue to both ET-1 and ET-2 was detected in autoradiographic studies. There appeared to be no difference between the peptides in the location nor the density of binding sites.

6 We conclude that contraction of human bronchial tissue by ET-1 is not dependent upon influence of extracellular calcium nor release of prostaglandins or thromboxane A_2. It is likely that the action of ET-1 in this tissue is due to binding of this peptide to specific receptors located on the smooth muscle.

Introduction

Endothelin is a 21 amino acid peptide which has extremely potent vasoconstrictor properties and is produced by vascular endothelial cells (Yanagisawa *et al.*, 1988). It is now apparent that there are three forms of endothelin (endothelin-1 (ET-1), ET-2 and ET-3), hence there is the possibility of more than one endothelin receptor subtype (Inoue *et al.*, 1989). It may be that endothelin, the most potent vasoconstrictor substance yet isolated, has properties which are relevant to respiratory diseases such as asthma. In a recent report, markedly elevated levels of endothelin were detected in the lavage fluid of a patient in 'status asthmaticus' (Nomura *et al.*, 1989). Little is known of the conditions under which endothelin is released but hypoxia (Hiley, 1989) or a decrease in temperature (Fyhrquist *et al.*, 1990) may be provoking stimuli, both of these factors being associated with asthma. Events occurring in the vasculature (where endothelin is probably produced), may affect not only the calibre of the airways themselves, but also local concentrations of bronchoactive mediators.

The effects and mechanism of action of ET-1 on the vasculature of many species have been investigated in some depth. Ohtsuka *et al.* (1989) have detailed the contractile action of ET-1 on human pulmonary artery strips and have also reported binding sites for this peptide on the smooth muscle of these vessels. Intravenous or aerosol administration of endothelin to guinea-pigs results in bronchoconstriction (Braquet *et al.*, 1989). *In vitro* studies have shown that both ET-1 and ET-3 induce contraction of guinea-pig isolated trachea and bronchus (Maggi *et al.*, 1989; 1990; Battistini *et al.*, 1990), but the mechanism by which this contraction is generated remains unclear. There are as yet no quantitative data available on the functional effects of endothelin in human isolated airways, although there is an isolated report of the contractile action on human bronchus (Uchida *et al.*, 1988).

The mode of action of endothelin has attracted considerable interest. Results of initial studies carried out in vascular tissue indicate that endothelin may act as an endogenous ligand for voltage-dependent calcium (VDC) channels, while others have found that it promotes calcium entry into the cell via activation of non voltage-dependent rather than voltage-dependent calcium channels. The results of a recent study suggest that contraction of guinea-pig trachea and bronchus may be due to release of thromboxane A_2 (Battistini *et al.*, 1990).

In the present study, we compared the contractile effects of ET-1 and ET-2 on human isolated pulmonary arterial and bronchial segments and used *in vitro* autoradiography to identify possible binding sites for both of these forms of endothelin in these tissues. In addition, we examined the contractile activity of ET-1 in human bronchus under conditions of calcium exclusion and in the presence of the VDC antagonist, verapamil, to investigate whether ET-1 acts as a modulator of the VDC channel in these tissue preparations. We also studied contractions generated in the presence of the cyclo-oxygenase inhibitor indomethacin, to determine if the action of ET-1 is due to the liberation of prostaglandins or thromboxane A_2.

Methods

Functional studies

Tissue was obtained after removal from patients undergoing lung resection for carcinoma as previously described (Armour *et al.*, 1986; Black *et al.*, 1986). Consent had been obtained prior to surgery and the protocol had been approved by the Medical Ethical Review Committee of The University of Sydney. Segments of bronchus and pulmonary artery 3–5 mm in internal diameter were dissected free of surrounding tissue and cut into rings measuring approximately 3–4 mm in length.

Subsequently, paired tissue preparations were suspended on stainless steel hooks under an optimal load of 1 g (bronchus) or 1–1.5 g (artery) (Armour *et al.*, 1988) in jacketed organ baths containing Krebs-Henseleit solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and D-glucose 11.1) maintained at 37°C and continuously aerated with 5% CO_2 in 95% O_2. During a 1 to 3 h equilibration period, the tension was readjusted to the load originally applied and the bath fluid was changed at 15–20 min intervals.

Changes in tension in response to the addition of an agonist to the bath were measured isometrically with Grass FT03

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transducers and were recorded on Grass polygraphs. Whenever possible the tissue pairs were studied in duplicate.

Bronchial tissue

Studies of the comparative potency and efficacy of endothelin-1 and endothelin-2 Initially, comparisons of two of the forms of endothelin were made by obtaining cumulative concentration-response curves (10 pM to 0.3 μM) in parallel in four tissues from the same patient. When baseline tone was stable, a bolus dose of acetylcholine (1 mM) was added to the bronchial preparations. When the contractile response to acetylcholine had reached a maximum, the tissues were washed repeatedly until a stable baseline tone had been re-established. Cumulative concentration-response curves were then obtained to either ET-1 or ET-2 in each tissue pair.

The effect of verapamil Following establishment of baseline tone, one of each of the pair of tissues received 10 μM verapamil and 30 min later the ET-1 cumulative concentration-response curve was started. After completion of the concentration-response curve, and with no intervening washing of the tissues, a bolus dose of carbachol (1 mM) was administered to each tissue.

The effect of calcium-free Krebs-Henseleit solution In this series of experiments, the contractile responses to both ET-1 and histamine were elicited in the presence and absence of extracellular calcium. The maximal contractile response generated as a result of the addition of 1 mM acetylcholine was determined as above. When the maximal response had been attained, half of the tissues were washed twice (at 15 min intervals) with Krebs-Henseleit solution from which the calcium had been omitted and to which 1 mM EGTA had been added. Subsequently, the bathing solution was changed to one with no calcium and no EGTA and the tissues were washed three times with this solution. The remaining tissues, which acted as controls, were washed as previously with Krebs-Henseleit solution of the usual composition. When baseline tone was stable in all tissues, cumulative concentration-response curves to either ET-1 or histamine were started.

The effect of indomethacin In these experiments, following addition of 1 mM acetylcholine and washout as above, indomethacin (25 μM) was added to half of the tissues, the other half being left as controls. After 30 min and when tone was stable, cumulative concentration-response curves to ET-1 were started in all tissues.

Vascular tissue

In this series of experiments, a comparison was made of the relative potency and efficacy of ET-1 and ET-2 in pulmonary arterial tissue. Following addition of 1 mM noradrenaline the tissues were washed and when baseline tone had been reestablished, cumulative concentration-response curves were obtained (10 pM to 0.3 μM) for ET-1 and ET-2 in paired tissues.

Autoradiographic studies

To investigate the location of binding sites for ET-1 and ET-2, autoradiographic studies were carried out on human bronchus ($n = 6$) and pulmonary artery ($n = 10$). The source of tissue for these experiments was generally the same as that for the functional studies. In some cases ($n = 2$) however, bronchial tissue was obtained at autopsy. Specimens were frozen in Tissue-Tek embedding medium over liquid nitrogen and stored at -70°C until required. Twelve serial sections of 10 μM thickness from each specimen were cut on a cryostat and thaw mounted onto glass slides with a gelatin coating. Slides of adjacent serial sections were formed into six pairs, one of each pair being used to designate 'total binding', while the other was used to estimate 'non-specific binding'. After three 5 min washes in Tris-HCl buffer (pH 7.4) containing NaCl 100 mM, $MgCl_2$ 5 mM, bacitracin 40 mg ml^{-1} and bovine serum albumin 0.3%, six slides from each patient (i.e. those labelled 'total binding') were incubated for 30 min at 21°C in buffer containing 25 pM [^{125}I]-ET-1 (specific activity: 1500 Ci mmol^{-1}).

'Non-specific binding' was assessed in the presence of 1 μM unlabelled ET-1, this concentration being in excess to ensure that all ET-1 binding sites were occupied by the unlabelled agonist. The six remaining slides from each patient were placed in this solution for 30 min.

At the end of the incubation period, the slides were washed in ice-cold Tris buffer, then in water and dried under a stream of cold air. The slides were placed in a dessicator and kept in the refrigerator overnight. Glass coverslips coated with Amersham LM-1 liquid emulsion (diluted 1 : 1 with water) were then placed in apposition to the slides the following day. The slide assemblies were stored in light-proof X-Ray boxes at 4°C for 14–16 days.

At the conclusion of this time, the slide/coverslip assemblies were developed with Ilford Phenisol developer and the sections stained with methyl-green pyronin. After dehydration of the sections in increasing concentrations of alcohol, the coverslips were attached to the slides with mounting medium.

The various histological structures were first identified under bright-field microscopy at which time the light condenser was changed to a dark-field one and the endothelin binding sites were visualised as illuminated silver grains over the tissue.

The above procedure was then repeated, this time with 25 pM [^{125}I]-ET-2 (specific activity: 2000 Ci mmol^{-1}) and 1 μM unlabelled ET-1.

Analysis of results

In each tissue preparation, contractile responses to each concentration of agonist were expressed as a percentage of the maximal response to acetylcholine, carbachol, or in the case of vascular tissue, to noradrenaline. A graph was then constructed relating cumulative concentration of agonist to response. From this an EC_{50} value was derived. Where preparations were studied in duplicate, a mean response curve was constructed for each experiment and a geometric mean EC_{50} value calculated. An overall mean curve was then constructed for all experiments for both control and treated tissues and a geometric EC_{50} value with 95% confidence limits obtained from individual experimental values. Responses to all concentrations of agonist obtained in the presence and absence of calcium and in the presence and absence of verapamil or indomethacin were then compared by a two-tailed Student's t test for paired data. Differences were considered significant when $P < 0.05$.

Drugs used

Both ^{125}I-radiolabelled and unlabelled ET-1 were obtained from Auspep (Australia); ET-2 from The Peptide Institute (Japan); [^{125}I]-ET-2 from Amersham (U.K.); acetylcholine, carbamylcholine chloride (carbachol), ethylene glycol-*bis*(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA), histamine acid phosphate, indomethacin and noradrenaline ((−)-arterenol) from Sigma (U.S.A.) and verapamil from Knoll (U.S.A.). Both forms of endothelin were dissolved in 0.1 M acetic acid and noradrenaline in 10 mM HCl. Stock solutions of other drugs were prepared in distilled water, except for indomethacin which was prepared in 5% $NaHCO_3$. Aliquots were kept frozen between experiments and diluted in Krebs-Henseleit solution on the day of the experiment. Where applicable, separate series of dilutions of ET-1 and histamine were made in calcium-free Krebs-Henseleit solution.

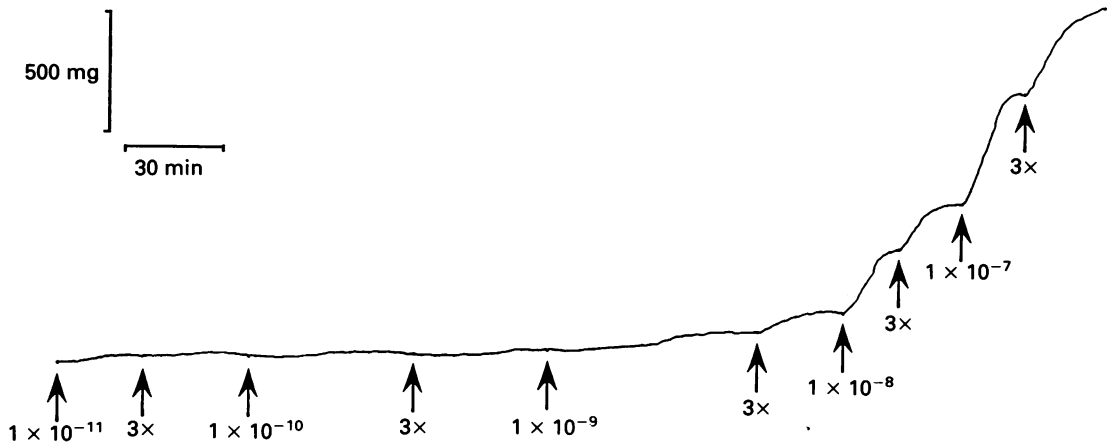


Figure 1 An example of the contractile effect of endothelin-1 on a human bronchial segment. Endothelin-1 was cumulatively added to the organ bath to give the final concentration shown (M).

Results

Functional studies

Endothelin produced protracted contractions of both the bronchial and arterial preparations. The contractile response to each concentration took up to 40 min to reach a plateau and thus a cumulative concentration-response curve took approximately 6 h to complete (Figure 1). Therefore, only one response curve was carried out in each tissue. In some experiments we attempted to reverse the effects of endothelin by repeated washing of the tissues. Washout was extremely slow and was incomplete up to 90 min.

Bronchial tissue

Studies of the comparative potency and efficacy of endothelin-1 and endothelin-2 Both forms of endothelin induced potent contractions of human isolated bronchial tissue (Figure 2). ET-1 and ET-2 proved to be equally efficacious in airway tissue, producing average contractions in response to 0.3 μM ET-1 or ET-2 that were equivalent to 106 ± 12% and 95 ± 11% ($n = 6$) of those produced by 1 mM acetylcholine. There did however, appear to be a difference in the threshold concentrations required for contraction and the EC_{50} values. The initial contraction resulting from addition of ET-1 to the organ bath occurred at 0.1 nM, whereas in the case of ET-2 the threshold contraction occurred at 1 nM. Parallel studies of ET-1 and ET-2 in bronchial tissue resulted in an EC_{50} value of 17.9 nM [95% confidence interval: 8.0, 40.3 nM] for ET-1 while that for ET-2 was 44.8 nM [95% CI: 20.5, 97.7 nM] and this difference was statistically significant ($P = 0.04$, $n = 6$).

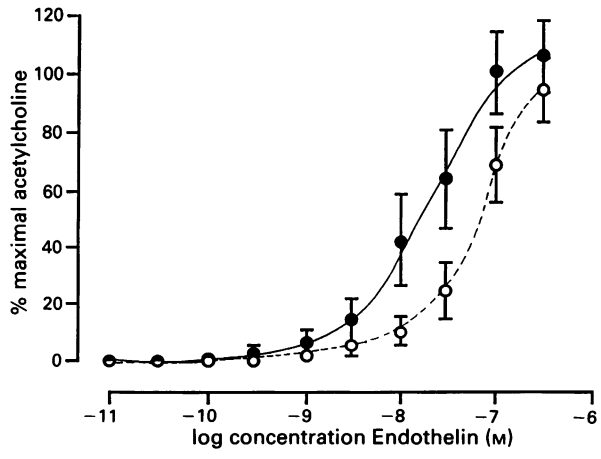


Figure 2 Mean cumulative concentration-response curves for endothelin-1 (●) and endothelin-2 (○) on human isolated bronchus. Mean responses from 6 experiments are expressed as a percentage of the maximal response to 1 mM acetylcholine. S.e.mean values are shown as vertical bars.

The effect of verapamil Blockade of the voltage-dependent calcium channels with 10 μM verapamil did not alter the potency or the efficacy of ET-1 (Table 1). Addition of verapamil to the bath did however, decrease the baseline tension by an average of 281 ± 72 mg ($n = 10$ tissues).

The effect of calcium-free Krebs-Henseleit solution The ET-1 cumulative concentration-response curve was also unaffected by removal of extracellular calcium (Figure 3a), the geometric mean EC_{50} in this instance being 10.8 nM [95% CI: 2.9, 40.0 nM ($n = 4$)] and the maximal contraction generated was 92 ± 25% of that induced by 1 mM acetylcholine (not significantly different from the values obtained in the presence of calcium). Contractile responses to histamine were however, markedly attenuated in a calcium-free environment obtained by the same method of calcium exclusion (Figure 3b). The mean maximal contraction to histamine in the presence of calcium was 127 ± 5% of the acetylcholine maximum whereas in the absence of calcium it was 41 ± 20% ($n = 4$).

The effect of indomethacin Neither the potency nor the efficacy of the contraction generated by ET-1 was affected by 25 μM indomethacin (Table 1). Addition of indomethacin to the bath induced a 306 ± 80 mg ($n = 3$) decrease in the baseline tone.

Vascular tissue

Contraction in response to addition of ET-1 to pulmonary artery rings occurred over the concentration-range 30 pM to 0.3 μM. For ET-2 however, the initial contraction was elicited at a bath concentration of 1 nM. At 0.3 μM, the highest concentration used, the cumulative concentration-response curves appeared to have reached plateaus, suggesting that the full contractile response of the tissue had been elicited (Figure 4). Neither the potency nor the efficacy of ET-1 was significantly different from those of ET-2. The geometric mean EC_{50} for ET-1 was 3.24 nM [95% CI: 0.44, 23.93 nM ($n = 5$)] while that for ET-2 was 8.04 nM [95% CI: 3.74, 17.22 nM]. The corresponding maximal contractions generated were 256 ± 71% and 226 ± 52% of the response to 1 mM noradrenaline in these tissues.

Autoradiographic studies

Binding sites appeared as uniformly sized white spots when dark field illumination was used. Dense, specific binding sites for [^{125}I]-ET-1 and [^{125}I]-ET-2 were localized on the smooth muscle of both the airway and pulmonary arterial tissue

Table 1 Mean values for maximum tension and sensitivity to endothelin in human bronchus in the presence and absence of verapamil or indomethacin

	Control	*Verapamil*	*Control*	*Indomethacin*
Mean T_{max} mg (±s.e.mean)	1260 (443)	1260 (415)	663 (100)	898 (204)
Mean $\%T_{max}$ (±s.e.mean)	67.1 (6.8)	70.3 (7.5)	129.1 (25.4)	139.0 (4.6)
Mean EC_{50} M (95% CI)	2.9×10^{-8} (1.3, 6.1)	2.3×10^{-8} (1.4, 3.8)	0.7×10^{-8} (0.1, 7.4)	1.4×10^{-8} (0.1, 19.0)
n	7	7	3	3

Maximum tension (T_{max}) is expressed as mg or as a % of the response to 1 mM carbachol/acetylcholine. Standard error of the mean (s.e.mean) values are shown in parentheses. Geometric mean EC_{50} values and the 95% confidence intervals (CI) are also shown; *n* refers to the number of experiments performed.

(Figure 5), in addition to binding over airway ganglia and alveoli. There appeared to be no binding associated with the epithelium or the endothelium. There was no obvious difference between either form of endothelin in the density or the location of binding. 'Non-specific binding' was uniformly low and was unassociated with histological structures for both peptides. The location and density of binding sites over the section appeared to be independent of the source of the tissue (i.e. whether it was from surgical or post mortem specimens).

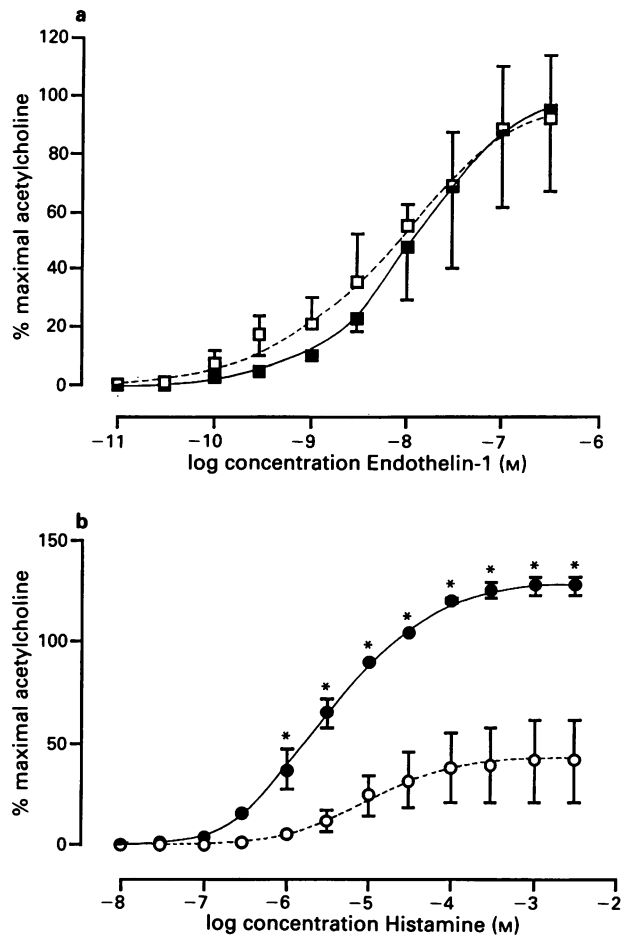


Figure 3 (a) Mean cumulative concentration-response curves for endothelin-1 in the presence (■) and absence (□) of extracellular calcium. Mean responses from 4 experiments are expressed as a percentage of the maximal response to 1 mM acetylcholine. S.e.mean values are shown as vertical bars. (b) Mean cumulative concentration-response curves for histamine in the presence (●) and absence (○) of extracellular calcium. Mean responses from 3 experiments are expressed as a percentage of the maximal response to 1 mM acetylcholine. S.e.mean values are shown as vertical bars. *indicates significant ($P < 0.05$) differences in the maximal responses.

Discussion

This study has shown that endothelin has an extremely potent contractile action on human airway and vascular tissue. Its action in isolated airways cannot be attributed to activation of voltage-dependent calcium channels or the liberation of cyclo-oxygenase products, and is independent of extracellular calcium. In addition, binding sites for two of the forms of endothelin (ET-1 and ET-2) have been visualized on sections of human bronchus and pulmonary artery suggesting that there are distinct receptors for endothelin on the smooth muscle of the vessels and bronchus of human lung.

The potency of ET-1 as a contractile agent of bronchial tissue in this study represented by a geometric EC_{50} of 18.3 nM was similar to that of leukotriene D_4 (Hanna *et al.*, 1981) and the thromboxane A_2-mimetic U46619 (Armour *et al.*, 1989), but far greater than that of carbachol and histamine (Black *et al.*, 1986). Its efficacy was however, less than that of carbachol and therefore of U46619 which produces a greater contractile response than carbachol in human airway tissue. Due to limited availability of the peptide, the maximal bath concentration attained in these experiments was 0.3 μM. At this point it was not possible to ascertain whether a maximal response had been reached. Thus, it is possible that the values representative of the efficacy of endothelin, namely T_{max} and $\%T_{max}$

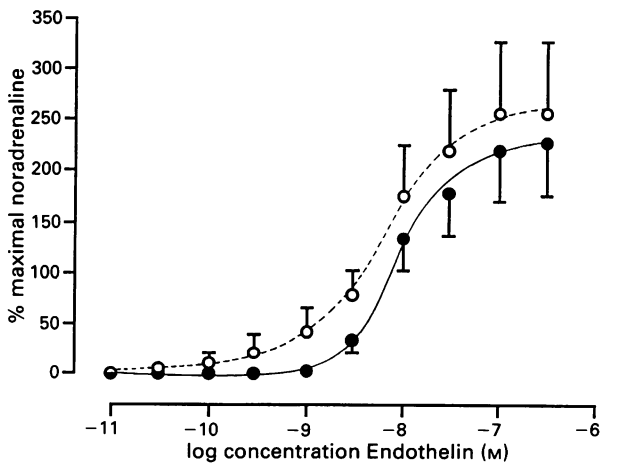


Figure 4 Mean cumulative concentration-response curves for endothelin-1 (○) and endothelin-2 (●) on human isolated pulmonary artery. Mean responses from 5 experiments are expressed as a percentage of the maximal response to 1 mM noradrenaline. S.e.mean values are shown as vertical bars.

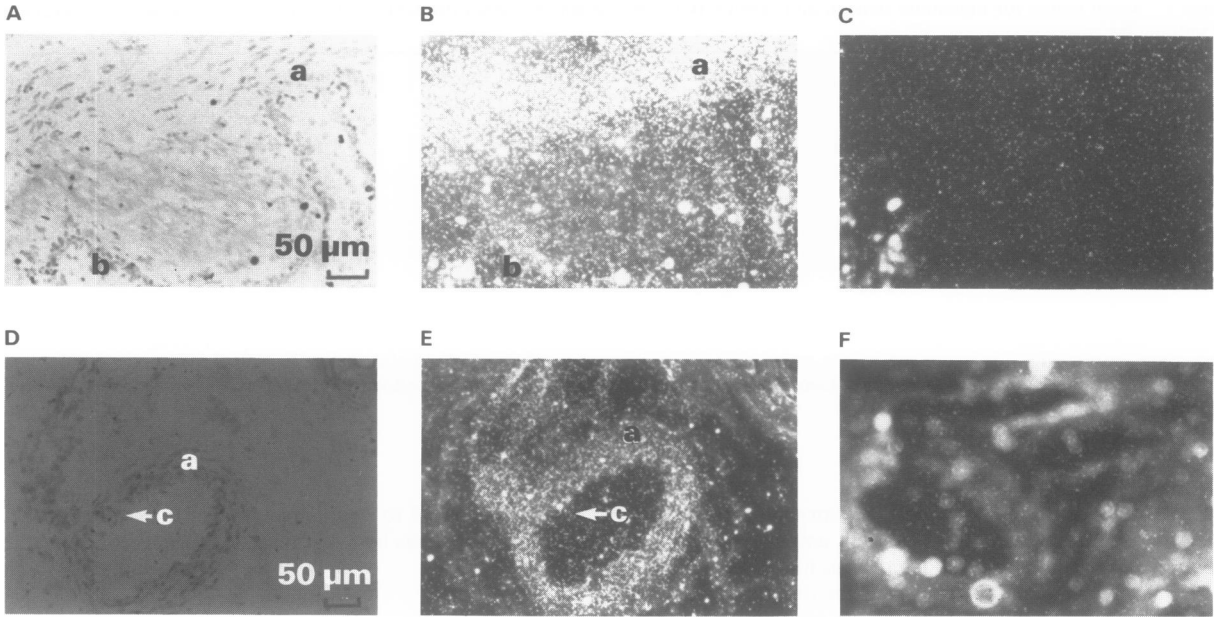


Figure 5 Photomicrographs of sections of human bronchus (A, B and C) and pulmonary artery (D, E and F). (A) and (D) are light field photomicrographs; central and right hand panels are dark field photomicrographs of adjacent sections incubated in [^{125}I]-endothelin-1 (B and C) and [^{125}I]-endothelin-2 (E and F) alone and in the presence of unlabelled endothelin-1 (C and F). (a) Smooth muscle, (b) epithelium and (c) endothelium.

may be underestimated in this preparation. If this were the case, then the mean values for EC_{50} may in fact be higher.

There is only one previous description of the contractile activity of ET-1 in human isolated airway tissue. Uchida *et al.* (1988), have briefly reported that endothelin contracts human bronchus. They commented that 'the dose response curve for endothelin was 2 orders of magnitude smaller than those of histamine and neurokinin A'. Whether this refers to the potency or the efficacy is unclear and as no data are presented, a comparison of experimental results in the two studies is not possible.

ET-1 and ET-2 have been shown to differ both in the potency and efficacy of their contractile action on porcine coronary artery strips (Inoue *et al.*, 1989). ET-1 was significantly more potent than ET-2, while ET-2 produced a far greater maximal tension in this tissue. From the results of the present study it would seem that these differences in activity are not as apparent in human isolated airways. Both forms of endothelin were equally efficacious in this tissue. There was however, a difference in the potency of the peptides, the geometric mean EC_{50} value for ET-2 being 2.5 times greater than that for ET-1 in human isolated bronchial tissue.

It has been suggested that the contractile action of endothelin on vascular smooth muscle results from an action of endothelin as an endogenous ligand for voltage-dependent calcium channels (Yanagisawa *et al.*, 1988). The results of recent studies have not conclusively supported or rejected this proposed mechanism. Maggi *et al.* (1989) demonstrated that in guinea-pig airways, partial inhibition of the endothelin-induced contraction can be achieved by pre-incubation with nifedipine, while in rat trachea the voltage-dependent calcium channel antagonist nicardipine was without effect (Turner *et al.*, 1989) although nifedipine slightly attenuated the response in this tissue (Borges *et al.*, 1989). The present study has shown that, in human isolated bronchial tissue, the presence of verapamil has no effect on the contractile activity of ET-1. Thus, it would seem unlikely that ET-1 acts as an endogenous ligand for the voltage-dependent calcium channel in human airways.

The finding that the action of ET-1 on human airway tissue was independent of extracellular calcium was unexpected. Yanagisawa *et al.* (1988) concluded that contraction of porcine coronary arteries in response to the application of endothelin was due, at least in part, to the influx of extracellular calcium into the cytoplasm of the smooth muscle cell. Removal of extracellular calcium from an *in vitro* preparation of rat trachea (Turner *et al.*, 1989) attenuated the contraction generated by endothelin. By contrast, in human omental vessels (Hughes *et al.*, 1989) as well as in the human bronchial tissue studied in this investigation, the removal of calcium from the extracellular environment was without effect on the resultant ET-1-induced contraction. It is difficult to compare results from these studies and others, as calcium-free conditions have been achieved by use of a number of different protocols. These include omission of calcium from the Krebs-Henseleit solution and in some cases, the addition of various concentrations of EDTA or EGTA. Moreover, these varying 'calcium-free solutions' remained in contact with the tissues for a range of time periods. Incubation of tissue in a calcium-free medium without chelating agents may not be sufficient to remove all the extracellular calcium (Keatinge, 1972). Conversely, prolonged exposure to EGTA may not only affect the residual extracellular calcium ions, it may also remove calcium from the intracellular stores. In the present study, the protocol described by Creese & Denborough (1981) was employed. Incubation of the tissue with EGTA for only 30 min and then the replacement of the EGTA solution with calcium-free Krebs-Henseleit solution, results in removal of the extracellular but not intracellular calcium, as evidenced by the markedly decreased contractile action of histamine in this extracellular calcium-free environment. It is thus apparent that this protocol is a suitable one for distinguishing those agonists with a contractile action dependent upon extracellular calcium from those able to elicit contractions in an extracellular calcium-free environment.

The contraction induced by the addition of endothelin to the organ baths was extremely slow in onset. Its prolonged nature was similar to the contraction which results from the application of leukotrienes to isolated bronchial tissue (Hanna *et al.*, 1981). Endothelin may therefore elicit contraction via endogenous leukotriene release. De Nucci *et al.* (1988), have demonstrated thromboxane A_2 and prostaglandin release from guinea-pig lung perfused with a solution containing ET-1. More recently, a decreased response to ET-1 in guinea-pig trachea and bronchus was observed when the contractions were generated in the presence of a thromboxane A_2 receptor

antagonist (Battistini *et al.*, 1990). There was therefore, a possibility that the endothelin contraction in human bronchus in the present series of experiments was mediated by the release of products of the arachidonic acid pathway. Incubation of the human bronchial rings with the cyclo-oxygenase inhibitor indomethacin, resulted in no change in the contraction induced by ET-1. On the basis of these results, the action of ET-1 in human bronchial tissue cannot therefore, be explained by the release of endogenous prostaglandins or thromboxane A_2. Indomethacin does not however, prevent the formation of products of the lipoxygenase pathway, so it is still possible that leukotrienes may be released as a result of addition of endothelin to the organ bath. Further studies using either leukotriene receptor antagonists or inhibitors of leukotriene release are indicated to address this issue.

The potent and efficacious nature of endothelin as a vasoconstrictor agent on human arterial vascular tissue has been confirmed by this study. Application of endothelin to rings of pulmonary artery resulted in a contraction that was both slow in onset and sustained over time. The geometric EC_{50} values of 3.24 nM (ET-1) and 8.04 nM (ET-2) establish that endothelin is as potent if not more potent a vasoconstrictor as a bronchoconstrictor agent. ET-1 and ET-2 were approximately 5 times as potent in the arterial preparations as in the bronchial tissues. This would seem to suggest that these forms of endothelin are acting at the same receptor subtype in both tissues. In contrast to the results obtained in bronchial tissue, the responses obtained at 0.3 μM ET-1 and ET-2 appeared to have reached a plateau in the arterial preparations and thus represent the true maximal responses in this tissue type.

In this study, the existence of binding sites for ET-1 on the smooth muscle of human airway tissue as reported by Power *et al.* (1989), was confirmed. Binding sites for ET-2 were also visualized on the smooth muscle as well as the parasympathetic ganglia associated with the bronchial tissue. This is the first reported localization of binding sites for ET-2 in any tissue from any species. In addition to the localization of ET-1 and ET-2 binding sites in human airway tissue, dense binding of both forms of endothelin was observed on sections of pulmonary arteries.

The results of autoradiographic studies carried out on a wide variety of tissue from many different species provide evidence for the suggestion of a distinct endothelin receptor. Studies in human tissue have localized ET-1 binding sites to coronary artery (Dashwood *et al.*, 1989), adrenal gland (Davenport *et al.*, 1989), and intrapulmonary vessels in addition to bronchus (Power *et al.*, 1989). This binding was not displaced by co-incubation with vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), atrial natriuretic peptide (ANP) or gastrin and was unaffected by nicardipine (Power *et al.*, 1989), verapamil or the presence of EDTA or EGTA (Kohzuki *et al.*, 1989). The evidence for the existence of a discrete receptor for endothelin is thus supported by the present results.

Future investigation into the mechanism of action of endothelin on human airway and vascular smooth muscle promises to be very exciting in the light of the finding of raised levels of endothelin in human lung during an acute asthma episode (Nomura *et al.*, 1989). Our results have demonstrated that it is one of the most potent contractile agents studied on human airway and vascular smooth muscle, its action is independent of the release of cyclo-oxygenase products and the influx of extracellular calcium into the cytoplasm of the smooth muscle cell, and there are specific receptors for endothelin on this muscle as well as on neural tissue. These findings suggest that in human airways, endothelin acts by binding at these receptors located on the smooth muscle and may subsequently alter the mobilization of intracellular calcium resulting in smooth muscle contraction. Since a number of events associated with an acute attack of asthma seem to be stimuli for endothelin release, further studies will clarify if and how endothelin has a role in the pathophysiology of asthma.

We are grateful to the following cardiothoracic surgeons for the supply of human lung tissue: Drs D. Baird, A. Grant, C. Hughes, B. Leckie, B. McCaughan, from Royal Prince Alfred Hospital and The Repatriation Hospital; Drs C. Deal, D. Ross from Royal North Shore Hospital: Drs V. Chang, A. Farnsworth, M. Shanahan from St Vincent's Hospital. The staff of the cardiothoracic theatres and the pathology departments of the above hospitals provided invaluable assistance with the collection of tissue. This study was supported by The Asthma Foundation of New South Wales.

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(Received May 31, 1990
Revised October 1, 1990
Accepted October 8, 1990)

Br. J. Pharmacol. (1991), **102**, 429–433 © Macmillan Press Ltd, 1991

Effects of acute paroxetine administration on tryptophan metabolism and disposition in the rat

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1 The effects of acute oral administration of paroxetine on tryptophan metabolism and disposition were examined in the rat.

2 Basal liver tryptophan pyrrolase activity was inhibited by paroxetine *in vitro* and after oral administration. Maximum inhibition was caused by a 1 mg kg^{-1} dose.

3 Paroxetine administration also inhibited pyrrolase activity that had previously been enhanced by hormonal induction by cortisol or cofactor activation by haematin. The cortisol induction of the enzyme was, however, not inhibited by pretreatment of rats with paroxetine.

4 Paroxetine increased tryptophan availability to the brain, because of the above pyrrolase-inhibitory mechanism. Cerebral 5-hydroxytryptamine (5-HT) synthesis was accordingly enhanced, though this was apparent only with doses of the drug of up to 1 mg kg^{-1}. With larger doses, decreased 5-HT turnover, probably as a result of 5-HT uptake inhibition, was the more dominant feature.

5 Paroxetine lowered circulating corticosterone concentration, but did not influence those of albumin, non-esterified fatty acids or glucose.

6 It is concluded that, in addition to inhibiting brain 5-HT turnover, paroxetine also, in common with 20 other antidepressants, enhances 5-HT synthesis by increasing brain tryptophan concentration secondarily to inhibition of liver tryptophan pyrrolase activity.

Introduction

Paroxetine [(−)-*trans*-4-(4′-fluorophenyl)3-(3′,4′-methylenedioxyphenoxymethyl)piperidine] is an antidepressant (Lund Laursen *et al.*, 1985; Feighner & Boyer, 1989; Rickels *et al.*, 1989) drug with a profile of a potent and selective 5-hydroxytryptamine (5-HT) uptake inhibitor (Thomas *et al.*, 1987). Preliminary studies (A.M. Johnson, personal communication) indicate that paroxetine can, as other 5-HT uptake inhibitors (Fuller *et al.*, 1974; Claassen *et al.*, 1977; Ross *et al.*, 1981), decrease 5-HT turnover in rat brain. However, it is not known whether it is capable of enhancing 5-HT synthesis by increasing tryptophan availability to the brain secondarily to inhibition of activity of the major tryptophan-degrading enzyme, liver tryptophan pyrrolase (tryptophan 2,3-dioxygenase, EC 1.13.11.11), as can 20 other antidepressants of different chemical structures and pharmacological classes (Badawy & Evans, 1981; 1982; Badawy, 1986). In the present work, we show that acute administration of paroxetine to rats both decreases turnover and enhances synthesis of brain 5-HT by the above mechanisms.

Methods

Animals and treatments

Locally bred male Wistar rats (150–170 g) were maintained on cube diet 41B (Oxoid) and water under a natural light:dark cycle and at 22 ± 1°C and were killed between 12 h 00 min and 13 h 30 min by stunning and cervical dislocation (for the determination of liver tryptophan pyrrolase activity) or by decapitation (for all other determinations). Paroxetine hydrochloride was administered orally in doses of 0.1–25 mg kg^{-1} body weight (dose calculated as salt) and appropriate control rats received an equal volume (2 ml kg^{-1}) of the vehicle (distilled water) by the same route. Cortisol acetate (20 mg kg^{-1}) or haematin HCl (5 mg kg^{-1}) was dissolved in dimethylformamide and administered intraperitoneally; control rats received an equal volume (1 ml kg^{-1}) of this latter vehicle by the same route.

Chemicals and drugs

Paroxetine hydrochloride was a gift from Smith Kline Beecham Pharmaceuticals; all other chemicals were purchased from BDH Chemicals and Sigma (both of Poole, Dorset) and were of the purest commercially available grades.

Chemical, enzymic and other determinations

Tryptophan pyrrolase activity was determined in fresh liver homogenates either in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added (2 μM) haematin (Badawy & Evans, 1975; see also the fuller description of Badawy, 1981 and additional comments by Badawy *et al.*, 1983). The activity of the apoenzyme (the haem-free predominant form in rat and human liver) was calculated by difference. Free (ultrafiltrable) serum, total (acid-soluble) serum, liver and brain tryptophan concentrations and those of brain 5-HT and its major metabolite 5-hydroxyindol-3-ylacetic acid (5-HIAA) and serum albumin, corticosterone, glucose and non-esterified fatty acids were all determined by standard procedures (for references, see Badawy *et al.*, 1984). Serum was isolated from clotted blood by centrifugation at 3000 *g* for 15–20 min, whereas brain was rapidly removed (within 10 s of the death of the animals), frozen in liquid nitrogen and then stored at −20°C overnight before analysis. For liver tryptophan determination, a portion of the tissue was also frozen in liquid nitrogen and then stored along with brain as described above. Statistical analysis of results was performed by Student's *t* test.

Results

Effects of paroxetine on the basal activity of rat liver tryptophan pyrrolase

The time course of the effects of acute oral administration of paroxetine (1 mg kg^{-1}) on the basal pyrrolase activity is

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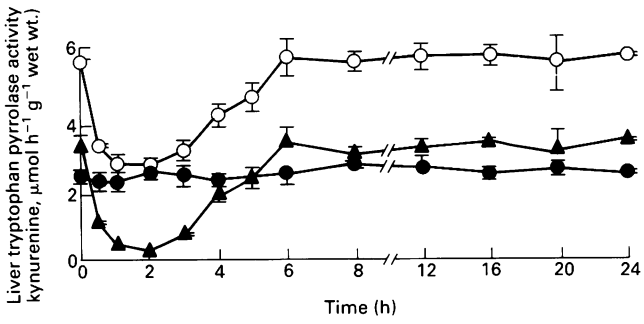


Figure 1 Time course of effects of acute oral administration of paroxetine (1 mg kg^{-1}) on the basal activity of rat liver tryptophan pyrrolase. Paroxetine was administered orally at zero-time and pyrrolase activity was determined at the various times shown as described in the Methods section either in the absence (holoenzyme activity; ●), or in the presence (total enzyme activity; ○) of added (2 μM) haematin. The apoenzyme activity (▲) was obtained by difference. Values are means (s.e.mean shown by vertical bars except where error lies within size of symbol) for each group of 4 rats.

shown in Figure 1. Whereas the activity of the holoenzyme (the haem-containing form) was not significantly altered at any of the time intervals examined, that of the total enzyme was significantly decreased by 42, 52, 54, 46 and 27% (P = 0.02–0.005) at 0.5, 1, 2, 3 and 4 h respectively. Activity of the apoenzyme (the haem-free form, obtained by difference) was therefore decreased by 68, 85, 94, 79 and 41% at the above time intervals respectively (P = 0.02–0.001) and also by 29% ($P < 0.05$) at 5 h. Pyrrolase activity then returned to the control (zero time) values at 6 h and remained unaltered during the subsequent 18 h.

We also found (results not shown) that a 0.5 mg kg^{-1} oral dose of paroxetine exerted similar time course effects, except that the extent of the pyrrolase inhibition was marginally smaller.

The results in Figure 2 show that maximum inhibition of basal liver tryptophan pyrrolase activity at 2 h after oral paroxetine administration was observed with the 1 mg kg^{-1} dose (45% and 89% for the total enzyme and apoenzyme activities respectively; $P < 0.001$) and that significant inhibition of these two activities (of 18% and 42% respectively; P = 0.02–0.01) was evident with a dose of the drug as small as 0.1 mg kg^{-1}.

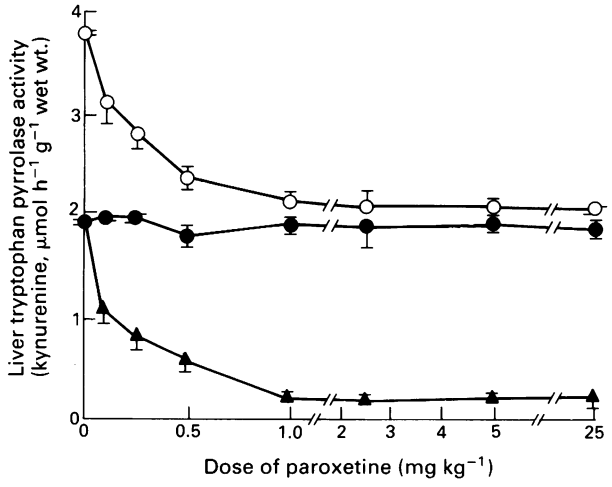


Figure 2 Effects of acute oral administration of various doses of paroxetine on the basal activity of rat liver tryptophan pyrrolase. Rats were treated orally with paroxetine (0.1–25 mg kg^{-1}) or an equal volume (2 ml kg^{-1}) of distilled water and were killed 2 h later. Liver tryptophan pyrrolase activity was determined as described in the Methods section either in the absence (holoenzyme activity; ●) or in the presence (total enzyme activity; ○) of added (2 μM) haematin. The apoenzyme activity (▲) was obtained by difference. Values are means (with s.e.mean shown by vertical bars) for each group of 4 rats.

Table 1 Effects of paroxetine *in vitro* on the basal activity of rat liver tryptophan pyrrolase

Paroxetine concentration	*Liver tryptophan pyrrolase activity* (μmol of kynurenine formed h^{-1} g^{-1} wet wt.) *Holoenzyme*	*Total enzyme*	*Apoenzyme*
0	1.6 ± 0.1	3.9 ± 0.2	2.3 ± 0.1
1 μM	1.7 ± 0.1	3.1 ± 0.1**	1.4 ± 0.1**
10 μM	1.6 ± 0.1	2.7 ± 0.2*	1.1 ± 0.1***
0.1 mM	1.7 ± 0.2	2.5 ± 0.2**	0.8 ± 0.1***
1 mM	1.6 ± 0.2	2.0 ± 0.1***	0.4 ± 0.0***

Paroxetine was added *in vitro* to liver homogenates from normal fed rats. Pyrrolase activity was determined as described in the Methods section either in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added (2 μM) haematin. The apoenzyme activity was obtained by difference. Values are means ± s.e.mean for each group of four separate experiments. The significance of the differences between paroxetine-supplemented and control homogenates is indicated as follows: * $P < 0.01$; ** $P < 0.005$; *** $P < 0.001$.

Addition of paroxetine to rat liver homogenates *in vitro* also inhibited the basal pyrrolase activity in a concentration-dependent fashion in the concentration range of 1 μM–1 mM (Table 1).

The results in Table 2 show that the inhibition of the basal total pyrrolase activity (that demonstrable in the presence of 2 μM added haematin) by paroxetine both *in vitro* (1 mM) and after oral administration (1 mg kg^{-1}) was completely reversed by the addition of an excess (4 and 6 μM) of this source of the haem cofactor of the enzyme.

Effects of paroxetine administration on rat liver tryptophan pyrrolase activity previously enhanced by hormonal induction or cofactor activation

The results in Table 3 show that paroxetine (0.5 or 5 mg kg^{-1}) caused a significant inhibition of liver tryptophan pyrrolase activity that had previously been enhanced by either hormonal induction by cortisol or cofactor activation by haematin. The effects of paroxetine on pyrrolase activity previously enhanced by tryptophan administration were not examined, because substrate activation of the pyrrolase would be an inappropriate model here, in view of the absence of any evidence for an increase in tryptophan availability in depressive illness.

Effects of pretreatment of rats with paroxetine on the cortisol induction of tryptophan pyrrolase

As shown in Table 4, pretreatment with paroxetine (0.5 or 5 mg kg^{-1}) failed to exert any significant influence on the extent of induction of liver tryptophan pyrrolase activity by subsequent cortisol administration. The smaller dose of paroxetine did not cause any significant changes in activity of the basal (un-induced) pyrrolase in control rats at the time interval (4.5 h) at which these experiments were performed, whereas the 5 mg kg^{-1} dose inhibited the basal total enzyme and apoenzyme activities by 25% and 43% respectively (these are similar to the 27% and 41% decreases observed at 4 h after administration of the 1 mg kg^{-1} dose; see Figure 1).

Effects of paroxetine administration on rat tryptophan metabolism and disposition and on concentrations of circulating corticosterone and other constituents

As shown in Table 5, free serum, total serum, liver and brain tryptophan concentrations were maximally increased by the 1 mg kg^{-1} dose of paroxetine at 3.5 h (the time interval at which we found brain tryptophan concentration to be maxi-

Table 2 Reversal by excess haematin *in vitro* of the inhibition of rat liver tryptophan pyrrolase activity by paroxetine added *in vitro* or administered orally

Expt No.	Line No.	Treatment	Liver tryptophan pyrrolase activity (μmol of kynurenine formed $h^{-1} g^{-1}$ wet wt.) Concentration of added haematin (μM) 0	2	4	6
1	1	Nil	1.5 ± 0.1	3.0 ± 0.2	3.0 ± 0.2	3.0 ± 0.3
	2	Paroxetine (1 mM)	1.5 ± 0.1	2.0 ± 0.1*	3.0 ± 0.1	3.0 ± 0.2
2	3	Water	1.6 ± 0.1	3.4 ± 0.2	3.4 ± 0.1	3.6 ± 0.1
	4	Paroxetine (1 mg kg^{-1})	1.6 ± 0.1	2.2 ± 0.1**	3.4 ± 0.1	3.4 ± 0.1

Paroxetine was either added *in vitro* (1 mM) or administered orally (1 mg kg^{-1}, 2 h previously). Control rats for the latter treatment received water (2 ml kg^{-1}) by the same route. Tryptophan pyrrolase activity was determined as described in the Methods section either in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added (2 μM) haematin. Additionally, activity was measured in the presence of an excess (4 and 6 μM) of added haematin. Values are means ± s.e.mean for each group of 3 (experiment 1) or 4 (experiment 2) rats. The values in line 2 have been compared with those in line 1, whereas those in line 4 were compared with those in line 3. The significance of the differences is indicated as follows: $*P < 0.01$; $**P < 0.005$.

Table 3 Effects of acute oral administration of paroxetine on liver tryptophan pyrrolase activity enhanced by pretreatment of rats with cortisol or haematin

Pretreatment	Treatment	Line No.	Liver tryptophan pyrrolase activity (μmol of kynurenine formed $h^{-1} g^{-1}$ wet wt.) Holoenzyme	Total enzyme	Apoenzyme	Pyrrolase haem saturation (%)
DMF	Water	1	3.0 ± 0.3	6.2 ± 0.4	3.2 ± 0.1	48 ± 3
DMF	Paroxetine (0.5 mg kg^{-1})	2	3.4 ± 0.2	4.9 ± 0.2††	1.5 ± 0.1***	NA
DMF	Paroxetine (5 mg kg^{-1})	3	2.9 ± 0.1	3.1 ± 0.1***	0.2 ± 0.1***	NA
Cortisol	Water	4	5.8 ± 0.3***	11.4 ± 0.4***	5.6 ± 0.3***	NA
Cortisol	Paroxetine (0.5 mg kg^{-1})	5	4.2 ± 0.2**	6.9 ± 0.5***	2.7 ± 0.5**	NA
Cortisol	Paroxetine (5 mg kg^{-1})	6	3.2 ± 0.3***	5.6 ± 0.4***	2.4 ± 0.2***	NA
Haematin	Water	7	5.4 ± 0.5*	6.4 ± 0.6	NA	84 ± 1***
Haematin	Paroxetine (0.5 mg kg^{-1})	8	3.4 ± 0.4††	5.2 ± 0.2†	NA	65 ± 1***
Haematin	Paroxetine (5 mg kg^{-1})	9	2.4 ± 0.2**	4.9 ± 0.3†	NA	49 ± 2***

Rats received an i.p. injection of cortisol acetate (20 mg kg^{-1}), haematin HCl (5 mg kg^{-1}) or an equal volume (1 ml kg^{-1}) of the vehicle dimethylformamide (DMF) and were killed 4 h later. The animals also received, 2 h after the above injection, an oral intubation of either paroxetine (0.5 or 5 mg kg^{-1}) or an equal volume (2 ml kg^{-1}) of water. Tryptophan pyrrolase activity was determined as described in the Methods section either in the absence (holoenzyme activity) or in the presence (total enzyme activity) of added (2 μM) haematin. The apoenzyme activity was obtained by difference. In the experiments with administered haematin it is more appropriate to express the effect of the cofactor as the percentage haem saturation (100 × holoenzyme activity/total enzyme activity), instead of apoenzyme activity. Values are means ± s.e.mean for each group of 4 rats. The values in lines 2, 3, 4 and 7 have been compared with those in line 1, those in lines 5 and 6 were compared with those in line 4, and those in lines 8 and 9 were compared with those in line 7. The significance of the differences is expressed as follows: $†P < 0.05$; $††P < 0.025$; $*P < 0.01$; $**P < 0.005$; $***P < 0.001$. NA denotes not applicable.

Table 4 Effect of pretreatment of rats with paroxetine on the cortisol induction of liver tryptophan pyrrolase activity

Pretreatment	Treatment	Line No.	Liver tryptophan pyrrolase activity (μmol of kynurenine formed $h^{-1} g^{-1}$ wet wt.) Holoenzyme	Total enzyme	Apoenzyme
Water	DMF	1	2.4 ± 0.1	5.2 ± 0.5	2.8 ± 0.3
Paroxetine (0.5 mg kg^{-1})	DMF	2	2.2 ± 0.1	4.9 ± 0.3	2.7 ± 0.3
Paroxetine (5 mg kg^{-1})	DMF	3	2.3 ± 0.1	3.9 ± 0.2†	1.6 ± 0.1*
Water	Cortisol	4	4.9 ± 0.3***	11.1 ± 0.9**	6.2 ± 0.7**
Paroxetine (0.5 mg kg^{-1})	Cortisol	5	5.1 ± 0.5	11.0 ± 1.1	5.9 ± 0.6
Paroxetine (5 mg kg^{-1})	Cortisol	6	4.8 ± 0.6	10.6 ± 0.9	5.8 ± 0.6

Rats were pretreated orally with either paroxetine (0.5 or 5 mg kg^{-1}) or an equal volume (2 ml kg^{-1}) of water. At 0.5 h after these intubations, the animals received an i.p. injection of either cortisol acetate (20 mg kg^{-1}) or an equal volume (1 ml kg^{-1}) of the vehicle dimethylformamide (DMF), and were killed 4 h after these latter injections. Values are means ± s.e.mean for each group of 4 rats. The values in lines 2, 3 and 4 have been compared with those in line 1, whereas those in lines 5 and 6 were compared with those in line 4. The significance of the differences is indicated as follows: $†P < 0.05$; $*P < 0.02$; $**P < 0.005$; $***P < 0.001$.

Table 5 Effects of acute oral administration of various doses of paroxetine on rat tryptophan metabolism and disposition and serum corticosterone concentration

Parameter	*Dose of paroxetine* ($mg\,kg^{-1}$) 0	0.5	1.0	5.0	25
Free serum Trp	1.2 ± 0.1	1.3 ± 0.1	1.7 ± 0.1***	1.8 ± 0.1**	1.8 ± 0.1***
Total serum Trp	18.6 ± 0.7	21.5 ± 1.4†	25.5 ± 1.7**	25.4 ± 0.9***	24.5 ± 0.9***
Free serum Trp (%)	6.2 ± 0.5	6.2 ± 0.3	6.7 ± 0.3	7.0 ± 0.6	7.3 ± 0.3†
Liver Trp	5.0 ± 0.1	5.6 ± 0.1**	6.3 ± 0.3**	6.2 ± 0.2***	6.6 ± 0.1***
Brain Trp	1.6 ± 0.0	1.8 ± 0.0†	2.1 ± 0.1***	2.0 ± 0.1**	2.1 ± 0.1***
Brain 5-HT	0.60 ± 0.03	0.72 ± 0.02††	0.68 ± 0.02†	0.52 ± 0.02†	0.48 ± 0.01*
Brain 5-HIAA	0.52 ± 0.01	0.52 ± 0.01	0.46 ± 0.02†	0.41 ± 0.02***	0.34 ± 0.02***
Serum corticosterone	59.9 ± 3.3	51.4 ± 4.7	44.3 ± 3.0*	36.9 ± 1.1***	49.9 ± 3.3†

Rats were killed at 3.5 h after oral administration of various doses of paroxetine or an equal volume ($2\,ml\,kg^{-1}$) of water. Other details are described in the Methods section. Values for serum and/or tissue tryptophan (Trp), 5-hydroxytryptamine (5-HT) and 5-hydroxyindol-3-ylacetic acid (5-HIAA) are expressed in $\mu g\,ml^{-1}$ or per g wet wt., whereas those for serum corticosterone are in nM. Values are means ± s.e.mean for each group of 6 rats. The significance of the differences between test and control values is indicated as follows: †$P < 0.05$; ††$P < 0.02$; *$P < 0.01$; **$P < 0.005$; ***$P < 0.001$. None of the above doses of paroxetine exerted any significant effect on concentrations of serum albumin, non-esterified fatty acids or glucose, the control values of which (means ± s.e.mean for 6 rats per group, in $g\,l^{-1}$, mM and $mg\,dl^{-1}$ respectively) were 39.3 ± 0.7, 0.39 ± 0.03 and 109 ± 3 respectively.

mally elevated by antidepressants; Badawy & Evans, 1982). Tryptophan binding to serum proteins (expressed as the percentage free serum tryptophan) was not significantly altered, except by the $25\,mg\,kg^{-1}$ dose, which caused only a 16% decrease. Concentrations of the main physiological binder (albumin) and displacers (non-esterified fatty acids) of serum tryptophan and also that of glucose were not significantly altered by paroxetine (results not shown), whereas that of serum corticosterone was decreased dose-dependently with doses of 0.5–5 $mg\,kg^{-1}$ with a partial recovery occurring after the $25\,mg\,kg^{-1}$ dose (Table 5). Brain 5-HT concentration was significantly increased only by the 0.5 and $1\,mg\,kg^{-1}$ doses of paroxetine, by 20% and 13% respectively, whereas decreases of 13% and 20% were observed with the 5 and $25\,mg\,kg^{-1}$ doses, respectively. By contrast, brain 5-HIAA concentration remained unaltered by the $0.5\,mg\,kg^{-1}$ dose but was decreased by 12–35% by the higher doses, of paroxetine (Table 5).

Discussion

The present results have demonstrated the effectiveness of the antidepressant drug paroxetine as an inhibitor of tryptophan pyrrolase activity in rat liver. In this respect, paroxetine resembles 20 other antidepressants of various chemical structures and pharmacological classes (Badawy & Evans, 1981; 1982; Badawy, 1986). Paroxetine is not, however, the only selective 5-HT uptake blocker to inhibit pyrrolase activity; fluvoxamine, fluoxetine and zimelidine also exert a similar effect (A.A.-B. Badawy & C.J. Morgan, unpublished work). Pyrrolase inhibition is therefore a property shared by at least 24 different antidepressants and it remains to be seen if other existing and potential antidepressants could be added to this list. Pyrrolase inhibition by antidepressants should increase circulating tryptophan availability to the brain, which may be important in the treatment of depression, in view of recent evidence for decreased plasma tryptophan levels in major depression (Cowen *et al.*, 1989) and for the reversal of antidepressant-induced remission by rapid depletion of plasma tryptophan (Delgado *et al.*, 1990).

That the pyrrolase inhibition by paroxetine administration is a direct effect is suggested by the ability of the drug to inhibit the basal enzyme activity *in vitro* (Table 1). The ability of an excess of added haematin to reverse the inhibition (Table 2) suggests that paroxetine acts by preventing the conjugation of the apoenzyme with its cofactor haem, as is the case with other antidepressants (Badawy & Evans, 1981). The inability of paroxetine to inhibit the cortisol induction of tryptophan pyrrolase (Table 4) suggests that the drug does not block enzyme (protein) synthesis and is thus free from this undesirable property.

Liver tryptophan pyrrolase activity could be enhanced by a variety of mechanisms (Badawy & Evans, 1975), of which hormonal induction by glucocorticoids may be of particular importance in depression, in view of disturbed hypothalamic-pituitary-adrenal function in this illness. It was therefore considered important to find out if paroxetine is also capable of inhibiting liver pyrrolase activity that had previously been enhanced by hormonal induction by cortisol or through another mechanism, such as cofactor activation by haematin. As the results in Table 3 show, pyrrolase activity enhanced by either mechanism was significantly inhibited by paroxetine administration, thus suggesting that this drug should be an effective pyrrolase inhibitor under conditions expected in depressed subjects.

Liver tryptophan pyrrolase is a major peripheral determinant of tryptophan availability (see, e.g., Badawy & Evans, 1983) and the results in Table 5 show clearly that tryptophan accumulation in liver, serum and brain was maximum with a dose of paroxetine ($1\,mg\,kg^{-1}$) known to cause the maximum inhibition of liver tryptophan pyrrolase activity (Figure 2). The changes in tryptophan disposition shown in Table 5 and the associated absence of altered binding of the amino acid to circulating proteins are therefore typical consequences of pyrrolase inhibition and may be explained simply by this inhibition by paroxetine decreasing the rate of hepatic tryptophan degradation, thus leading to accumulation of the amino acid in the liver and a consequent increase in its availability in the circulation and, hence, to the brain.

Cerebral 5-HT synthesis is controlled mainly by brain tryptophan concentration, because the rate-limiting enzyme of the 5-HT-biosynthetic pathway, tryptophan hydroxylase, exists unsaturated with its tryptophan substrate (Fernstrom & Wurtman, 1971; Carlsson & Lindqvist, 1978; Curzon, 1979). The increases (Table 5) in brain [5-HT] after administration of the 0.5 and $1\,mg\,kg^{-1}$ doses of paroxetine may therefore be explained by the drug enhancing 5-HT synthesis secondarily to the associated increase in brain [tryptophan]. If the effect of paroxetine on brain 5-HT were confined to enhancement of its synthesis, then one would have expected an increase in [5-HIAA] and also that in [5-HT] to be maintained with the larger doses of the drug. This is clearly not so (Table 5) and the observed decreases in [5-HIAA] are consistent with a decreased turnover of 5-HT almost certainly as a consequence of uptake inhibition (see Thomas *et al.*, 1987). Kinetic data obtained by these latter authors show that 5-HT uptake inhibition is demonstrable with the doses of paroxetine used in the present work. The brain indolylamine data shown in Table 5 may therefore be explained by paroxetine both enhancing synthesis, and decreasing turnover, of 5-HT.

The results in Table 5 also show that serum corticosterone concentration was decreased at 3.5 h after paroxetine administration. Whether an earlier increase occurs, as shown with fluoxetine (Stark *et al.*, 1985), has yet to be determined. A possible decrease in circulating cortisol concentration in patients with depression would be an additional advantage of therapy with paroxetine or other antidepressants, as this should limit excessive hepatic tryptophan degradation and also that of the tyrosine precursor of brain catecholamines.

We thank Mr A.M. Johnson and Smith Kline Beecham Pharmaceuticals for helpful discussions, a gift of paroxetine HCl and other support.

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(Received May 21, 1990
Revised August 6, 1990
Accepted September 25, 1990)

Br. J. Pharmacol. (1991), **102**, 434–438 © Macmillan Press Ltd, 1991

Evidence for nitric oxide as mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut

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1 The effects of haemoglobin, and the nitric oxide (NO) biosynthesis-inhibitors N^G-monomethyl-L-arginine (L-NMMA), its enantiomer D-NMMA, and N^G-nitro-L-arginine (L-NNA) were investigated on non-adrenergic non-cholinergic (NANC)-mediated relaxation of circular muscle strips of the canine terminal ileum and ileocolonic junction induced by electrical stimulation, adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA) and NO.

2 Tetrodotoxin, L-NMMA and L-NNA, but not D-NMMA, inhibited the relaxations induced by electrical stimulation, ATP and GABA, but not those in response to NO.

3 The inhibitory effect of L-NMMA and L-NNA was prevented by L-arginine, but not by D-arginine. L-Arginine did not potentiate any of the NANC relaxations.

4 Haemoglobin reduced the relaxation induced by electrical stimulation, ATP and GABA, and abolished those in response to NO.

5 Our results demonstrate that the ATP- and GABA-induced relaxations resulting from stimulaton of intramural NANC neurones, in addition to those induced by electrical impulses, are mediated by NO or a NO releasing substance and thus provide further evidence in support of the proposal that NO is the final inhibitory NANC neurotransmitter in the canine terminal ileum and ileocolonic junction.

Introduction

In the gastrointestinal tract, a major part of the inhibitory autonomic innervation appears to be provided by non-adrenergic non-cholinergic (NANC) nerves (Burnstock & Costa, 1973). However, the exact nature of the inhibitory neurotransmitter released by inhibitory NANC neurones still remains controversial. Depending on the tissue and/or species studied, mainly vasoactive intestinal polypeptide (VIP) (Goyal & Rattan, 1980; Grider *et al.*, 1985) or adenosine 5'-triphosphate (ATP) (Burnstock, 1972; 1981) have been suggested as the putative NANC neurotransmitter. Previously, we demonstrated the presence of an inhibitory NANC innervation in the canine terminal ileum and ileocolonic junction; stimulation of NANC neurones either by electrical impulses or by nicotinic receptor stimulation resulted in relaxations resistant to adrenoceptor and cholinoceptor blockade (Pelckmans *et al.*, 1989). The neurotransmitter released by these neurones is not VIP, ATP, γ-aminobutyric acid, 5-hydroxytryptamine, an opioid, somatostatin or substance P (Pelckmans *et al.*, 1989; Boeckxstaens *et al.*, 1990b,c,d,e). However, ATP, as well as GABA, stimulated purinoceptors and $GABA_A$ receptors respectively located on inhibitory NANC neurones resulting in the release of an unknown NANC neurotransmitter (Boeckxstaens *et al.*, 1990c,d).

Recently, we provided evidence in support of the proposal that nitric oxide (NO) or a related substance, which accounts for the biological activity of the vascular endothelium-derived relaxing factor (Palmer *et al.*, 1987), is the inhibitory NANC neurotransmitter in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990a; Bult *et al.*, 1990). The NANC relaxations induced by electrical impulses or acetylcholine were blocked by inhibitors of NO biosynthesis, N^G-monomethyl-L-arginine (L-NMMA) (Palmer *et al.*, 1988) and N^G-nitro-L-arginine (L-NNA) (Ishii *et al.*, 1990; Moore *et al.*, 1990; Mülsch & Busse, 1990) and in addition, haemoglobin, which avidly binds NO (Ignarro *et al.*, 1987; Kelm *et al.*, 1988) reduced these NANC relaxations (Boeckxstaens *et al.*, 1990a). Similarly, in the rat and mouse anococcygeus muscle, NO or a NO releasing substance has been proposed as the transmitter of NANC nerves (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989; Gillespie & Sheng, 1990; Gibson *et al.*, 1990). Furthermore, we were able to demonstrate the release of NO in response to NANC nerve stimulation by electrical impulses and 1,1-dimethyl-4-phenylpiperazinium (DMPP) (Bult *et al.*, 1990).

In the present study, we investigated whether the NANC relaxations induced by ATP and GABA were also mediated by NO or a NO releasing substance by means of L-NMMA, its enantiomer D-NMMA, L-NNA and haemoglobin.

Methods

Tissue preparation

Mongrel dogs of either sex (body weight 10–30 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and a laparotomy was performed. The ileum and colon were resected 10 cm above and 3 cm below the ileocolonic junction respectively. After the resected specimen was cleaned and rinsed, the mucosa was removed from the ileum and ileocolonic junction by means of sharp dissection. Circular muscle strips were cut, approximately 1.5 cm long and 0.3 cm wide and mounted in organ baths (25 ml) (Pelckmans *et al.*, 1989) filled with a modified Krebs-Ringer solution (mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, CaEDTA 0.026 and glucose 11.1). The solution was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

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Isometric tension recording

One end of each muscle strip was connected to a metal rod while the other end was attached to a strain gauge transducer (Statham UC2) for continuous recording of isometric tension. After the muscle strips were brought to the optimal point of their length-tension relationship (Pelckmans *et al.*, 1989), they were washed three times and then allowed to equilibrate for at least 45 min before experimentation.

Experimental protocols

All experiments were performed on muscle strips maximally contracted with noradrenaline (Pelckmans *et al.*, 1990) (30 μM; 2.7 $\pm$ 0.6 g in the ileum and 4.3 $\pm$ 0.5 g in the ileocolonic junction, mean $\pm$ s.e.mean; n = at least 6) and in the presence of 0.3 μM atropine. After each noradrenaline-induced contraction, the muscle strips were washed at least three times with an interval of 5 min, and a cycle time of at least 20 min.

In the first series of experiments, the effects of tetrodotoxin, haemoglobin, L-NMMA, its enantiomer D-NMMA, and L-NNA were studied on the relaxations (submaximal) induced by electrical stimulation (4 Hz, 1 ms), ATP (100 μM), GABA (100 μM) and NO (10 μM). In the second series of experiments, the inhibitory effect of L-NMMA and L-NNA was re-examined in the presence of L-arginine or D-arginine (2 mM). Electrical impulses (rectangular waves, 100 mA, 9 V) were delivered by a GRASS stimulator and a direct current (d.c.) amplifier in trains of stimuli of 10 s. Pharmacological agents were added at least 10 min prior to stimulation of the tissue.

Drugs used

The following drugs were used: adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA), D-arginine, L-arginine, bovine haemoglobin (Sigma Chemical Co., St. Louis, MO., U.S.A.), atropine sulphate (Federa, Brussels, Belgium), N^G-nitro-L-arginine (L-NNA), tetrodotoxin (Janssen Chimica, Beerse, Belgium), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland).

All drugs were administered as aqueous solutions except L-NNA which was dissolved in 65 mM HCl. Ascorbic acid (57 mM) was added to the stock solution of noradrenaline. These were prepared on the day of experimentation and administered in volumes not exceeding 0.5% of the bath volume. The stock solution of tetrodotoxin (1 mM, in sodium citrate, pH 4.8) was stored at -20°C. NO solutions and haemoglobin were prepared as described by Kelm *et al.* (1988). L-NMMA and D-NMMA were generously supplied by Dr S. Moncada (Wellcome Research Labs, Beckenham, Kent).

Presentation of results and statistical analysis

Results are expressed as percentage decrease of the noradrenaline-induced contraction and are shown as mean $\pm$ s.e.mean for the number of dogs indicated.

For statistical analysis, a two tailed Student's t test for paired observations was used. P values of less than 0.05 were considered to be significant.

Results

Effect of tetrodotoxin, L-NMMA, D-NMMA, L-NNA and haemoglobin on NANC relaxations induced by electrical stimulation, ATP, GABA or NO

During a noradrenaline (30 μM)-induced contraction and in the presence of 0.3 μM atropine, electrical stimulation (4 Hz, 1 ms), ATP (100 μM), GABA (100 μM) and NO (10 μM) induced submaximal relaxation of the canine terminal ileum and ileocolonic junction. These relaxations were qualitatively very similar as they were fast in onset and transient (Figures 1 and 2). Comparable to previous results, tetrodotoxin (0.6 μM), L-NMMA (100–300 μM), L-NNA (30–100 μM) and haemoglobin (10–30 μM) increased the basal tension in the muscle strips of the canine ileocolonic junction, but not in those of the terminal ileum (Figures 1 and 2). In both tissues, tetrodotoxin (0.6 μM) abolished and L-NMMA (100–300 μM) and L-NNA (30–100 μM) concentration-dependently inhibited the relaxations induced by electrical stimulation, ATP and GABA, whereas those induced by NO remained unaffected (Figures 1 and 2, Table 1). The inhibitory effect of L-NNA on these NANC relaxations was greater compared to L-NMMA in both the canine terminal ileum and ileocolonic junction (Figure 1b,c, Table 1). The enantiomer of L-NMMA, D-NMMA (100–300 μM), however, did not affect the relaxations (n = 3) (Figure 1d). Haemoglobin (10–30 μM) concentration-dependently reduced the relaxations in response to electrical stimulation, ATP and GABA, and abolished those induced by NO (Figure 2c, Table 2).

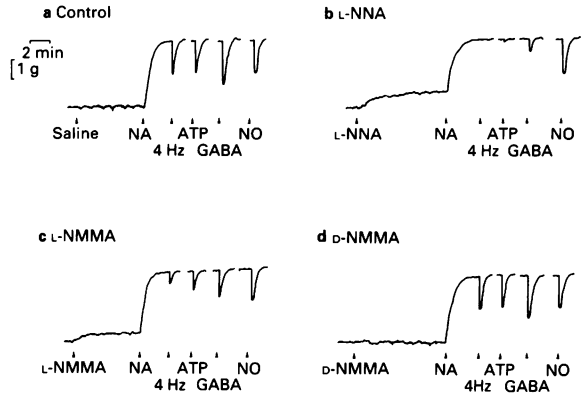


Figure 1 Isometric tension recordings showing (a) the control and the effect of (b) N^G-nitro-L-arginine (L-NNA, 100 μM), (c) N^G-monomethyl-L-arginine (L-NMMA, 300 μM) and (d) D-NMMA (300 μM) on NANC relaxations induced by electrical stimulation (4 Hz, 1 ms), adenosine 5'-triphosphate (ATP, 100 μM), γ-aminobutyric acid, (GABA, 100 μM) and nitric oxide (NO, 10 μM) in a muscle strip of the canine ileocolonic junction. L-NMMA and L-NNA, but not D-NMMA, inhibited the relaxations in response to electrical stimulation, ATP and GABA, but not those to NO. All effects were on a noradrenaline (NA, 30 μM)-induced contraction (4.3 $\pm$ 0.5 g, n = 6) in the presence of 0.3 μM atropine. Breaks in the tracings represent periods of tissue equilibration. Similar results were obtained in tissues from five other dogs and in the terminal ileum (n = 6).

After a wash-out period of 30–40 min, the effect of the inhibitors was removed as the amplitude of the NANC relaxations returned to their initial control values.

Effect of L-arginine or D-arginine on the inhibitory effect of L-NMMA and L-NNA

The presence of L-arginine (2 mM) prevented the inhibitory effect of both L-NMMA (100–300 μM) and L-NNA (30–100 μM) on the NANC relaxations induced by electrical stimulation, ATP and GABA in both the terminal ileum and ileocolonic junction (Table 1). In contrast, in the presence of D-arginine (2 mM), L-NMMA and L-NNA still inhibited these relaxations (Table 1). Preincubation with L-arginine did not potentiate any of the studied relaxations (n = 4, data not shown).

Discussion

In previous studies, we provided evidence for NO, a related substance or a NO releasing substance as the inhibitory NANC neurotransmitter in the canine terminal ileum and ileocolonic junction (Boeckxstaens *et al.*, 1990a; Bult *et al.*,

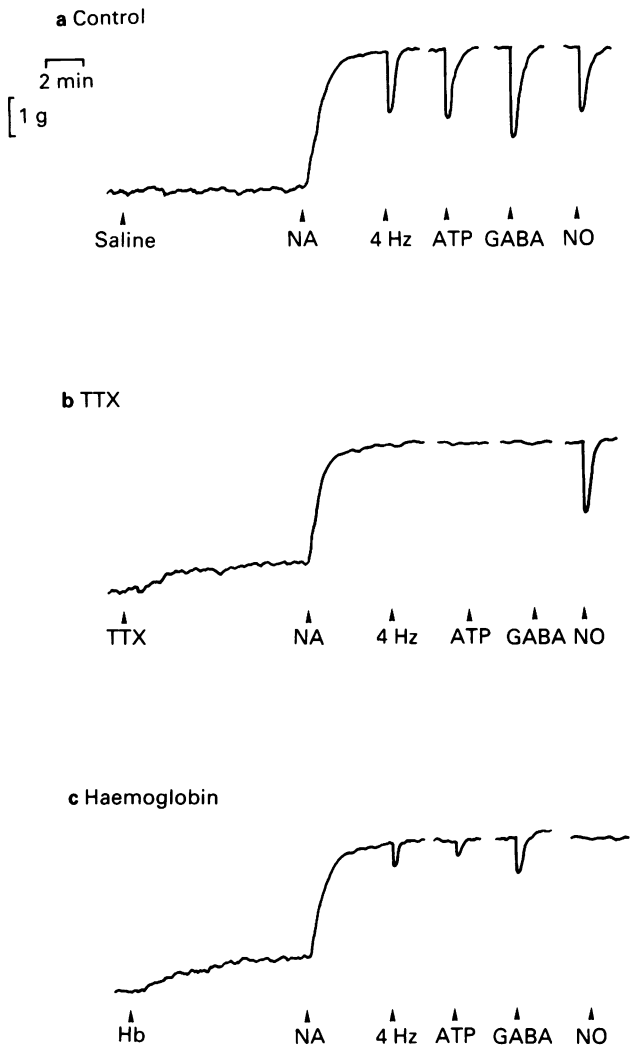


Figure 2 Isometric tension recordings showing (a) the control and the effect of (b) tetrodotoxin (TTX, 0.6 μM) and (c) haemoglobin (Hb, 30 μM) on NANC relaxations induced by electrical stimulation (4 Hz, 1 ms), adenosine 5'-triphosphate (ATP, 100 μM), γ-aminobutyric acid (GABA, 100 μM) and nitric oxide (NO, 10 μM) in a muscle strip of the canine ileocolonic junction. Tetrodotoxin abolished the relaxations in response to electrical stimulation, ATP and GABA, whereas those induced by NO remained unaffected. Haemoglobin reduced the relaxations in response to electrical impulses, ATP and GABA, whereas it abolished those to NO. All effects were on a noradrenaline (NA, 30 μM)-induced contraction (4.1 $\pm$ 0.4 g, $n = 6$) in the presence of 0.3 μM atropine. Breaks in the tracings represent periods of tissue equilibration. Similar results were obtained in tissues from five other dogs and in the terminal ileum ($n = 6$).

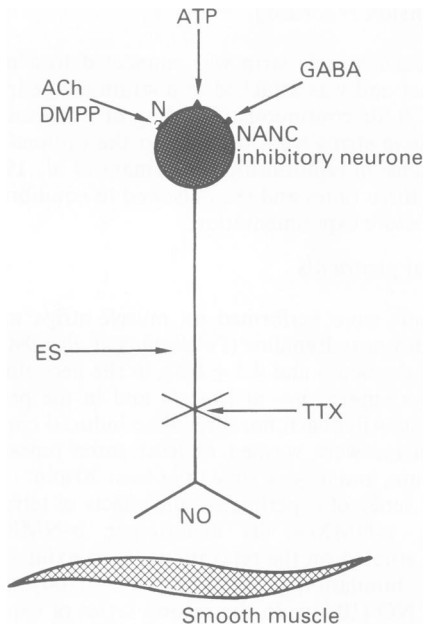


Figure 3 Schematic summary illustrating nitric oxide (NO) as the inhibitory neurotransmitter mediating the NANC relaxations in response to electrical stimulation (ES), adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA) and nicotinic receptor (N) stimulation by acetylcholine (ACh) and the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) in the canine terminal ileum and ileocolonic junction.

1990). The NANC relaxations evoked by electrical impulses or nicotinic receptor stimulation were blocked by inhibitors of NO biosynthesis and by haemoglobin. In addition, we demonstrated the release of NO in response to NANC nerve stimulation. The present study illustrates that the neurally-mediated NANC relaxations induced by ATP or GABA are also mediated by NO or a NO releasing substance, suggesting that NO is the common final NANC neurotransmitter in the canine terminal ileum and ileocolonic junction (Figure 3).

As previously reported (Boeckxstaens *et al.*, 1990c,d), the relaxations induced by ATP or GABA, like those in response to electrical stimulation of the inhibitory NANC neurones, were abolished by the blocker of nerve conduction tetrodotoxin which indicates mediation by nerves. Both L-NMMA and L-NNA inhibited these relaxations in a stereospecific and competitive manner, since their effect was only prevented by the substrate of NO biosynthesis, L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988) and not by D-arginine. Also the enantiomer of L-NMMA, D-NMMA, was without effect. Furthermore, as illustrated by the lack of effect of L-NMMA and L-NNA on the relaxation produced by NO, the blockade of the NANC-mediated relaxation was on the transmitter system rather than on the postjunctional effector cells. In addition, haemoglobin abolished the NO-induced relaxations and reduced the neurally-mediated responses. Considering the molecular size of haemoglobin, only a small proportion may have reached the neuromuscular junction which may explain the discrepancy in efficacy. Thus, those findings further favour NO or a NO releasing substance as mediator of the NANC relaxations induced by ATP and GABA in the canine terminal ileum and ileocolonic junction.

Finally, the rise in basal tension in the canine ileocolonic junction induced by tetrodotoxin and by inhibition of NO biosynthesis might suggest a constant background release of NO (Boeckxstaens *et al.*, 1990a). In contrast to our results, GABA released from myenteric neurones stimulates $GABA_A$ receptors on VIP and acetylcholine containing neurones to enhance the descending relaxation of the colonic peristaltic reflex via a predominant release of VIP (Grider & Makhlouf, 1990). In the rat duodenum on the other hand, $GABA_A$ receptor stimulation of intramural NANC neurones induced relaxation, possibly via the release of ATP (Maggi *et al.*, 1984; Manzini *et al.*, 1985). In the canine terminal ileum and ileocolonic junction however, ATP desensitization (Boeckxstaens *et al.*, 1990c) or trypsin (unpublished results) did not affect the GABA-induced relaxations and VIP failed to induce relaxation (Pelckmans *et al.*, 1989), indicating that it is unlikely that ATP or VIP mediates the responses to GABA.

NO or a related substance, which accounts for the biological activity of the vascular endothelium-derived relaxing factor (Palmer *et al.*, 1987), is synthesized from L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988). This so-called L-arginine: NO pathway has been demonstrated in a variety of cells and thus might represent a more general pathway for the regulation of cell function and communication (Moncada *et al.*, 1989). In the canine ileocolonic junction, relaxation resulting from stimulation of NANC neurones by either elec-

Table 1 Effect of N^G-monomethyl-L-arginine (L-NMMA, 300 μM) and N^G-nitro-L-arginine (L-NNA, 100 μM) on relaxations induced by electrical stimulation, adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA) or nitric oxide (NO) in the absence or presence of L- or D-arginine (2 mM)

	Electrical stimulation (4 Hz, 1 ms)	*ATP* (100 μM)	*GABA* (100 μM)	*NO* (10 μM)
(a) *Ileum*				
Control	40 ± 6	49 ± 7	70 ± 6	53 ± 7
L-NMMA	11 ± 4*	27 ± 9*	47 ± 11*	48 ± 9
L-NMMA/L-arginine	35 ± 9	61 ± 5	78 ± 6	58 ± 8
L-NMMA/D-arginine	14 ± 5*	20 ± 9*	41 ± 7*	60 ± 8
L-NNA	0*	8 ± 8*	29 ± 14*	60 ± 8
L-NNA/L-arginine	38 ± 6	42 ± 6	78 ± 8	52 ± 12
L-NNA/D-arginine	1 ± 1*	0*	0*	46 ± 9
(b) *Ileocolonic junction*				
Control	39 ± 7	37 ± 7	74 ± 8	65 ± 7
L-NMMA	16 ± 2*	18 ± 2*	41 ± 9*	65 ± 7
L-NMMA/L-arginine	40 ± 10	33 ± 5	65 ± 8	55 ± 6
L-NMMA/D-arginine	27 ± 6*	25 ± 7*	52 ± 8*	63 ± 10
L-NNA	1 ± 1*	3 ± 2*	9 ± 5*	61 ± 8
L-NNA/L-arginine	33 ± 8	31 ± 5	51 ± 10*	61 ± 8
L-NNA/D-arginine	0*	0*	4 ± 2*	66 ± 6

The experiments were performed during a noradrenaline (30 μM)-induced contraction in the presence of 0.3 μM atropine. Results are shown as mean ± s.e.mean for 5–7 experiments and expressed as percentage decrease of the noradrenaline-induced contraction.
* $P < 0.05$, significantly different from controm, Student's t test for paired observations.

Table 2 Effect of haemoglobin on relaxations induced by electrical stimulation, adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA) or nitric oxide (NO)

	Electrical stimulation (4 Hz, 1 ms)	*ATP* (100 μM)	*GABA* (100 μM)	*NO* (10 μM)
(a) *Ileum*				
Control	50 ± 9	46 ± 6	79 ± 7	58 ± 6
Haemoglobin 10 μM	29 ± 5*	16 ± 7	30 ± 11*	0*
Haemoglobin 30 μM	14 ± 6*	5 ± 3*	21 ± 8*	0*
(b) *Ileocolonic junction*				
Control	49 ± 6	56 ± 1	71 ± 7	65 ± 9
Haemoglobin 10 μM	22 ± 5*	36 ± 10*	50 ± 12*	0*
Haemoglobin 30 μM	17 ± 1*	31 ± 10*	41 ± 12*	0*

The experiments were performed during a noradrenaline (30 μM)-induced contraction in the presence of 0.3 μM atropine. Results are shown as mean ± s.e.mean for 6–7 experiments and expressed as percentage decrease of the noradrenaline-induced contraction.
* $P < 0.05$, significantly different from control, Student's t test for paired observations.

trical impulses or chemical agents (acetylcholine, DMPP (unpublished results), ATP, GABA) are inhibited by blockade of the NO biosynthesis or by haemoglobin. Although we cannot exclude the possibility that another, as yet unidentified, NANC neurotransmitter induces the release of the NO-like factor from some non-neuronal cell, e.g. the smooth muscle, we favour the view that NO is the transmitter itself and represents the common pathway mediating the NANC relaxations in the canine ileocolonic junction (Figure 3). This mechanism is not confined to this region of the gastrointestinal tract and/or species as we obtained similar results in the rat gastric fundus (Boeckxstaens *et al.*, 1990f) and canine lower oesophageal sphincter (De Man *et al.*, unpublished observations). Furthermore, evidence for NO or a substance releasing NO as the transmitter of NANC nerves was also provided in the rat and mouse anococcygeus muscle (Gillespie *et al.*, 1989; Ramagopal & Leighton, 1989; Gillespie & Sheng, 1990; Gibson *et al.*, 1990). In addition, in the central nervous system, a role for NO in neurotransmission has already been postulated (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989), further accentuating the importance of this L-arginine:NO pathway.

In conclusion, we provide evidence that the neurally-mediated relaxations induced by GABA or ATP are mediated by NO or a NO-releasing substance in a similar way as those in response to electrical impulses. Therefore, we propose NO as the common final neurotransmitter that mediates the NANC relaxations in the canine terminal ileum and ileocolonic junction (Figure 3).

This work was supported by the Belgian Fund for Medical Research (Grant 3.0014.90). G.E.B. is a Research Assistant of the National Fund for Scientific Research, Belgium (NFWO). The authors gratefully acknowledge F.H. Jordaens for the technical assistance and Mrs L. Van de Noort for typing the manuscript. We also wish to thank Dr S. Moncada (Wellcome Research Labs, Beckenham, Kent, U.K.) for the generous supply of L-NMMA and D-NMMA.

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(Received August 13, 1990
Revised September 24, 1990
Accepted September 26, 1990)

Br. J. Pharmacol. (1991), **102**, 439–445 © Macmillan Press Ltd, 1991

Mechanisms underlying the differential sensitivity to α_1-adrenoceptor activation in the bisected rat vas deferens

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1 The factors underlying the different responsiveness of the prostatic and epididymal portions of rat vas deferens to α_1-adrenoceptor stimulation were investigated.

2 The α_1-adrenoceptors in membranes of both halves of rat vas deferens were labelled with [^{3}H]-prazosin and the affinities of agonists and antagonists for these receptors were determined. In saturation studies, the B_{max} and K_D values for [^{3}H]-prazosin in membranes of both portions were the same.

3 In competition studies, the inhibition curves for phentolamine were biphasic and consistent with the presence of both α_{1a}- and α_{1b}-adrenoceptor subtypes. The proportions of binding sites with high and low affinity for phentolamine in both halves of rat vas deferens were similar and in good agreement with the percentages of binding sites for WB-4101 and phentolamine previously reported in the whole rat vas deferens.

4 The phenylethylamines displaced [^{3}H]-prazosin with a shallow inhibition curve. The data are compatible with the assumption of two affinity states for the binding sites. For the imidazoline compounds no such distinct affinity states could be demonstrated.

5 The affinity for, and the relative intrinsic efficacy on postsynaptic α_1-adrenoceptors of both portions of rat vas deferens were studied for noradrenaline, phenylephrine and methoxamine by irreversible inactivation of the α_1-adrenoceptors by phenoxybenzamine. The parameters for partial agonists were determined by comparing the responses to the partial agonist to those of a full agonist in the same tissue. Homogeneous estimates of the equilibrium dissociation constants (K_a) were obtained, indicating that these agonists bind to the receptors of both tissues in an identical manner. Further, estimation of the intrinsic efficacy of agonists relative to noradrenaline, indicated no differences between the two halves of rat vas deferens.

6 K_a values for agonist activation of the functional α_1-adrenoceptors were compared with K_I values for agonist inhibition of specific [^{3}H]-prazosin binding. The K_a values were well correlated with the low affinity K_I values for phenylethylamines in both portions of rat vas deferens, suggesting that the initial event in signal transduction by α_1-adrenoceptors is the binding to the low affinity state of the receptor.

7 There was a non-linear relationship between response and receptor occupancy in both halves of rat vas deferens but the occupancy-response coupling was more efficient in the epididymal than in the prostatic portion. This fact may account for the differences observed in the functional responses.

Introduction

The rat vas deferens has been commonly considered as a suitable preparation for studying α-adrenoceptors. However, there is considerable evidence to substantiate that prostatic and epididymal portions of this tissue differ not only in their motor transmission (McGrath, 1978; Brown *et al.*, 1983) but in their postsynaptic sensitivity to α-adrenoceptor agonists (Vardolov & Pennefather, 1976; Kasuya & Suzuki, 1979; MacDonald & McGrath, 1980; Moore & Griffiths, 1982). In this regard it has been demonstrated that the imidazoline α_1-adrenoceptor agonist St-587 behaves both as a partial agonist at α_1-adrenoceptors in the epididymal half of the rat vas deferens and as an antagonist of prostatic α_1-adrenoceptors, suggesting that a different efficiency of stimulus-response mechanism is operating along the tissue (Badia & Sallés, 1989). These differences in the sensitivity of each portion of rat vas deferens to α-adrenoceptor activation may therefore depend on factors such as variations in receptor density, changes in the affinity of agonists or to the presence or absence of spare receptors. In addition, recent binding studies reported the existence of two α_1-adrenoceptor subtypes in the rat vas deferens (Han *et al.*, 1987; Hanft & Gross, 1989), so that heterogeneous distribution of these subtypes may also be involved in the different responsiveness of each portion of the tissue to agonists. In order to elucidate the factors responsible for these functional differences, the binding parameters for α_1-adrenoceptors and the contractile responses to different agonists in each half of rat vas deferens were determined. Thus, the equilibrium dissociation constants for agonists were calculated by two independent methods. In addition, it was possible to determine receptor occupancy and to analyze its relationship with response.

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Methods

Animals

Adult male Sprague-Dawley rats weighing 250 to 350 g were used for the experiments. Both vasa deferentia were removed, carefully cleaned and bisected into prostatic and epididymal portions. These preparations were used for functional or radioligand binding assays.

Radioligand binding studies

For binding assays, the epididymal and prostatic portions from several animals were pooled separately, placed in 10 ml of ice cold 50 mM Tris/HCl buffer pH 7.5, and homogenized with a polytron type homogenizer (Rafer$^{10/20}$, setting 6, 2 × 15 s). Homogenates were filtered through a double layer of surgical gauze and centrifuged at 49,000*g* for 20 min. Pellets were washed twice by resuspension and centrifugation under the same conditions. Final pellets were resuspended in the same buffer at 2 mg protein per ml approximately. For

saturation experiments aliquots (100 μg protein) of membrane suspensions were incubated in triplicate with increasing concentrations (0.05 to 4 nM) of [^{3}H]-prazosin in 0.25 ml (final volume) of 50 mM Tris buffer pH 7.5 at 25°C for 45 min. The potencies of drugs in competing for the specific [^{3}H]-prazosin binding was determined by incubation of a single concentration of [^{3}H]-prazosin (0.3–0.4 nM) in the presence or absence of 15–20 concentrations of competing drug (range: 0.01 nM–1 mM). Incubations were stopped by rapid filtration through Whatman GF/C glass fibre filters which were subsequently washed 3 times with 5 ml of the same buffer. Radioactivity retained in the filters was determined at 48–50% efficiency by liquid scintillation spectrometry. Nonspecific binding was defined as that insensitive to 10 μM phentolamine. Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Functional studies

For the measurement of contractile responses, prostatic and epididymal halves of rat vas deferens were set up in isolated organ baths containing 20 ml of Krebs bicarbonate solution composition (in mM): NaCl 112.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1. The solution was maintained at 32 ± 0.5°C and gassed with 95% O_2 and 5% CO_2. Propranolol (1 μM), desmethylimipramine (0.1 μM) and normetanephrine (1 μM) were continuously present in the medium throughout the experiment to block β-adrenoceptors, and neuronal and extraneuronal uptake respectively. The preparations were equilibrated for at least 45 min at a resting tension of 0.5 g. Thereafter, in order to obtain a maximal response the tissues were exposed twice to 100 μM of noradrenaline and thoroughly washed. Contractions were registered by means of an isometric transducer (UF-1) on an OmniScribe pen-recorder.

Agonist-induced increases in isometric tension were determined by use of a non-cumulative dosage schedule with 6 min intervals between doses. Responses were measured as the peak tension developed while the preparation was exposed to the agonist and are expressed as a percentage of the maximal contraction induced by noradrenaline. Thus, noncumulative concentration-response curves of isometric contractions were obtained for the agonists in each tissue and the EC_{50} (molar concentration of agonist inducing 50% of its maximal effect) was determined by linear regression of all points between 20–80% of the maximal response to that agonist.

To evaluate the antagonist potency of prazosin and phentolamine, cumulative agonist concentration-response curves were constructed in the absence and presence of the test antagonist. The maintained ('tonic') component of the contraction to the agonist was measured. Antagonists were allowed to equilibrate for 30 min before addition of the agonist. α_1-Adrenoceptor antagonist potency was expressed as a pA_2 value which was determined by the method of Arunlakshana & Schild (1959).

Exposure to phenoxybenzamine

After performing the initial non-cumulative concentration-response curve for agonist-induced contractions, preparations were washed three times. Fifteen minutes later, phenoxybenzamine at the selected concentration was added, and at the end of the incubation period (10 min) the tissues were washed three times successively and then six times over the following 60 min. Ten minutes later, agonist concentration-response curves were repeated. In some experiments the contralateral half was used as a control to correct the time-dependent changes in sensitivity of the tissue to agonist (Furchgott, 1972).

The equilibrium activation constants (K_A values) and relative efficacies of full agonists were determined by the method developed by Furchgott (Furchgott, 1966; Furchgott & Bursztyn, 1967). This method employs the use of an irreversible antagonist to produce both a shift and a depression of the concentration-response curve of the agonist. To obtain K_A values in an experiment of the type outlined, the reciprocal of the concentrations of an agonist to produce given responses before phenoxybenzamine exposure (1/[A]) were plotted against the reciprocals of the corresponding equieffective concentrations after the treatment with the irreversible antagonist (1/[A']). The values for K_A and for the fraction of active receptors remaining (q) was calculated from the slope and intercept of the straight line fitting the points, on the basis of the equation (Furchgott, 1966):

$$\frac{1}{[A]} = \frac{1}{q[A']} + \frac{1-q}{qK_A}$$

according to which K_A equals (slope-1)/intercept, and q equals 1/slope. For each experiment, the agonist concentration-response curves before and after phenoxybenzamine treatment were analyzed by linear regression of all points between 20% and 80% of the maximal response obtained in each case. Using the linear functions obtained in this manner, the concentration of agonist necessary to give 20, 30, 40, 50, 60% of the maximal response before and after phenoxybenzamine treatment was calculated. Linear regression of the reciprocals of these calculated values resulted in K_A and q values as described above.

The equilibrium activation constants of partial agonists (K_P) were also determined by a second method in which the responses to the partial agonist are compared to those of a full agonist in the same tissue (Waud, 1969). In this method the reciprocals of the concentrations of a full agonist to produce given responses (1/[A]) are plotted against the reciprocals of the concentrations of a partial agonist which produce the same responses (1/[P]). The K_P values of the partial agonists were calculated by the equation (Waud, 1969)

$$K_P = \frac{\text{slope}}{\text{intercept}}$$

Once the K_A and K_P values for full and partial agonists had been calculated by the methods mentioned above, the fractional receptor occupancy for a given response in each half of rat vas deferens could be determined from the equation:

$$[R]/[R_t] = [A]/(K_a + [A])$$

where [A] is the concentration of agonist producing a given response, $[R]/[R_t]$ is the fractional receptor occupancy and K_a is the dissociation constant of the receptor-agonist complex.

Occupancy-response curves for agonists in each half of rat vas deferens were plotted on a logarithmic abscissa of % occupancy. Since absolute efficacy cannot be determined, it is usual to assign relative efficacies within a series of agonists, a full agonist being used as reference (Furchgott & Bursztyn, 1967). A relative efficacy of 1.0 was assigned for noradrenaline. The antilog of the distance between the two occupancy-response curves along the abscissa scale was taken as the ratio of the intrinsic efficacy of the second agonist to that of noradrenaline (Furchgott & Bursztyn, 1967), and thus it was a measure of the relative efficacy (e_r) of the second agonist as compared to noradrenaline.

Statistical analysis

The data were expressed as means ± s.e. of the mean. Student's two-tailed t test was used for statistical evaluations. Correlation coefficients were calculated by the least squares method. Significance level was set as $P < 0.05$. Ligand binding data were analyzed with computerized curve-fitting programs as described by McPherson (1983), F-test analysis was used to decide whether a model of one or two binding sites was more appropriate ($P < 0.05$).

Drugs

Drugs used and their sources were: noradrenaline bitartrate (Sigma), phenylephrine hydrochloride (Sigma), methoxamine hydrochloride (Gayoso-Wellcome), St-587 (2-(2-chloro-5-trifluoromethylphenylimino)imidazolidine nitrate) (Boehringer Ingelheim), prazosin hydrochloride (Pfizer), propranolol hydrochloride (Fides), desmethylimipramine hydrochloride (Sigma), normetanephrine hydrochloride (Calbiochem), yohimbine hydrochloride (Sigma), phentolamine methansulphonate (Ciba-Geigy), phenoxybenzamine hydrochloride (Smith, Kline and French), tolazoline hydrochloride (Ciba-Geigy) and [^{3}H]-prazosin (76 Ci mmol^{-1}, New England Nuclear).

Results

Binding studies

Measurement of [^{3}H]-prazosin binding to α_1-adrenoceptors in epididymal and prostatic halves of rat vas deferens The B_{max} and K_D values were not different in membranes of both portions (prostatic half: $K_D = 0.46 \pm 0.04$ nM and $B_{max} = 132.3 \pm 7.2$ fmol mg^{-1} of protein, $n = 6$; epididymal half: $K_D = 0.44 \pm 0.04$ nM and $B_{max} = 130.2 \pm 9.4$ fmol mg^{-1} of protein, $n = 6$). Specific binding was saturable and of high affinity. Scatchard analysis of saturation isotherms resulted in straight lines suggesting that the [^{3}H]-prazosin labels a homogeneous population of receptors in both portions of rat vas deferens. A Hill coefficient of 0.97 ± 0.03 and 0.95 ± 0.02 was obtained in the prostatic and epididymal portions respectively, indicating a lack of cooperativity and reinforcing that [^{3}H]-prazosin binds to a single population of receptors. Specific binding determined by 10 μM phentolamine was approximately 70% of the total binding at a concentration of [^{3}H]-prazosin close to the K_D value.

Displacement of specific [^{3}H]-prazosin binding by α-adrenoceptor antagonists and agonists Specific binding of [^{3}H]-prazosin to membranes of both halves of rat vas deferens was inhibited by several α-adrenoceptor antagonists (Table 1). The order of potency was prazosin > phentolamine ≫ yohimbine = tolazoline in each portion, a pattern similar to that expected for the interaction of these ligands with an α_1-adrenoceptor. As shown in Table 1, prazosin, yohimbine and tolazoline inhibited the specific binding of [^{3}H]-prazosin monophasically with pseudo-Hill coefficients (n_H) close to unity and with similar K_I values in both tissues. In contrast, phentolamine inhibited [^{3}H]-prazosin binding in both portions and resulted in shallow competition curves with pseudo-Hill coefficients significantly lower than unity. Iterative non-linear curve-fitting analysis (Munson & Rodbard, 1980) of competition curves obtained for phentolamine in both portions revealed two different components for the interaction of these agents at [^{3}H]-prazosin sites. The two populations of sites were present in a proportion approximately equal in both halves of rat vas deferens (Table 1).

As shown in Table 2, the parameters estimated by nonlinear regression of the competition curves of the agonists were similar in both halves of rat vas deferens. [^{3}H]-prazosin binding was inhibited monophasically with pseudo-Hill coefficients (n_H) close to unity by the imidazoline compounds St-587 and clonidine, and with similar K_I values in both portions. In contrast, the phenylethylamine compounds, noradrenaline, phenylephrine and methoxamine inhibited [^{3}H]-prazosin binding in a biphasic way, resulting in shallow inhibition curves with pseudo-Hill coefficients significantly lower than unity in both portions. Two-site analysis of the data suggests the existence of two affinity states of α_1-adrenoceptors for agonists in these tissues.

Functional studies

Concentration-response curves of agonists In the epididymal portion all the agonists studied were able to induce increases

Table 1 Inhibition of [^{3}H]-prazosin binding to α_1-adrenoceptors of the bisected rat vas deferens by antagonists

Antagonist	n	n_H	K_{High}	K_{Low}	% R_{High}
Prostatic half					
Prazosin	5	0.93 ± 0.03	0.56 ± 0.03 nM		100
Phentolamine	4	0.64 ± 0.02	6.10 ± 0.15 nM	78.15 ± 3.30 nM	57.16 ± 3.40
Yohimbine	4	1.02 ± 0.03	2.86 ± 0.15 μM		100
Tolazoline	4	0.92 ± 0.03	7.08 ± 0.52 μM		100
Epididymal half					
Prazosin	5	0.99 ± 0.03	0.52 ± 0.02 nM		100
Phentolamine	6	0.66 ± 0.02	6.80 ± 0.50 nM	80.40 ± 3.28 nM	50.70 ± 3.20
Yohimbine	6	1.10 ± 0.11	3.15 ± 0.37 μM		100
Tolazoline	4	0.96 ± 0.04	8.05 ± 0.55 μM		100

Data are expressed as means ± s.e.mean of *n* experiments.

Table 2 Inhibition of [^{3}H]-prazosin binding to α_1-adrenoceptors of the bisected rat vas deferens by agonists

Agonists	n	n_H	K_{High} (μM)	K_{Low} (μM)	%R_{High}
Prostatic half					
Noradrenaline	5	0.58 ± 0.02	0.19 ± 0.01	3.51 ± 0.15	45.30 ± 3.50
Phenylephrine	4	0.60 ± 0.03	1.47 ± 0.21	11.99 ± 0.82	50.15 ± 3.67
Methoxamine	3	0.62 ± 0.02	0.71 ± 0.05	36.58 ± 1.96	50.20 ± 3.76
St-587	4	0.99 ± 0.02	1.14 ± 0.06		100
Clonidine	4	1.06 ± 0.02	1.48 ± 0.05		100
Epididymal half					
Noradrenaline	5	0.59 ± 0.03	0.22 ± 0.03	5.68 ± 0.48	44.25 ± 6.03
Phenylephrine	4	0.71 ± 0.03	1.47 ± 0.03	11.03 ± 1.03	52.30 ± 2.60
Methoxamine	3	0.62 ± 0.03	0.72 ± 0.05	42.20 ± 2.36	53.20 ± 2.45
St-587	4	0.96 ± 0.02	1.26 ± 0.07		100
Clonidine	4	1.08 ± 0.02	1.41 ± 0.03		100

Data are expressed as means ± s.e.mean of *n* experiments.

in the tension in a concentration-dependent manner between 0.1 to 100 μM. However, only the three phenylethylamines were full agonists, while the imidazoline compounds behaved as partial agonists achieving 50% of the maximal effect of full agonists (Figure 1b). In the prostatic portion (Figure 1a) phenylephrine and methoxamine elicited a maximal response only 69.2 ± 1.0% and 60.3 ± 2.0% of that induced by noradrenaline respectively, and the two imidazoline compounds were only able to produce rhythmic contractions at high concentrations (100 nM) (Figure 1a). As shown in Figure 1, each agonist was more potent in the epididymal than in the prostatic portion.

To determine whether the actions of the agonists in each portion were mediated through activation of α_1-adrenoceptors, the selective and nonselective α_1-adrenoceptor antagonists, prazosin and phentolamine were used. As shown in Table 3, the pA_2 values for each antagonist in both portions of rat vas deferens were virtually identical against the three phenylethylamines and St-587 in the epididymal portion. In all cases prazosin was thirty times more potent than phentolamine for each agonist. However the dissociation constant of prazosin was higher in functional studies compared with binding studies. Such differences may be due to the fact that incubation time was very short to establish equilibrium in the functional studies as has been previously reported (McGrath, 1984). The Schild regression analysis resulted in straight lines, their slopes being not significantly different from the theoretical value of 1. This indicates a reversible competition between the antagonist and the agonist for a single binding site which has the pharmacological characteristics of an α_1-adrenoceptor.

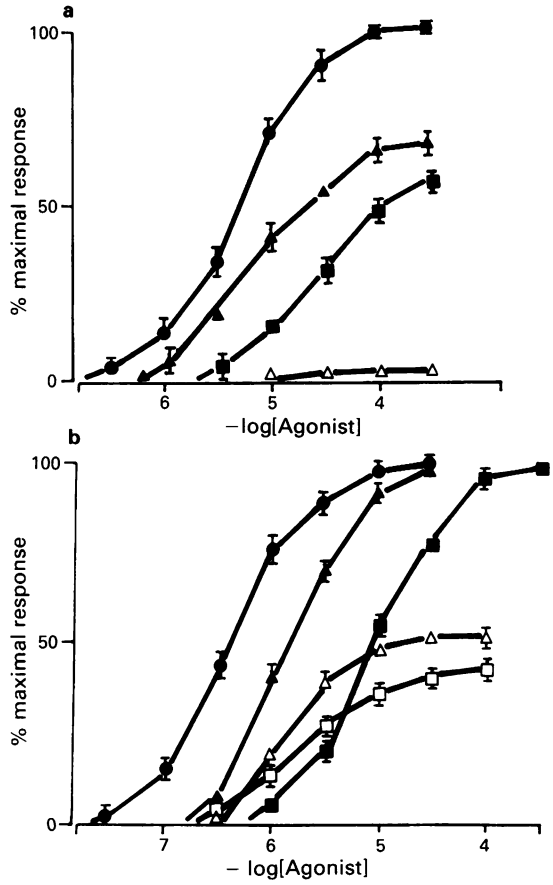


Figure 1 Concentration-response curves of α-adrenoceptor agonists in (a) prostatic and (b) epididymal portions of rat vas deferens. Responses to noradrenaline (●, $n = 10$), phenylephrine (▲, $n = 6$), methoxamine (■, $n = 6$), St-587 (△, $n = 6$), clonidine (□, $n = 6$) for each tissue. Vertical bars show s.e.mean.

Effect of phenoxybenzamine exposure on noradrenaline concentration-response curves in each portion of rat vas deferens In order to determine the contribution of spare receptors in the different sensitivity to the agonists in each portion of rat vas deferens, some experiments were designed to obtain a controlled partial alkylation of a certain proportion of receptors. Firstly, each portion was exposed to increasing concentrations of phenoxybenzamine (3 nM–1 μM) for 10 min. The analysis of concentration-response curve to noradrenaline after phenoxybenzamine incubation showed that low concentrations of the alkylating agent (3 and 10 nM) depressed by 40% and 80% the maximal contractile response to the agonist in the prostatic portion. In contrast, in the epididymal portion higher concentrations of phenoxybenzamine (0.03–0.3 μM) were required to obtain a similar decrease in the maximal contractile response to the agonist. In the receptor analysis the selected concentrations of phenoxybenzamine were 0.1 μM and 3 nM for the epididymal and prostatic portions respectively.

Determination of K_a values for full agonists (K_A) Table 4 shows the maximal responses (E_{max}') and the EC_{50}' values for the agonists after phenoxybenzamine incubation as compared with control experiments. The K_A and q determined for each agonist are also shown. Phenoxybenzamine (0.1 μM) after 10 min incubation caused a decrease in potency (17–19 times) and in maximal contractile response (30–40%) for the three agonists in the epididymal portion. In contrast, in the prostatic portion, phenoxybenzamine (3 nM) after 10 min of incubation induced only a moderate decrease in potency (1.4 times) and a marked depression of maximal response (40%) to noradrenaline.

Determination of K_a values for partial agonists (K_P) Since the E_{max} values induced by phenylephrine and methoxamine were smaller than those induced by noradrenaline in the prostatic half, the former compounds were considered as partial

Table 3 Mean pA_2 values of prazosin and phentolamine against α-adrenoceptor agonists in the bisected rat vas deferens

		Antagonist				
		Prazosin			*Phentolamine*	
Agonist	n	*Slope*	*pA_2*	n	*Slope*	*pA_2*
Prostatic half						
Noradrenaline	6	0.98 ± 0.03	8.47 ± 0.05	6	1.02 ± 0.03	7.65 ± 0.05
Phenylephrine	5	1.01 ± 0.02	8.43 ± 0.02	6	0.99 ± 0.05	7.61 ± 0.06
Methoxamine	6	0.95 ± 0.02	8.50 ± 0.05	6	1.04 ± 0.06	7.58 ± 0.05
Epididymal half						
Noradrenaline	6	0.95 ± 0.03	8.48 ± 0.02	6	0.92 ± 0.04	7.65 ± 0.03
Phenylephrine	6	1.08 ± 0.08	8.45 ± 0.03	6	1.01 ± 0.02	7.70 ± 0.04
Methoxamine	6	0.98 ± 0.03	8.50 ± 0.05	6	0.95 ± 0.02	7.54 ± 0.06
St-587	5	0.92 ± 0.05	8.52 ± 0.05			—

Data are expressed as means ± s.e.mean of n experiments.

Table 4 EC_{50}' and $\%E_{max}'$ values for agonists after phenoxybenzamine treatment and estimated values of K_A and q in the bisected rat vas deferens

Agonist	EC_{50}' (μM)	$\%E_{max}'$	EC_{50}'/EC_{50}	K_A(μM)	*q*	n
Prostatic half						
Noradrenaline	7.12 ± 0.52	60.04 ± 1.01	1.4	9.45 ± 0.30	40.6 ± 0.9	6
Epididymal half						
Noradrenaline	8.33 ± 0.32	70.03 ± 2.03	19	8.65 ± 0.2	4.5 ± 0.01	6
Phenylephrine	26.68 ± 1.43	64.94 ± 1.01	18	28.30 ± 0.3	6.8 ± 0.03	6
Methoxamine	161.21 ± 8.32	62.56 ± 1.27	17	118.36 ± 11.3	5.2 ± 0.04	6

Data are expressed as mean ± s.e.mean of *n* experiments.

agonists in this tissue. In the same way St-587 and clonidine were partial agonists in the epididymal half. The equilibrium activation constants of the partial agonists (K_P) in both halves were calculated as described by Waud (1969). The plot of the reciprocals of equieffective concentrations between the full and the partial agonist resulted in a straight line that allowed to estimate the K_P values for each partial agonist in the corresponding portion. The EC_{50}, E_{max} and K_P values are summarized in Table 5.

Relative efficacies of the agonists and relationships between occupancy and response The relative efficacies of the agonists were estimated by the method of Furchgott and Bursztyn (1967) in both portions of rat vas deferens. A plot of the relative responses to noradrenaline and the other agonists against the logarithm of the theoretical percentage of receptor occupancy yielded sigmoid curves. The antilog of the distance along the abscissa scale between each one of the agonists curves and the noradrenaline curve represents the relative efficacy (e_r) of each compound. As shown in Table 6, the e_r values for each compound were the same in the two portions. The plot of the fractional responses for noradrenaline against the theoretical percentage of receptor occupancy resulted in hyperbolic curves (Figure 2). As shown, noradrenaline induces the maximal effect in the epididymal half when only 50% of the receptors were occupied, but in the prostatic portion 100% of the receptors were required to elicit the maximal response.

Table 5 EC_{50} and $\%E_{max}$ values of partial agonists and estimated K_P values in the bisected rat vas deferens

Agonist	EC_{50} (μM)	$\%E_{max}$	K_P (μM)	n
Prostatic half				
Phenylephrine	7.68 ± 0.04	67.25 ± 1.65	22.64 ± 0.07	6
Methoxamine	25.72 ± 0.75	57.39 ± 3.30	100.80 ± 1.70	6
Epididymal half				
St-587	1.35 ± 0.03	51.49 ± 2.68	1.33 ± 0.02	6
Clonidine	2.88 ± 0.06	44.31 ± 1.70	1.53 ± 0.06	6

Data are expressed as mean ± s.e.mean of *n* experiments.

Comparison of K_I (K_{High}, K_{Low}) *values for binding and the calculated* K_a (K_A *or* K_P) *values for* α_1*-adrenoceptor agonists in both halves of rat vas deferens* For the imidazoline compounds there was a good similarity between the K_P values obtained in functional studies and the K_I values for inhibiting [^{3}H]-prazosin binding in the epididymal portion. In turn, the results obtained with the phenylethylamine compounds showed that the affinity rank order of these agonists (K_A, K_P) in functional studies in both portions of rat vas deferens, was

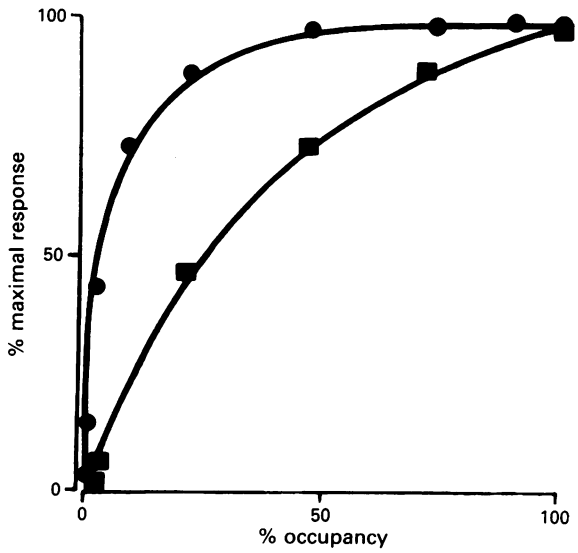


Figure 2 Curves of percentages of response of noradrenaline to maximum contractile effect against their percentage of receptor occupancy in the (■) prostatic and (●) epididymal portions of rat vas deferens.

Table 6 Comparison of equilibrium dissociation constants calculated by different methods and relative efficacies of α-adrenoceptor agonists in the bisected rat vas deferens

	Prostatic		*Epididymal*	
Agonists	K_a (μM)	e_r	K_a (μM)	e_r
Noradrenaline	9.45 ± 0.30[a]	1	8.65 ± 0.22[a]	1
Phenylephrine	22.64 ± 1.32[b]	0.67 ± 0.02	28.30 ± 1.61[a]	0.66 ± 0.03
Methoxamine	100.80 ± 10.31[b]	0.53 ± 0.03	118.23 ± 11.30[a]	0.52 ± 0.01
St-587	1.31 ± 0.02[c]	—	1.33 ± 0.05[b]	0.032 ± 0.002
Clonidine	ND		1.53 ± 0.06[b]	0.025 ± 0.002

Data are expressed as mean ± s.e.mean of at least 6 experiments. ND = not determined. [a]Determined by the method of Furchgott. [b]Determined with the method of Waud. [c]Determined by the method of Schild (Badia & Sallés, 1989).

similar to the rank order of their dissociation constants (K_I) for the low-affinity state of the receptor (Tables 2 and 6). Moreover, an excellent correlation for prostatic ($r = 0.9931$, $P < 0.001$) and epididymal portions ($r = 0.9951$, $P < 0.001$) was obtained between the calculated dissociation constants from functional and binding studies.

Discussion

Several studies performed in the whole vas deferens have demonstrated that the increases in the isometric tension elicited by noradrenaline and other compounds are due to an interaction with α_1-adrenoceptors and that this tissue lacks postsynaptic α_2-adrenoceptors (Minneman *et al.*, 1983; Minneman & Abel, 1984). A functional reserve of α_1-adrenoceptors for phenylethylamines, and the existence of a non-linear relationship between the fractional receptor occupancy and the fractional response elicited by these agonists (Minneman *et al.*, 1983; Minneman & Abel, 1984; Diaz-Toledo & Martí, 1988) has also been determined. On the other hand, accumulating evidence supports the idea that the prostatic and epididymal portions of rat vas deferens differ in their postsynaptic sensitivity to α-adrenoceptor agonists (Vardolov & Pennefather, 1976; Kasuya & Suzuki, 1979; Moore & Griffiths, 1982), the epididymal portion being more susceptible to the excitatory effects of imidazolines and phenylethylamines (MacDonald & McGrath, 1980; Badia & Sallés, 1989) than the prostatic portion.

Our results also confirm and extend the above mentioned findings; in the epididymal portion all the phenylethylamines tested behaved as full agonists and the imidazoline compounds showed only partial agonist activity. In contrast, in the prostatic portion only noradrenaline was a full agonist, while phenylephrine and methoxamine were partial agonists and the two imidazolines were unable to contract the tissue. The pA_2 values obtained for prazosin and phentolamine against all the agonists tested support the assumption that these compounds elicit the contractile responses by an interaction with α_1-adrenoceptors in each portion. In addition, it has been previously demonstrated that St-587 is an α_1-adrenoceptor antagonist in the prostatic portion with a pA_2 value similar to pEC_{50} value obtained in the epididymal half (Badia & Sallés, 1989), supporting an interaction of this compound with the same α_1-adrenoceptor activated by phenylethylamines.

The existence of gradients in responsiveness to agonists which coincide with anatomical orientation has been extensively demonstrated in different tissues. For example, rabbit aorta is not uniformly sensitive to β-adrenoceptor agonists (Altura & Altura, 1970) and in the rabbit basilar artery a graded responsiveness to noradrenaline, increasing from the distal to the proximal portions has also been reported (Gintautas *et al.*, 1980). This variation in the response may be ascribed to the heterogeneous distribution of receptor types, as occurs in the urinary bladder of the rabbit: in the bladder body, α-adrenoceptors predominate over β-adrenoceptors whereas in the bladder base the opposite predominance occurs (Levin & Wein, 1979). Thus, the responses of both halves of rat vas deferens to selective α-adrenoceptor agonists may depend on a heterogeneous distribution of postsynaptic α_1-adrenoceptors, as previously suggested (MacDonald & McGrath, 1980). Other possibilities include changes in affinity of the agonists for the receptors, a heterogeneous distribution of the recently proposed α_1-adrenoceptor subtypes or differences in the efficiency of receptor coupling to response. All these possibilities were analyzed.

In binding studies, [^{3}H]-prazosin labels an apparently homogeneous population of α_1-adrenoceptors in each half of rat vas deferens. The maximal number of binding sites and the K_D values for [^{3}H]-prazosin were not significantly different between the two tissues. The order of potency of α_1-adrenoceptor antagonists to displace [^{3}H]-prazosin specific binding to membranes of both portions was similar to that expected for an interaction with an α_1-adrenoceptor (Minneman *et al.*, 1983). However, the competition curves for phentolamine were biphasic, indicating two distinct binding sites. The percentages of high- and low-affinity sites in both portions as defined by the use of this antagonist were not different. Similar results have been previously reported in other tissues (including the rat vas deferens) with [^{3}H]-prazosin used as radioligand. In these studies, on the basis of the affinities of several antagonists, the concept of α_1-adrenoceptor subtypes was introduced (Morrow & Creese, 1986; Gröss *et al.*, 1988; Hanft & Gröss, 1989; Hanft *et al.*, 1989): the binding sites with high affinity for WB-4101 and phentolamine were designated as α_{1A} (or α_{1a}) and those with low affinity for these antagonists α_{1B} (or α_{1b}) (Morrow & Creese, 1986). On the basis of our present results it is very difficult to ascribe the differences in functional responsiveness between the two portions of rat vas deferens to a heterogeneous distribution of α_1-adrenoceptor subtypes.

Analysis of competition experiments with imidazoline compounds yielded monophasic curves with pseudo-Hill coefficients (n_H) close to unity and with similar K_I values in both portions. In contrast, phenylethylamines inhibited [^{3}H]-prazosin binding with pseudo-Hill coefficients significantly lower than unity in each portion of rat vas deferens. Two-site analysis of the data suggests the existence of two α_1-adrenoceptor binding sites in these tissues. K_I values for the high (K_{High}) and for the low affinity sites (K_{Low}) for these drugs were in good agreement in both portions. However, several observations suggest that the two affinity states for agonists are distinct from the α_{1A}/α_{1B} binding sites above mentioned. It has been demonstrated that full agonists, like adrenaline and noradrenaline, can bind to α_1-adrenoceptors with two affinity constants in various tissues (Ernsberger & U'Prichard, 1987; Gross *et al.*, 1988). Moreover, the addition of guanosine 5'-triphosphate (GTP) or its non hydrolizable analogues can convert high affinity binding sites of α_1-adrenoceptor for agonists into the low affinity state without affecting the antagonist binding (Gross *et al.*, 1988). In any case, the differences in responsiveness after α_1-adrenoceptor activation observed in each portion of rat vas deferens are not associated with differences in the characteristics of binding.

In functional studies the equilibrium dissociation constants (K_A) for full agonists were determined by the method described by Furchgott (1966), and for partial agonists (K_P) by the method of Waud (1969). In our study the K_A obtained for the full agonists in both portions are similar to those previously reported in the whole vas deferens (Minneman & Abel, 1984; Díaz-Toledo & Martí, 1988). Similarly, the K_P values for the partial agonists phenylephrine and methoxamine in the prostatic portion were similar to the K_A obtained in the epididymal half. Moreover, the K_P for St-587 in the epididymal portion was coincident with the pA_2 previously reported in the prostatic portion (Badia & Sallés, 1989). In addition the dissociation constants of phenylethylamines for the low affinity state (K_{Low}) are well correlated with the K_a (K_A and K_P) for these agonists in both portions of rat vas deferens. Therefore, the initial step for the activation of the response in both portions might be the interaction of agonists with the low affinity state of the α_1-adrenoceptor. Finally, another drug-related parameter considered was efficacy. In our studies no differences in the relative efficacies of agonists, as estimated by the method of Furchgott & Bursztyn (1967) were observed in the two portions of rat vas deferens.

The equilibrium dissociation constants K_a (K_A or K_P) for phenylethylamines were higher than the respective EC_{50} values in each portion (Table 6). These discrepancies could imply the existence of spare receptors in both halves. However, the assumption of the existence of spare receptors in a tissue based exclusively on the differences between K_A and EC_{50} for a certain agonist may lead to erroneous conclusions. A different non-linear relationship between occupancy and

response exists for noradrenaline in the two portions. In the epididymal half the maximal effect for noradrenaline could be obtained when only 50% of the α_1-adrenoceptors were occupied. In contrast, in the prostatic portion 25% of the receptors were required to elicit half maximal responses but all the receptors need to be occupied to induce the maximal effects. Thus, in the prostatic portion there is no indication of spare receptors at maximal response of noradrenaline, though differences between K_A and EC_{50} seem to exist. Our results also demonstrate that the relationship between occupancy and response in the epididymal portion is more efficient than in the prostatic half. Thus, the greater amplification of stimuli in the epididymal portion, as compared with the prostatic portion, is reflected in the steeper occupancy-response curve for the former tissue, as appears in the Figure 2.

In conclusion the differences observed in the sensitivity to α-adrenoceptor agonists along the rat vas deferens could not be explained by a heterogeneous distribution of receptors, changes in the affinity constants for agonists, or a different proportion of the postulated α_1-adrenoceptor subtypes. The most suitable explanation of these differences may involve a tissue factor such as receptor coupling to stimulus-response mechanisms.

We would like to thank all the firms who generously donated samples of the drugs. J.S. was recipient of a fellowship from MEC, Spain.

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(Received May 17, 1990
Revised October 9, 1990
Accepted October 15, 1990)

Br. J. Pharmacol. (1991), **102**, 446–452 © Macmillan Press Ltd, 1991

Very high affinity interaction of DPI 201-106 and BDF 8784 enantiomers with the phenylalkylamine-sensitive Ca^{2+}-channel in *Drosophila* head membranes

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1 Piperazinylindoles (DPI 201-106, BDF 8784), drugs known to act on voltage-dependent Na^+-channels, bind with very high affinity to a Ca^{2+}-channel-associated phenylalkylamine receptor in *Drosophila melanogaster* head membranes. These compounds and (+)-tetrandrine, a naturally occurring Ca^{2+}-antagonist, were the most selective inhibitors for phenylalkylamine-labelled *Drosophila* Ca^{2+}-channels compared to mammalian L-type Ca^{2+}-channels.

2 Replacement of the cyano group by a methyl group in (+)-DPI 201-106 ((+)-BDF 8784) increases the IC_{50} value for inhibition of phenylalkylamine labelling of *Drosophila* Ca^{2+}-channels from 0.29 to 2.1 nM but decreases the IC_{50} value for inhibition of phenylalkylamine labelling of mammalian skeletal muscle Ca^{2+}-channels from 3480 to 49 nM.

3 DPI 201-106 enantiomers completely block (at 0.1 μM) phenylalkylamine photolabelling of a 136 K polypeptide in *Drosophila* head membranes whereas 10 μM aconitine or lidocaine are without effect.

4 Assessment of the Ca^{2+}-antagonist effects of the substituted DPI 201-106 enantiomers in K^+-depolarized taenia strips from guinea-pig caecum yielded pA_2 values of 6.33 ± 0.07 for (−)-BDF 8784 and 6.99 ± 0.17 for (+)-BDF 8784, respectively.

5 Piperazinylindoles, previously believed to act nonspecifically on voltage-dependent mammalian L-type Ca^{2+}-channels, therefore have stereoselectivity for a novel binding site and chemical selectivity unrelated to local anaesthetic activity.

6 It is proposed that a very high affinity piperazinylindole-selective site is coupled to the phenylalkylamine receptor of *Drosophila* Ca^{2+}-channels. These sites are still present on mammalian L-type Ca^{2+}-channels but have lower affinity and/or are less tightly coupled to phenylalkylamine receptors on the α_1-subunit.

Introduction

Drosophila melanogaster head membranes contain at least eight distinct voltage-regulated Ca^{2+}-channels after reconstitution in phospholipid bilayers (Pelzer *et al.*, 1989). There is a Ca^{2+} channel, permeable to Ba^{2+} (13 pS conductance) which is extremely sensitive to phenylalkylamines (transient stimulation and subsequent block by nM concentration of gallopamil or (−)-desmethoxyverapamil) but is completely insensitive to 1,4 dihydropyridines. As *Drosophila melanogaster* head membranes also contain 1,4 dihydropyridine-sensitive Ca^{2+}-channels (with 21 and 31 pS conductances, respectively), that are insensitive to phenylalkylamines, in addition to Ca^{2+} channels that are not blocked by either class of Ca^{2+} antagonists, they offer a unique system to study the evolution of drug receptor domains and the phylogeny of Ca^{2+}-channels. The mammalian L-type Ca^{2+}-channel, e.g. that from skeletal muscle transverse tubule membranes, possesses several distinct but allosterically coupled drug receptors (e.g. for 1,4 dihydropyridines, phenylalkylamines, benzothiazinones, benzothiazepines, diphenylbutylpiperidines and indolizinsulfones) which are all localized on the α_1-subunit as shown by photolabelling (see e.g. Hosey & Lazdunski, 1988; Schmid *et al.*, 1989; Glossman & Striessnig, 1990). The precise location of these drug domains within the primary amino acid sequence of the α_1-subunit is not yet known but the possibility exists that mammalian L-type Ca^{2+}-channels evolved by gene duplication and fusion, assembling 1,4-dihydropyridine and phenylalkylamine receptors on one continuous peptide chain.

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High-affinity phenylalkylamine binding sites are found in *Drosophila melanogaster* head membranes (Pauron *et al.*, 1987; Pelzer *et al.*, 1989; Greenberg *et al.*, 1989). These sites are not coupled (as are mammalian L-type Ca^{2+}-channel phenylalkylamine receptors) to 1,4 dihydropyridine sites (Pauron *et al.*, 1987; Greenberg *et al.*, 1989) and can be specifically photolabelled by the arylazide phenylalkylamine [N-methyl-^{3}H]-LU 49888 ((−)-5-[(3-azido-phenethyl)-[N-methyl-^{3}H]methyl-amino-2-(3,4,5-trimethoxy-phenyl)-2-isopropylvalero-nitrile)]. [N-methyl-^{3}H]-LU 49888 photolabels a 135 K polypeptide in *Drosophila* head membranes (Pauron *et al.*, 1987; Greenberg *et al.*, 1989; Pelzer *et al.*, 1989) with a sedimentation coefficient of 12 S upon sucrose gradient centrifugation after solubilization with digitonin (Greenberg *et al.*, 1989). The *Drosophila melanogaster* phenylalkylamine-sensitive Ca^{2+}-channel seems to be unique, as Ca^{2+}-antagonists from other chemical classes (benzothiazepines e.g. (+)-*cis*-diltiazem; 1,4 dihydropyridines e.g. nitrendipine) interacted only very weakly or not at all with the radiolabelled receptor (Pauron *et al.*, 1987; Greenberg *et al.*, 1989). DPI 201-106 (4-3(4-diphenyl-methyl-1-piperazinyl)-2-hydroxy- propoxy-1H-indole-2-carbonitrile) is a new cardiotonic agent (Scholtysik *et al.*, 1985). The two enantiomers of DPI 201-106 have opposite effects on the voltage-dependent Na^+-channel in neuronal or heart cells, but allosterically inhibit with apparently equal affinity, [^{3}H]-batrachotoxinin A20-α-benzoate binding (Romey *et al.*, 1987). (S)- and (R)-DPI 201-106 seem to interact at distinct receptor sites on the voltage-dependent Na^+-channel (Wang *et al.*, 1989). A methyl-indole derivative of DPI 201-106 (BDF 8784, 2-methyl-4-3′-(4″-benzhydryl-1″-piperazinyl) - 2′ - hydroxypro - poxy-1H-indole) is not cardiotonic and both enantiomers

block in a noncompetitive manner the activation of Na^+-channels by DPI 201-106 (Honerjäger *et al.*, 1988; Armah *et al.*, 1989). In the following study we show that these piperazinylindoles interact with very high affinity at the *Drosophila* phenylalkylamine receptors. These compounds define a previously unrecognized class of drug receptors that are linked to the phenylalkylamine-sensitive Ca^{2+}-channel in *Drosophila* head membranes. Piperazinylindoles also define a novel drug receptor domain on mammalian L-type Ca^{2+}-channels that is allosterically coupled to 1,4-dihydropyridine-, phenylalkylamine- and benzothiazepine-selective sites, but with lower affinity than the *Drosophila* Ca^{2+}-channel.

Methods

Membrane isolation

Membrane extracts from Canton-S *Drosophila melanogaster* heads were prepared as described (Greenberg *et al.*, 1989). Briefly, 100 mg (wet weight) of fly heads per ml ice-cold 0.2 M sucrose, 10 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 50 mM HEPES (pH 7.0) were homogenized in a motor-driven glass-teflon homogenizer (30 strokes at 1700 r.p.m.). The homogenate was centrifuged (3000 *g*, 10 min, 4°C), the supernatant saved and the pellet re-homogenized as above. After repeating the centrifugation, the pooled supernatants were centrifuged at 48,000 *g* for 40 min and the upper, lighter portion of the pellet resuspended by homogenization (4 strokes in 50 mM HEPES, pH 7.0) at a final head membrane protein concentration of 4 mg ml^{-1}. Membranes were flash frozen and stored in liquid nitrogen until use. Partially purified transverse tubule membranes from guinea-pig skeletal muscle and guinea-pig cerebral cortex membranes were isolated as described by Glossmann & Ferry (1985).

Binding assays

Na^+-channel labelling [^{3}H]-batrachotoxinin A 20-α-benzoate (6.2–6.5 nM final concentration) was incubated for 60 min at 37°C with 0.14 to 0.23 mg ml^{-1} guinea-pig cerebral cortex membrane protein in 50 mM Tris-HCl (pH 7.4), 65 mM choline chloride, 0.2 mg ml^{-1} bovine serum albumin, 0.1 mM phenylmethylsulphonylfluoride (PMSF) and 1.6 mg ml^{-1} of crude *L. quinquestratus* toxin. The assay volume was 0.125 or 0.25 ml. The blank binding definition was 0.4 mM aconitine. Drugs were dissolved and diluted in dimethylsulphoxide (DMSO) and 2 or 5 μl added to the assay tubes as described (Boer *et al.*, 1989). The final DMSO concentration never exceeded 2% (v/v).

Ca^{2+}-channel labelling Interaction of drugs with the phenylalkylamine-selective domain of the L-type Ca^{2+}-channel in guinea-pig membranes was investigated with (−)-[^{3}H]-desmethoxyverapamil (Goll *et al.*, 1984). The ligand concentrations varied between 0.66 to 1.2 nM, and the definition of nonspecific binding was by 2 μM (±)-desmethoxyverapamil. The assay volume was 0.5 ml. Incubations were at 25°C for 60 min in 50 mM Tris-HCl, pH 7.4 supplemented with 0.1 mM PMSF. Skeletal muscle transverse tubule membrane protein was 0.04 to 0.12 mg ml^{-1}, cerebral cortex membrane protein varied from 0.07 to 0.16 mg ml^{-1}. Drugs were added in DMSO as above (5 μl); the final DMSO concentration never exceeded 1% (v/v). Saturation analysis with (−)-[^{3}H]-desmethoxyverapamil and skeletal muscle membranes was performed and data analysed as described (Knaus *et al.*, 1990) in the absence and presence of different concentrations of (+)-BDF 8784, to yield dissociation constants (K_D values) and maximal densities of sites (B_{max}). Interaction of drugs with the 1,4-dihydropyridine-selective domain of L-type Ca^{2+}-channels in guinea-pig membranes was investigated with (+)-[^{3}H]-isradipine. Cerebral cortex membrane protein (0.07–0.08 mg ml^{-1}) was incubated with 0.2–0.25 nM radioligand for 60 min at 25°C; for skeletal muscle membranes 0.03 to 0.06 mg ml^{-1} protein and 0.53–0.89 nM radioligand was employed and the incubation time was 30 min at 25°C. The assay volume, drug additions in DMSO and buffers were as above; the definition of nonspecific binding was 1 μM (±)-isradipine. Dissociation experiments to assess the type of interaction of the (+)-BDF 8784 enantiomer were performed as described by Knaus *et al.* (1990). Briefly, skeletal muscle membranes (0.005 mg protein ml^{-1}) were incubated at 37°C with 3 μM (+)-*cis* dilitazem and 50–90 pM (+)-[^{3}H]-isradipine for 90 min. Dissociation was initiated by action of unlabelled (±)-isradipine (3 μM), (+)-BDF 8784 (5 μM) or by simultaneous addition of both drugs and followed for 30 min by measuring specifically bound radioligand in duplicates at 1 min intervals for 15 min and at 20, 25 and 30 min. Dissociation data from 3 independent experiments was fitted by Graphpad™ to the monoexponential decay function to yield dissociation rate constants.

The benzothiazepine-selective domain of the Ca^{2+}-channel in skeletal muscle membranes was probed at 4°C and 30°C with (+)-*cis*-[^{3}H]-diltiazem as follows: 0.06–0.17 mg ml^{-1} membrane protein was incubated with and without drugs (added as above) with 0.5–1.75 nM radioligand and incubated either for 60 min (30°C) or 12 h (4°C) in an assay volume of 0.5 ml in 50 mM Tris-HCl, pH 7.4, 0.1 mM PMSF with 10 μM (+)-*cis*-diltiazem used for definition of nonspecific binding.

Binding-inhibition experiments with the phenylalkylamine receptor in *Drosophila* membranes were performed as described by Greenberg *et al.* (1989), with assay volumes of 0.5 or 1.0 ml and 1 μM (±)-desmethoxyverapamil used for definition of nonspecific binding. [N-methyl ^{3}H]-LU 49888 (0.52–0.84 nM) or (−)-[^{3}H]-desmethoxyverapamil (0.66–1.21 nM) were used in 50 mM Tris-HCl (pH 7.4) buffer, supplemented with 0.1 mM PMSF and 0.051 to 0.091 mg ml^{-1} membrane protein. The incubation time at 25°C was 60 min. Drugs (with the exception of heparin) were added in DMSO as above.

Separation of bound and free ligand After incubation, bound and unbound radioligands were separated (after dilution with 3.5 ml ice-cold 10% polyethylene glycol 6000, 10 mM Tris HCl, 10 mM $MgCl_2$ (pH 7.4)) by rapid filtration over Whatman GF/C glass fibre filters followed by two washes (3.5 ml each). Dried filters were counted for radioactivity in a liquid scintillation counter. Specific binding was defined as the differences between radioligand bound in the absence and presence, respectively of the appropriate blank definition.

Photoaffinity labelling

For photoaffinity labelling of the phenylalkylamine receptor in *Drosophila* head membranes, 0.12 to 0.13 mg ml^{-1} protein was incubated with 3.0–3.5 nM [N-methyl ^{3}H]-LU 49888 (with or without added drugs) in 50 mM Tris-HCl (pH 7.4) buffer at 25°C in the dark for 60 min. Incubation mixtures were transferred to Petri dishes on ice, irradiated for 30 min with ultraviolet light as described by Greenberg *et al.* (1989) and centrifuged for 10 min (4°C) at 40,000 *g*. Membrane pellets were solubilized (at 56°C) in electrophoresis sample buffer (10 mM dithiothreitol, reducing conditions) in the presence of a protease inhibitor mix (Vaghy *et al.*, 1988) and electrophoresed on 9% SDS polyacrylamide gels. Fixed gels were either incubated with Amplify™ (Amersham) and fluorography performed (Greenberg *et al.*, 1989) or radioactivity was quantified by cutting the gel into 3 mm slices, extracted with Econofluor™, containing 3% (v/v) Protosol™ (both New England Nuclear) and liquid scintillation counting.

Data analysis

Binding-inhibition curves were analysed by fitting the experimental data to the general dose-response equation (De Lean *et al.*, 1978), yielding maximal inhibition (as %), IC_{50} value and Hill slopes (nH).

Assessment of Ca^{2+} antagonist effects

Ca^{2+}-antagonism of the BDF 8784 enantiomers was assessed as described by Spedding (1982). Briefly, strips of taenia obtained from the caecum of male guinea-pigs (300–350 g) (1–2 mm diameter, relaxed length 1.8–2.5 cm) were set up in an organ bath (30 ml) containing the K^+-depolarized Tyrode solution (Spedding, 1982) at 37°C and gassed with 95% O_2 and 5% CO_2. Cumulative concentration-response curves were obtained to $CaCl_2$ (0.03–1 mM) by increasing the Ca^{2+} concentration at 3–5 min intervals in logarithmic increments. Drugs were preincubated for 2 h and dose-ratios calculated by the method of Arunlakshana & Schild (1959).

Drugs and chemicals

DPI 201-106 enantiomers were a gift from Prof. Scholtysik (Sandoz AG Basle, Switzerland), (+)-tetrandrine was obtained from Dr Kaczorowski (Merck Sharp and Dohme, Rahway N.J., U.S.A.), (R)-(+) BDF 8784 (BDF 9158) (called (+)-BDF 8784 here) and (S)-(−)-BDF 8784 (BDF 9157) (named (−)-BDF 8784 here) were provided by one of us (B.I.A.), HOE-166 (R-(±)-3,4-dihydro-2-isopropyl-4-methyl- 2-[2-[4-[4-[2-(3,4,5-tri-methoxyphenyl)ethyl]piperazinyl]- butoxy]phenyl]- 2H-1, 4-benzothiazin-3-on- dihydrochloride) and its (S)-enantiomer (HOE-167) were gifts from Prof. Schölkens (Hoechst AG, Frankfurt, F.R.G.), *trans*-diclofurime was a gift from Dr Spedding, (Syntex Lab, Edinburg, UK), (−)-cyanopindolol a gift from Dr Engel, Sandoz AG, Basle, Switzerland. Crude *L. quinquestratus* toxin and high molecular weight heparin (average molecular weight: 13600) were from Sigma (Munich, F.R.G.). (+)-[^{3}H]-isradipine (70 Ci mmol^{-1}), (−)-[^{3}H]-desmethoxyverapamil (76 Ci mmol^{-1}), [^{3}H]-batrachotoxinin A 20-α-benzoate (44 or 42.7 Ci mmol^{-1}) and (+)-*cis*-[^{3}H]-diltiazem (136 Ci mmol^{-1}) were from Amersham, Vienna, Austria. [N-methyl ^{3}H]-LU 49888 (85 Ci mmol^{-1}) as well as the optically pure enantiomers of the phenylalkylamines were gifts from Knoll AG (Ludwigshafen, F.R.G.).

Results

Pharmacological profile of the phenylalkylamine receptor in Drosophila *head membranes*

In previous studies (Pauron *et al.*, 1987; Greenberg *et al.*, 1989) the high affinity and selectivity of the (13 pS) Ca^{2+}-channel-associated receptor (Pelzer *et al.*, 1989) in *Drosophila* membranes for phenylalkylamines was noted. The data in Table 1 and the examples shown in Figure 1, however illustrate that drugs from different chemical classes, including compounds that are not classified as L-type Ca^{2+}-channel blockers, can inhibit phenylalkylamine (e.g. (−)-[^{3}H]-desmethoxyverapamil and [N-methyl ^{3}H]-LU 49888) binding to *Drosophila* membranes. Most notable was the very high affinity interaction of the piperazinylindoles. The (+)-enantiomer of DPI 201-106 was 3 times more potent than the (−)-enantiomer. An eudismic ratio around unity was found for the methylindole-substituted DPI 201-106 derivatives, (+) BDF 8784 and (−) BDF 8784. The importance of the side chain substitution of the piperizinylindoles is underlined by (−)-cyanopindolol which inhibited phenylalkylamine binding with an IC_{50} value three orders of magnitude higher than (+)-DPI 201-106. (+)-Tetrandrine, an L-type Ca^{2+}-channel blocker, which binds in a competitive manner (K_i = 550 nM at 25°C) to the (+)-*cis* [^{3}H]-diltiazem-labelled benzothiazepine-selective domain in mammalian heart membranes (King *et al.*, 1988) interacted with an IC_{50} value of 9.5 nM. The diltiazem diastereomers were inhibitory with Hill slopes smaller than unity, indicating an inhibitory mechanism that was not simple

Table 1 Pharmacological profile of phenylalkylamine receptors in *Drosophila melanogaster* head membranes in comparison to mammalian skeletal muscle transverse tubule membranes

		Drosophila melanogaster		*Mammalian skeletal muscle*	
Chemical class	*Drug*	IC_{50} [nM]	*nH*	IC_{50} (nM)	*Ratio*
Piperazinylindoles[1]	(+)-DPI 201-106	0.29 ± 0.003	0.90 ± 0.16	1230	4240
	(−)-DPI 201-106	0.72 ± 0.1	0.97 ± 0.12	3480	4833
	(+)-BDF 8784	2.1 ± 0.5	0.97 ± 0.12	49.4	23.5
	(−)-BDF 8784	3.1 ± 0.3	1.02 ± 0.11	1790	577
Phenylalkylamines	(−)-Verapamil	0.66 ± 0.12	1.1 ± 0.21	40.2	61
	(+)-Verapamil	16.7 ± 1.8	0.93 ± 0.14	70.4	4.2
	(−)-Gallopamil	0.96 ± 0.07	0.89 ± 0.12	12.5	13.0
	(+)-Gallopamil	20.5 ± 3.7	1.0 ± 0.08	55.3	2.7
	(−)-Desmethoxyverapamil	0.66 ± 0.09	0.97 ± 0.08	4.0	6.1
	(+)-Desmethoxyverapamil	5.9 ± 1.2	0.94 ± 0.12	34.7	5.9
	(−)-Emopamil	5.4 ± 1.3	0.84 ± 0.13	37.2	6.9
	(+)-Emopamil	7.8 ± 1.6	1.17 ± 0.28	33.5	4.3
Benzothiazinones[2]	HOE 167	30.1 ± 2.5	0.89 ± 0.06	5.94	0.2
	HOE 166	88.4 ± 5.9	1.14 ± 0.08	1.04	0.01
Benzothiazepines	(+)-*cis*-Dilitiazem	750 ± 133	0.63 ± 0.11	164	0.22
	(−)-*cis*-Diltiazem	146 ± 80	0.71 ± 0.14	9140	62.6
Diphenylbutylpiperidines[3]	Fluspirilene	15.8 ± 5.0	0.75 ± 0.16	0.4	0.02
	Pimozide	84.4 ± 1.4	1.3 ± 0.30	2.0	0.02
Miscellaneous[4]	(+)-Tetrandrine	9.5 ± 1.8	1.3 ± 0.3	>10000	>100
	(−)-Cyanopindolol	330.4 ± 69.4	0.8 ± 0.11	ND	ND
	trans-Diclofurime	35 ± 6.8	0.93 ± 0.14	39.1	1.1
	Heparin	>10000		64	<0.01

IC_{50} values are means from 4–5 experiments (±s.d.) each carried out with 8 to 11 different concentrations of drugs in duplicates employing [N-methyl ^{3}H]-LU 49888 as ligand for *Drosophila* phenylalkylamine receptors, except for the benzothiazinone data which were obtained with (−)-[^{3}H]-desmethoxyverapamil. All drugs inhibited binding to *Drosophila* membranes by 100%. 'Ratio' defined as: IC_{50} value for mammalian skeletal muscle divided by the IC_{50} value for the *Drosophila* phenylalkylamine receptor. Data for skeletal muscle Ca^{2+}-channel-linked phenylalkylamine receptors were obtained as described in Methods. (ND = not determined).

[1] see Table 4 for details.

[2] Benzothiazinone data for *Drosophila* are taken from Grassegger *et al.* (1989).

[3] Fluspirilene and pimozide IC_{50} values for mammalian skeletal muscle are taken from Gallizi *et al.* (1986).

[4] Heparin inhibition data for mammalian skeletal muscle are taken from Knaus *et al.* (1990) using the average molecular weight given by the manufacturer.

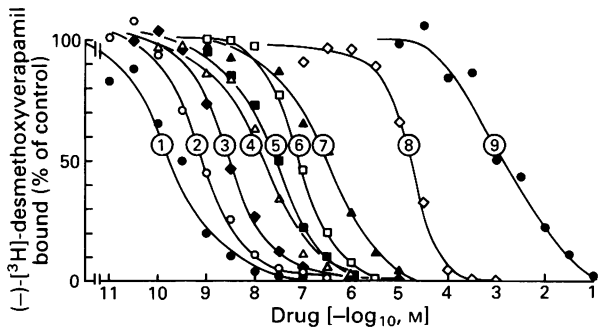


Figure 1 Binding-inhibition profile of (−)-[^{3}H]-desmethoxyverapamil-labelled phenylalkylamine receptors in *Drosophila melanogaster* head membranes. The figure shows representative experiments where points are means from duplicate determinations. Under the experimental conditions (see Methods) the concentration of total bound ligand varied from 48.9 to 66.9 pM, nonspecifically bound ligand from 4.8 to 6.8 pM. (1) (+)-DPI 201-106; (2) (−)-DPI 201-106; (3) (+)-BDF 8784; (4) fluspirilene; (5) HOE-167; (6) HOE-166; (7) (−)-cyanopindolol; (8) Ca^{2+}; (9) La^{3+}.

competition. Compared to the mammalian phenylalkylamine receptors, the stereoselectivity is reversed for these benzothiazepines as was reported for the benzothiazinones previously (Grassegger *et al.*, 1989). *Trans*-diclofurime, suggested to interact preferentially with the benzothiazepine-selective site of L-type Ca^{2+}-channels (Mir & Spedding, 1987; Spedding *et al.*, 1987) inhibited phenylalkylamine binding to *Drosophila* membranes with an IC_{50} value of 35 nM. For comparison, IC_{50} values for inhibition of the phenylalkylamine-selective domain of the Ca^{2+}-channel from mammalian skeletal muscle membranes are shown in Table 1 together with a calculated ratio of the IC_{50} values.

Photoaffinity labelling experiments

The fluorogram in Figure 2 shows that 0.1 μM (+)- or (−)-DPI 201-106 completely inhibited photolabelling of the 135 K phenylalkylamine receptor in *Drosophila* head membranes,

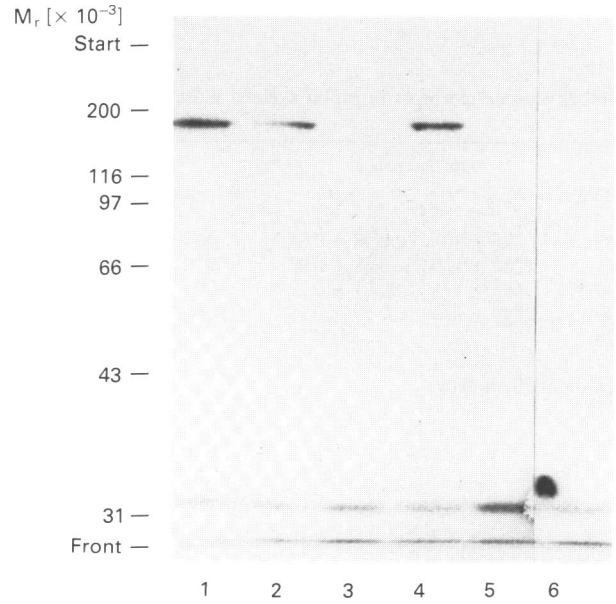


Figure 2 Photoaffinity labelling of *Drosophila* head membranes. The fluorogram of the SDS-gel shows in lane (1) control photoincorporation, (2) lidocaine 10 μM present, (3) 0.1 μM (+)-DPI 201-106 present, (4) 10 μM aconitine present, (5) 0.1 μM (−)-DPI 201-106 present, (6) 0.3 μM (−)-desmethoxyverapamil present. Membrane protein concentration for photolabelling was 0.13 mg ml^{-1}, [N-methyl ^{3}H]-LU 49888 was 3.1 nM and the film exposure time of the gel was 2 weeks at −80°C. The mobility of marker proteins is shown on the left side.

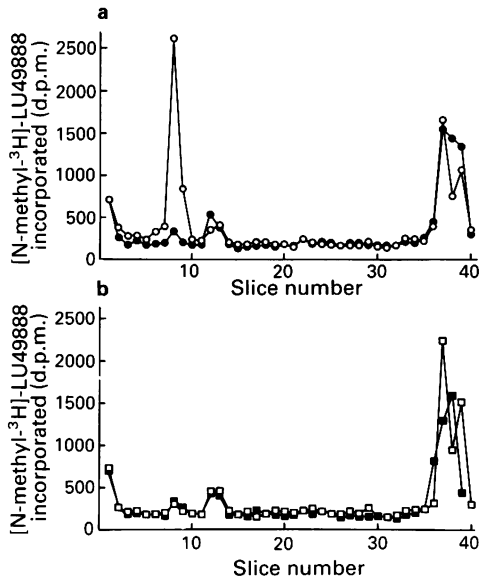


Figure 3 Quantitation of photoincorporated radioactivity in *Drosophila* head membranes. (a) Photolabelling was as in Figure 2 except that 0.11 mg ml^{-1} of membrane protein and 2.8 nM [N-methyl ^{3}H]-LU 49888 was used. Gels were processed as described in Methods. Open symbols: control photoincorporation (no drug added); closed symbols: photoincorporation in the presence of 0.3 μM (−)-desmethoxyverapamil. (b) As above but with (+)-DPI 201-106 (open symbols) or with closed symbols (−)-DPI 201-106 present (each at 0.1 μM).

whereas lidocaine or aconitine were without effect. *Drosophila* head membranes also contain low affinity, high capacity phenylalkylamine sites which are not stereoselective but are photolabelled by [N-methyl ^{3}H]-LU 49888 (Greenberg *et al.*, 1989). Photoincorporation into this 27 K polypeptide can be suppressed by high (μM) concentrations of (+)- or (−)-verapamil (Greenberg *et al.*, 1989). Quantitative analysis of photoincorporated radioactivity (Figure 3) shows that the DPI 201-106 enantiomers do not inhibit photolabelling of the 27 K band.

Interaction of piperazinylindoles with mammalian Na^+- and Ca^{2+}-channel associated drug receptors

Table 2 confirms for guinea-pig cerebral cortex membranes results previously reported for rat brain synaptosomes (Romey *et al.*, 1987), namely that both DPI 201-106 enantiomers inhibit specific [^{3}H]-batrachotoxin A 20-α-benzoate binding with similar IC_{50} values. The BDF 8784 enantiomers were slightly less potent. When the piperazinylindoles were diluted in buffer (instead of DMSO) as described by Glossmann & Ferry (1985), IC_{50} values were higher and apparent Hill slopes ($n = 3$) significantly greater than unity: (±)-BDF 8784 (IC_{50}: 460 ± 55 nM, nH 1.5 ± 0.2), (+)-BDF 8784 (IC_{50}: 510 ± 66 nM, nH = 1.88 ± 0.33), (−)-BDF 8784 (IC_{50}:

Table 2 Interaction of piperazinylindoles with [^{3}H]-batrachotoxinin A 20-α-benzoate labelled Na^+-channels in guinea-pig cerebral cortex membranes

Drug	IC_{50} (nM)	*nH*	*Maximal inhibition* (%)
(−)-DPI 201-106	90.7 ± 23.8	1.0 ± 0.2	100
(+)-DPI 201-106	93.8 ± 14.2	1.1 ± 0.17	100
(+)-BDF 8784	149.3 ± 28.9	0.99 ± 0.17	100
(−)-BDF 8784	200.6 ± 42.8	1.1 ± 0.25	100

$n = 3$. Values are means ± s.d. For details, see Methods.

692 ± 83 nM, nH 1.89 ± 0.34), (±)-DPI 201-106 (IC_{50}: 458 ± 31 nM, nH 1.6 ± 0.15). It was recently shown that DPI 201-106 interacted allosterically with the three classical Ca^{2+}-antagonist drug receptor domains to downregulate 1,4-dihydropyridine, phenylalkylamine and benzothiazepine binding. However, because the inhibition was not stereoselective it was suggested that DPI 201-106 binds to a local anaesthetic-like site, common to both Ca^{2+}- and Na^{+}-channels (Siegl *et al.*, 1988). From the results shown in Table 2 we expected that the BDF 8784 enantiomers would have similar affinities for Ca^{2+}-antagonist drug receptors. Data summarized in Tables 3, 4 and 5 show that this is clearly not the case. In general the DPI 201-106 enantiomers (with the exception of (+)-DPI 201-106 and phenylalkylamine labelling) were more potent inhibitors of brain Ca^{2+}-channel labelling than in skeletal muscle. The reverse was observed for the BDF 8784 enantiomers. Enantioselectivity was marginal or absent for the DPI 201-106 enantiomers when the 1,4-dihydropyridine-selective site was studied but (−)-DPI 201-106 was 3.7 times more potent than (+)-DPI 201-106 in inhibiting the brain phenylalkylamine receptor. Clear evidence for enantioselectivity was obtained when the phenylalkylamine-selective site of the skeletal muscle Ca^{2+}-channel was investigated with the BDF 8784 enantiomers. (+)-BDF 8784 was 36 times more potent than the (−)-enantiomer. The racemate inhibited with an IC_{50} value approximately twice that obtained for the eutomer. The piperazinylindoles were very potent inhibitors at the benzothiazepine-selective domain of skeletal muscle Ca^{2+}-channels. (+)-BDF 8784 was 4 (at 30°C) or 7 (at 4°C) times more potent than the (−)-enantiomer. Although the IC_{50} value of the BDF eutomer is in the range observed for (+)-*cis*-diltiazem (see Table 5) the steep Hill slope and kinetic experiments performed with (+)-BDF 8784 (Glossmann & Striessnig, 1990) clearly prove that the inhibition is allosteric and not occurring by direct binding to the (+)-*cis*-[^{3}H]-diltiazem labelled receptor domain, confirming for skeletal muscle Ca^{2+}-channels previous findings with heart Ca^{2+}-channel labelling (Siegl *et al.*, 1988). Study of dissociation kinetics is a powerful tool to distinguish competitive from allosteric inhibition (Romey *et al.*, 1987). When the 1,4-dihydropyridine-selective domain of the skeletal muscle membrane Ca^{2+}-antagonist receptors was labelled with (+)-[^{3}H]-PN 200-110 in the presence of 3 μM (+)-cis diltiazem the following dissociation rate constants (K_{-1} values) were obtained: isradipine, 0.11 ± 0.006 min^{-1}; (+)-BDF 8784, 0.24 ± 0.007 min^{-1}; isradipine plus (+)-BDF 8784: 0.24 ± 0.008 min^{-1}. Clearly this methylindole-substituted DPI 201-106 enantiomer interacts allosterically with the dihydropyridine-selective site as previously shown for (±)-DPI 201-106 (Siegl *et al.*, 1988). Saturation analysis of the phenylalkylamine-selective domain of Ca^{2+}-antagonist receptors in skeletal muscle membranes with (−)-[^{3}H]-desmethoxyverapamil (performed in the absence and presence of different concentrations of (+)-BDF 8784) demonstrated a concentration-dependent reduction of B_{max}

Table 3 Interaction of piperazinylindoles with Ca^{2+}-channel-linked 1,4 dihydropyridine receptors in mammalian skeletal and guinea-pig brain cortex membranes labelled with (+)-[^{3}H]-isradipine

	Skeletal muscle			*Brain cortex*		
Drug	IC_{50} (nM)	*nH*	*MI* (%)	IC_{50} (nM)	*nH*	*MI* (%)
(−)-DPI 201-106	3410 ± 1330	0.98 ± 0.2	79	1810 ± 380	0.98 ± 0.17	87
(+)-DPI 201-106	5210 ± 1370	0.94 ± 0.2	67	1970 ± 540	1.14 ± 0.3	92
(+)-BDF 8784	155 ± 28.4	0.92 ± 0.14	99	1080 ± 390	1.13 ± 0.08	95
(−)-BDF 8784	492 ± 140	0.58 ± 0.15	88	960 ± 280	0.81 ± 0.16	89

$n = 4$. Values are mean ± s.d. MI = maximal inhibition at 10 μM drug.

Table 4 Interaction of piperazinylindoles with Ca^{2+}-channel-linked phenylalkylamine receptors in rabbit skeletal muscle and guinea-pig brain cortex membranes labelled with (−)-[^{3}H]-desmethoxyverapamil

	Skeletal muscle			*Brain cortex*		
Drug	IC_{50} (nM)	*nH*	*MI* (%)	IC_{50} (nM)	*nH*	*MI* (%)
(−)-DPI 201-106	3480 ± 810	0.99 ± 0.19	75	346 ± 36	0.46 ± 0.08	96
(+)-DPI 201-106	1230 ± 330	0.98 ± 0.21	91	1280 ± 130	0.68 ± 0.09	97
(+)-BDF 8784	49.4 ± 8	1.41 ± 0.2	100	666 ± 135	1.1 ± 0.19	100
(−)-BDF 8784	1790 ± 110	0.89 ± 0.2	100	2840 ± 290	0.59 ± 0.10	100
(±)-BDF 8784	97.9 ± 26	0.97 ± 0.2	100		ND	

$n = 3$; values are means ± s.d. ND = not determined. MI = maximal inhibition at 10 μM drug.

Table 5 Interaction of piperazinylindoles with Ca^{2+}-channel-linked benzothiazepine receptors in rabbit skeletal muscle membranes labelled with (+)-*cis*-[^{3}H]-diltiazem

	30°C			4°C		
Drug	IC_{50} (nM)	*nH*	*MI* (%)	IC_{50} (nM)	*nH*	*MI* (%)
(−)-DPI 201-106	61.1 ± 11	0.72 ± 0.07	100	ND		
(+)-DPI 201-106	43.1 ± 15	0.80 ± 0.2	100	ND		
(+)-BDF 8784	56.5 ± 5.9	1.5 ± 0.2	100	24.5 ± 7	1.3 ± 0.2	100
(−)-BDF 8784	228 ± 46	1.0 ± 0.2	100	184 ± 26	0.96 ± 0.2	100
(+)-*cis*-Diltiazem	57 ± 13	0.9 ± 0.1	100	44 ± 8	1.0 ± 0.1	100

$n = 4$. Values are means ± s.d. MI = maximal inhibition at 10 μM drug. ND = not determined.

values and increases in apparent K_D values by the drug (results not shown).

Ca^{2+} antagonist effects of the BDF 8784 enantiomers

Having obtained evidence for enantioselective interactions of the BDF 8784 compounds with L-type Ca^{2+} channel-associated drug receptors we were prompted to study their effects in K^+ depolarized smooth muscle. Using taenia strips from guinea-pig caecum the following apparent pA_2 values ($n = 4$) were determined: (+)-BDF 8784, 6.99 ± 0.17 (slope 0.89 ± 0.07); (−)-BDF 8784, 6.33 ± 0.07 (slope 1.03 ± 0.04).

Discussion

Our interest in investigating the interaction of piperazinylindoles with *Drosophila* Ca^{2+}-channels, characterized by radiolabelled phenylalkylamines, originated from the observation that (+)-DPI 201-106 was 10 times more potent in inhibiting phenylalkylamine labelling of Ca^{2+}-channels in mammalian brain than in skeletal muscle membranes. Furthermore, having available DPI 201-106 derivatives (i.e. the BDF 8784 enantiomers) we realized that the postulate of a low affinity, nonspecific, local anaesthetic site (Siegl *et al.*, 1988) for the piperazinylindoles on L-type Ca^{2+}-channels could not be upheld. Replacement of the cyano group in DPI 201-106 by a methyl group dramatically increased the potency of (+)-DPI 201-106 to inhibit 1,4-dihydropyridine and phenylalkylamine binding in skeletal muscle whereas modest increases (1,4-dihydropyridine binding-inhibition in guinea-pig cerebral cortex membranes) or decreases (phenylalkylamine binding-inhibition in guinea-pig cerebral cortex membranes) were observed for the methyl-substituted (−)-DPI 201-106. The investigated piperazinylindoles are among the most potent agents to inhibit allosterically (+)-*cis*-[^{3}H]-diltiazem labelling of the skeletal muscle Ca^{2+}-channels. The BDF derivatives had eudismic ratios of 4 or 7, respectively, depending on temperature. An eudismic ratio of 4.5 was also established for Ca^{2+} antagonism in a well established functional assay system (Spedding, 1982). Taken together this pointed to a well defined domain on L-type Ca^{2+}-channels which discriminated between optical antipodes and displayed chemical specificity. The chemical specificity was unrelated to local anaesthetic properties which were similar or even weaker for the methyl-substituted DPI 201-106 enantiomers as shown by batrachotoxinin A 20-α-benzoate labelling of the voltage-dependent Na^+-channel. It should be noted that the piperazinylindoles as well as some other compounds listed in Table 1 exhibited very steep Hill slopes and higher IC_{50} values when diluted in a conventional manner (see Glossmann & Ferry, 1985) in plastic tubes with aqueous buffer. However, when drugs were dissolved and diluted in DMSO and glass tubes, inhibition-potency increased and Hill slopes decreased, exactly as previously described by Boer *et al.* (1989) for hydrophobic 1,4-dihydropyridines. This was strong evidence for partitioning of the drugs into plastic upon dilution in buffer, henceforth all experiments reported above were performed with the DMSO dilution protocol.

The discovery of a (13 pS) voltage-dependent Ca^{2+}-channel (Pelzer *et al.*, 1989) in *Drosophila* head membranes, where stereoselective phenylalkylamine receptors have higher affinity than observed for the mammalian L-type Ca^{2+}-channel (Pauron *et al.*, 1987; Greenberg *et al.*, 1989), prompted us to search for compounds which are allosteric regulators of phenylalkylamine binding to the mammalian L-type channel and display some preference for the neuronal subtype. As mentioned above, the piperazinylindoles were promising candidates and much to our surprise they were among the most potent drugs. It was beyond the scope of the present investigation to characterize the mechanism of inhibition (competitive vs allosteric) in *Drosophila* membranes. With the exception of the phenylalkylamines themselves, all drugs in Table 1 are allosteric inhibitors for the phenylalkylamine-selective domain of the L-type Ca^{2+}-channel. Thus the ratios in Table 1 are not ratios of true affinities. Nevertheless the data are consistent with the assumption that a drug receptor domain for piperazinylindoles which is coupled to a phenylalkylamine-selective domain is present on both mammalian L-type Ca^{2+}-channels and (with very high affinity or perhaps much tighter coupling) on (13 pS) *Drosophila* Ca^{2+}-channels. For the latter we could demonstrate that the DPI 201-106 enantiomers at very low concentration prevent photolabelling of a 135 KDa polypeptide. This polypeptide has a pharmacological profile (Pauron *et al.*, 1987; Greenberg *et al.*, 1989) which strongly suggests that it is a subunit of the reconstituted phenylalkylamine-sensitive 13 pS Ca^{2+}-channel (Pelzer *et al.*, 1989). We believe that this is further evidence for the structural relationship of mammalian L-type and the *Drosophila* Ca^{2+}-channels.

Interestingly, the only naturally occurring Ca^{2+} antagonist that we investigated, (+)-tetrandine, was a very potent inhibitor at the *Drosophila* phenylalkylamine receptor. This alkaloid is isolated from the Chinese medicinal plant *Stephania tetrandra* and we suggest that it could perhaps belong to the family of plant insecticides as do the pyrethroids. Heparin, on the other hand, which is a very potent allosteric regulator of Ca^{2+}-antagonist receptor labelling in skeletal muscle from carp or rabbit (Knaus *et al.*, 1990) was completely ineffective at the *Drosophila* receptor. This indicates that the heparin binding domain is either not present or not coupled to the phenylalkylamine receptors of *Drosophila* head Ca^{2+}-channels.

Beside its proven interaction with the voltage-dependent Na^+-channel, DPI 201-106 has well-documented (but weak) Ca^{2+}-channel blocking activity (Hof & Hof, 1985; Takahashi *et al.*, 1987; Siegl *et al.*, 1988; Holck & Osterrieder, 1988). Direct evidence for inhibition of reconstituted Ca^{2+}-channels in *Drosophila* brain membranes by DPI 201-106 is missing. However, we have documented stereospecific Ca^{2+} antagonist effects of the methylindole substituted DPI 201-106 derivatives. Our findings may give clues for development of novel insecticides, Ca^{2+}-channel blocking agents or new high affinity labels for Ca^{2+}-channels in *Drosophila* head membranes. In addition probes such as the piperazinylindoles may be useful to study the evolution of drug receptor domains on Ca^{2+}-channels.

The authors would like to thank their colleagues from other drug companies for supplying them with compounds. H.G. was supported by grants (S45-01, S45-02) from Fonds zur Förderung der wissenschaftlichen Forschung (Austria) and by a grant from Bundesministerium für Wissenschaft und Forschung (Austria).

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(Received July 6, 1990
Revised September 24, 1990
Accepted October 17, 1990)

Br. J. Pharmacol. (1991), **102**, 453–461 © Macmillan Press Ltd, 1991

Effect of phorbol ester on the release of atrial natriuretic peptide from the hypertrophied rat myocardium

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1 To determine the cellular mechanisms of atrial natriuretic peptide (ANP) release from ventricular cardiomyocytes, the secretory and the cardiac effects of a phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), known to stimulate protein kinase C activity in heart cells, were studied in isolated, perfused heart preparations from 2- and 21-month-old Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. TPA was added to the perfusion fluid for 30 min at a concentration of 46 nM after removal of atrial tissue. Additionally, atrial and ventricular levels of immunoreactive ANP (IR-ANP) and ANP mRNA, the distribution of ANP within ventricles as well as the relative contribution of atria and ventricles in the release of ANP were studied.

2 Ventricular hypertrophy that gradually developed in hypertensive rats resulted in remarkable augmentation of ANP gene expression, as reflected by elevated levels of immunoreactive ANP and ANP mRNA. The total amount of IR-ANP in the ventricles of the SHR rats increased 41 fold and ANP mRNA levels 12.9 fold from the age of 2 to 21 months. At the age of 21 months, levels of IR-ANP and ANP mRNA in the ventricles of SHR rats were 5.4 fold and 3.7 fold higher, respectively, than in the normotensive WKY rats. Immunohistochemical studies demonstrated ANP granules within the hypertrophic ventricles of the old SHR rats, but not within normal ventricular tissue.

3 In isolated perfused heart preparations, the severely hypertrophied ventricular tissue of SHR rats after atrialectomy secreted more ANP into the perfusate than did the control hearts. The phorbol ester TPA caused a marked vasoconstriction in both the 2-month and 21-month old normal and hypertrophied hearts. Interestingly, the ANP release from the hypertrophied ventricles of the old SHR rats increased considerably (from 413 ± 30 to the maximum of 623 ± 75 pg ml^{-1}, $F = 10.8$, $P < 0.001$, two-way analysis of variance), whereas only a small increase was seen in old WKY rats and no effect was observed in young animals of either strain. When intact rat hearts (without atrialectomy) were used, infusion of phorbol ester also increased the ANP secretion into the perfusate in young animals.

4 Our present results indicate that the phorbol ester TPA increases the release of ANP from the hypertrophied, but not from normal rat myocardium. Thus, hypertrophied rat ventricular myocytes appear to possess the cellular mechanisms necessary to secrete ANP by a regulated pathway. The results further suggest that protein kinase C activity may be involved in the the regulation of ANP secretion from ventricular cells, as has been shown earlier for atrial myocytes.

Introduction

The major site of biosynthesis of atrial natriuretic peptide (ANP), a hormone that regulates salt and water balance and blood pressure, is the atria of the heart, where it constitutes 1–3% of the total RNA (mRNA) (de Bold, 1985; Ballerman & Brenner, 1986; Needleman *et al.*, 1989). Biosynthesis of ANP also occurs in other tissues, particularly in cardiac ventricles (Gutkowska & Nemer, 1989). Ventricular expression of the ANP gene is high in the late foetal and early neonatal period (Bloch *et al.*, 1986; Wei *et al.*, 1987; Zeller *et al.*, 1987; Tsuchimochi *et al.*, 1988; Wu *et al.*, 1988; Gardner *et al.*, 1989), decreases rapidly after birth (Bloch *et al.*, 1986; Wei *et al.*, 1987; Gardner *et al.*, 1989) and remains low in the adult ventricle. ANP gene expression in the adult ventricle, however, has been shown to be activated in patients with congestive heart failure (Tsuchimochi *et al.*, 1988; Edwards *et al.*, 1988; Saito *et al.*, 1989) and in animal models of cardiac hypertrophy (Needleman *et al.*, 1989; Gutkowska & Nemer, 1989).

In the atria, the release of ANP is regulated primarily by atrial stretch (Lang *et al.*, 1985), but there is evidence for a role in atrial cardiocytes of secretagogues (for review see Ruskoaho *et al.*, 1987). Recent *in vitro* studies have shown that ANP is also released from ventricular tissue. Ventricular cardiomyocytes in culture secrete ANP into the medium (Bloch *et al.*, 1986), and when perfused heart preparations are used, ANP is released into the perfusion fluid, the amount of the peptide released depending on the degree of ventricular hypertrophy (Ruskoaho *et al.*, 1989a; Thibault *et al.*, 1989; Kinnunen *et al.*, 1990). Moreover, *in vivo* physical excercise causes release of ANP from the hypertrophic ventricle with depletion of endocardial left ventricular stores (Ruskoaho *et al.*, 1989a). The mechanism by which ventricular cells secrete ANP is, however, not yet clear. Bloch *et al.* (1986) have suggested that neonatal ventricular cells utilize a constitutive pathway for ANP release because they lack secretory granules characteristic of atrial cardiocytes, which store the peptide before secretion. However, the presence of ANP granules in hypertrophied ventricular tissue (Ding *et al.*, 1987; Tsuchimochi *et al.*, 1988; Edwards *et al.*, 1988; Gu *et al.*, 1989; Mercadier *et al.*, 1989; Takemura *et al.*, 1989) suggests that a segretagogue could enhance ANP release from ventricular cells by a regulatory pathway analogous to that in atria.

The aim of the present study was to determine the ability of normal and hypertrophic ventricular tissue *in vitro* to secrete ANP in response to infusion of the phorbol ester TPA, which is known to stimulate ANP secretion from perfused rat hearts (Ruskoaho *et al.*, 1985) and atrial myocytes in culture (Matsubara *et al.*, 1988; Iida & Page, 1989; Shields & Glembotski, 1989). In addition, immunoreactive ANP and ANP mRNA levels in the atria and ventricles of young and old normotensive and genetically hypertensive rats as well as the distribution of ANP measured by immunohistochemistry in normal and hypertrophic ventricular tissue were examined.

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These studies now show that severe cardiac hypertrophy in hypertensive rats results in formation of secretory granules within the ventricles of rats and that the release of ANP from the hypertrophic ventricular cells can be stimulated by phorbol ester.

Animals

Male spontaneously hypertensive rats (SHR) of the Okamoto-Aoki strain (F65) and age-matched Wistar-Kyoto (WKY) (F63) rats from the Department of Pharmacology colony at the University of Oulu, Finland, were used. Both strains were originally obtained from Mollegaards Avslaboratorium, Skensved, Denmark. The rats were housed in plastic cages in a room with controlled 40% humidity at a temperature of 22°C. At 06 h 00 min lights were switched on and at 18 h 00 min they were switched off.

The animals were examined at ages ranging from 1 to 2 months (young) and from 20 to 22 months (old). The experimental design was approved by the Animal Experimentation Committee of the University of Oulu.

Rat isolated perfused hearts

The rat isolated, perfused heart preparation used in this study was similar to that previously described (Ruskoaho *et al.*, 1989a). Briefly, the rats were given heparin (500 iu kg^{-1} body weight i.p.) and were decapitated 20 min later. The abdominal cavity was immediately opened, the diaphragm transected, lateral incisions made along both sides of the rib cage, and the heart cooled with perfusion fluid (4–10°C). The aorta was cannulated above the aortic valve and retrograde perfusion was begun with a modified Krebs-Henseleit bicarbonate buffer, pH 7.4, equilibrated with 95% O_2 and 5% CO_2, at 37°C. Final concentrations of the salts in the buffer (mmol $litre^{-1}$) were: NaCl 113.8, $NaHCO_2$ 22.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.1, $CaCl_2 \cdot 2H_2O$ 2.5 and glucose 11.

Variations in perfusion pressure, arising from changes in coronary vascular resistance, were recorded on a Grass polygraph (model 7DA, Grass Instruments, Quincey, MA, U.S.A.) by way of a pressure transducer (model MP-15, Micron Instruments, Los Angeles, CA, U.S.A.) situated on a side-arm of the aortic cannula. Isometric force of contraction was recorded by a strain gauge transducer (model FT03, Grass Instruments) connected to the Grass polygraph. The output was damped to give the mean contractile force. The heart rate was counted from contractions by means of the Grass tachograph. The hearts were placed under a resting tension of 2 g and stimulated (10 V, 0.5 ms) with a Harvard Apparatus Stimulator model 345 (Harvard Apparatus, Millis, MA, U.S.A.) to increase heart rate 15–20% above the spontaneous beating rate. During the equilibration period (60 min) the hearts were perfused by means of a peristaltic pump (Ismatec SA801, Ismatec Instruments, Zürich, Switzerland) at an initial flow rate of 8 ml min^{-1} later reduced to a constant flow of 5 ml min^{-1}.

Each experiment was started by perfusing hearts for 20 min with the constant flow. Next, all atrial tissue was carefully cut away along the ventricular surface, and remaining tissue perfused again for 20 min. Finally, the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA), at a concentration of 46 nM, was added into the aortic perfusion cannula as a continuous infusion via an infusion pump (Perfusor ED, B. Braun Melsungen AG, Melsungen, F.R.G.) at a rate of 0.5 ml min^{-1} for 30 min. TPA was dissolved in dimethyl sulphoxide (DMSO) and then diluted with Krebs-Henseleit buffer. The final concentration of the solvent was less than 0.03%. Control experiments were run with the Krebs-Henseleit buffer. The coronary venous effluents were collected at 2 min intervals throughout the observation period, and were placed immediately on dry-ice and stored at −20°C until assayed.

The phorbol ester concentration (46 nM) used in this study was chosen because TPA at this concentration is known to stimulate protein kinase C activity (Yuan *et al.*, 1987), increase IR-ANP secretion (Mäntymaa *et al.*, 1990) and cause coronary vasoconstriction (Ruskoaho, 1988) in the Langendorff preparation. To validate the effects of the phorbol ester on ANP release and haemodynamic variables in this study, we examined the effect of TPA on haemodynamics and the perfusate IR-ANP concentration by use of isolated, spontaneously beating intact rat hearts as described previously (Ruskoaho *et al.*, 1985). In these experiments, vehicle ($n = 10$) or 46 nM TPA ($n = 8$) was added to the perfusion cannula at a rate of 0.5 ml min^{-1} for 30 min. The phorbol ester increased perfusion pressure from 26 ± 1 mmHg to 156 ± 9 mmHg ($F = 60.6$, $P < 0.001$, two-way analysis of variance), heart rate from 231 ± 8 beats min^{-1} to 298 ± 19 beats min^{-1} ($F = 5.2$, $P < 0.001$) and IR-ANP secretion into the perfusate from 204 ± 52 pg ml^{-1} to 307 ± 81 pg ml^{-1} ($F = 16.2$, $P < 0.001$), and decreased tension from 1.95 ± 0.03 to 0.81 ± 0.10 g ($F = 36.7$, $P < 0.001$), results which are in agreement with our previous studies (Ruskoaho, 1988; Mäntymaa *et al.*, 1990).

Preparation of tissues

After perfusion, the aorta and pulmonary artery were carefully cut close to the ventricular surface. The right and left ventricles were not separated because only 2.8% and 0.9% of the IR-ANP released from the ventricles in WKY and SHR, respectively, would be generated in the right ventricle (Ruskoaho *et al.*, 1989a; Kinnunen *et al.*, 1990). To avoid the possible contamination of the ventricular sample by atrial tissue, ventricles were cut into the superior (about 15–20% of total weight) and inferior part. Both ventricular samples, as well as the atrial tissue removed, were blotted dry, weighed and stored at −70°C until assayed.

ANP messenger RNA determination

RNA was isolated from fresh atrial and ventricular tissues of WKY and SHR rats by the guanidine thiocyanate-LiCl method (Cathala *et al.*, 1983). As shown previously (Ruskoaho *et al.*, 1989a), Northern blot hybridization analysis using full-length rat cDNA probe identified a single 0.9 kb mRNA species in the atria and ventricles of these strains. For the RNA dot blot analysis, 1 μg and 20 μg samples of the RNA from atria and ventricles, respectively, were transferred to a nitrocellulose membrane (Kinnunen *et al.*, 1990). The full-length rat ANP cDNA probe Car-55 (Flynn *et al.*, 1985) (a generous gift from Dr Peter L. Davies, Queen's University, Kingston, Canada) was labelled with [^{32}P]-dCTP (Amersham, Buckinghamshire, U.K.) with a random primed labelling kit (Boehringer-Mannheim GMBH, Mannheim, F.R.G.). The membrane was hybridized overnight at 65°C in 6 × SSC, 0.8% sodium dodecyl sulphate (SDS), 5 × Denhardt's solution, and 100 μg ml^{-1} sheared herring sperm DNA. After hybridization, the membrane was washed in 0.4 × SSC, 0.2% SDS 2–3 times for 20 min at room temperature, and once for 45 min at 65°C. The membrane was exposed at −70°C to XAR-5 X-ray film (Eastman Kodak, Rochester, NY, U.S.A.) with a Cronex Lightning Plus intensifying screen (DuPont, Wilmington, Delaware, U.S.A.) for three days. To quantify the amount of RNA loaded on membranes, a ^{32}P-labelled oligonucleotide probe complementary to rat 18S ribosomal RNA was hybridized to all filters as described by Albretsen *et al.* (1988). To estimate tissue ANP mRNA concentration, the ANP:18S ratio was determined by dividing the absorbance corresponding to the ANP probe by the absorbance corresponding to 18S probe hybridization.

Assay of immunoreactive ANP in the perfusate and tissues

For the radioimmunoassay, the atrial and ventricular guanidine thiocyanate extracts were diluted 3×10^5 and 1.2×10^3 fold, respectively. Tissue ANP is expressed either as

a concentration per mg wet weight or as content per organ. For the ANP radioimmunoassay the tissue extracts as well as the unextracted perfusate samples were incubated in duplicates of 100 μl with 100 μl of the middle specific rabbit ANP antiserum in the final dilution of 1:25.000 (Vuolteenaho *et al.*, 1985). Synthetic rat ANP_{1-28}, ranging from 0–500 pg per tube, was incubated as standard. The ANP tracer was rat-[^{125}I]-ANP_{1-28} from Amersham (Buckinghamshire, U.K.). After incubation for 48 h at +8°C the immunocomplexes were precipitated with sheep antiserum against rabbit gammaglobulin in the presence of 500 μl of 1.2 M ammonium sulphate, pH 7, followed by centrifugation for 40 min at 3000*g*. The sensitivity of the assay was 0.8 pg per tube. The 50% displacement of the standard curve was at 20 pg per tube. The inter-assay and the intra-assay variations were 14% and 5%, respectively. Serial dilutions of perfusate and tissue extracts showed parallelism to the synthetic ANP standard.

Immunohistochemistry

The hearts for the immunohistochemical evaluations were obtained from 21-month old WKY and SHR rats. The hearts were first perfused with Krebs-Henseleit buffer for 4 to 5 min as described above. Then, atria and ventricles were separated and immersed in the fixative (4% paraformaldehyde in 0.2 M Na-phosphate buffer, pH 7.4) for 4 h at room temperature. The samples were embedded in paraffin and cut into 3 μm sections.

The Vectastain ABC kit (from Vector Laboratories Inc., Burlingame, California, U.S.A.) was used in immunohistochemical staining. The deparaffinized sections were incubated with 0.3% H_2O_2 in methanol, washed with distilled water, incubated in 20% swine serum, anti-ANP serum (1:200–1:400) (Vuolteenaho *et al.*, 1985), biotinylated anti-rabbit immunoglobulins (1:20), and avidin-biotinylated peroxidase complex, with phosphate-buffered saline (PBS)-washes between incubations. The peroxidase label was visualized by 3,3′-diaminobenzidine-H_2O_2-reaction and the nuclei were stained with haematoxylin. The specificity of the immunochemical staining was controlled by replacing the primary antiserum with normal rabbit serum or with PBS.

Materials

The chemicals used in this study were: guanidine thiocyanate (Fluka Chemie AG, Switzerland); LiCl and formaldehyde (JT Baker Chemicals BV, Holland); Agarose NA (Pharmacia, Sweden), heparin (Medica, Helsinki, Finland); synthetic rat ANP_{1-28} and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma Chemical Company, St. Louis, Missouri, U.S.A.). Unless otherwise stated, other chemicals were from Sigma.

Statistical analysis

The results are expressed as mean ± s.e.mean. The data were analyzed by two- or one-way analysis of variance (ANOVA). For the comparison of statistical significance between two groups, Student's *t* test for unpaired or paired data was used. For the multiple comparison, one-way analysis of variance followed by Bonferroni's *t* test was used. Differences at the 95% level were considered statistically significant.

Results

Body and cardiac weights of WKY and SHR rats

Ventricular ANP expression and release was examined in 2- and 21-month old WKY and SHR rats. Table 1 shows the body and cardiac weights as well as the ages of the animals in each group. As expected, absolute ventricular weight as well as the ventricular-weight to body-weight ratio were higher in the SHR when compared to their age-matched controls. There were no significant differences between the TPA-treated and the control groups, except for higher ventricular weight in the TPA group of the old WKY rats and higher ventricular-weight to body-weight ratio in the TPA group of the young WKY rats (Table 1).

Atrial and ventricular levels of immunoreactive ANP and ANP mRNA in WKY and SHR rats

Immunoreactive ANP was present at higher concentrations in the atria than in the ventricles of both strains (Table 2). In the atria, IR-ANP concentrations (expressed as ng mg^{-1}) increased with age in the normotensive rats, but decreased in hypertensive rats; the IR-ANP concentration was 74% greater in the atria of the 21-month old WKY rats compared to the 2-month old animals, but 35% lower in the atria of SHR rats, respectively (Table 2). When normotensive animals were compared to those from the hypertensive rat strain, the IR-ANP concentration was greater in the atria of the young SHR than in the age-matched WKY rats. In contrast, the concentration of IR-ANP was significantly higher in the atria of the 21-month old WKY than in the age-matched SHR rats (Table 2). The total amount of IR-ANP in the atria (μg per atria) of the young WKY did not differ significantly from that in the young SHR rats but was significantly higher in the atria of the 21-month-old WKY than in the age-matched SHR rats (Table 2).

Ventricular hypertrophy that gradually developed due to chronically elevated blood pressure caused a remarkable augmentation of ventricular ANP levels in SHR rats. The total amount of IR-ANP in the ventricles of the SHR rats increased 41 fold and the IR-ANP concentration 9.8 fold between the ages of 2 and 21 months (Table 2). Corresponding changes in the WKY rats were considerably lower, 6.1 fold and 2.5 fold, respectively. At the age of 21 months total IR-ANP content in the ventricles of SHR rats was 5.4 fold and the IR-ANP concentration 3.6 fold greater than in the WKY rats (Table 2). In contrast, no substantial differences were found either in the ventricular IR-ANP content or in the IR-ANP concentration between the young WKY and SHR rats (Table 2).

Table 1 Body and cardiac weights in Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats

	Young				*Old*			
	WKY		SHR		WKY		SHR	
	Control $n = 5$	*TPA* $n = 7$	*Control* $n = 6$	*TPA* $n = 5$	*Control* $n = 6$	*TPA* $n = 7$	*Control* $n = 4$	*TPA* $n = 4$
Age (months)	1.4 ± 0.2	1.4 ± 0.2	1.7 ± 0.1	1.6 ± 0.3	19.5 ± 0.1	21.1 ± 1.1	21.5 ± 0.5	20.6 ± 0.9
Body weight (g)	153 ± 3	152 ± 8	180 ± 13	180 ± 18	514 ± 9	495 ± 15	397 ± 15	426 ± 33
Atria (mg)	122 ± 10	118 ± 15	133 ± 7	134 ± 10	213 ± 4	264 ± 18	269 ± 18	324 ± 90
Ventricles (mg)	483 ± 14	541 ± 28	654 ± 49	704 ± 57	1373 ± 17	1557 ± 20*	2213 ± 100	2356 ± 166
Ventr wt/body wt (mg g^{-1})	3.15 ± 0.05	3.57 ± 0.04*	3.64 ± 0.06	3.93 ± 0.08	2.68 ± 0.07	3.16 ± 0.07	5.59 ± 0.27	5.58 ± 0.39

Atria = total weight of atrial tissue, including weight of both auricles and non-auricular part of the atrium. TPA = hearts perfused with TPA-infusion. The results are expressed as means ± s.e.mean. * $P < 0.001$ control vs. TPA (Student's *t* test, unpaired).

Table 2 Atrial and ventricular immunoreactive atrial natriuretic peptide (IR-ANP) levels in Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats

	Young		*Old*	
	WKY $n = 6$	SHR $n = 6$	WKY $n = 6$	SHR $n = 6$
Atria				
IR-AP ($ng\,mg^{-1}$)	51.4 ± 10.3	81.8 ± 4.7**	89.2 ± 14.6	50.6 ± 9.5*
Total IR-ANP (μg/atria)	5.49 ± 1.94	8.22 ± 1.69	23.2 ± 3.7	13.4 ± 1.6*
Ventricles				
IR-ANP ($ng\,mg^{-1}$)	0.12 ± 0.01	0.11 ± 0.02	0.30 ± 0.11	1.08 ± 0.10***
Total IR-ANP (ng/ventricles)	59.2 ± 5.1	47.5 ± 14.6	358 ± 117	1951 ± 246***

$ng\,mg^{-1}$ = $ng\,mg^{-1}$ wet weight. The results are expressed as means ± s.e.mean.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ WKY vs. SHR (Student's t test, unpaired).

The induction of the ANP coding gene in the hypertrophic ventricular tissue of hypertensive rats was also seen when cardiac ANP mRNA levels were measured by dot blot analysis. The ANP mRNA:18 S ratio in the ventricles of the 21-month-old SHR was 12.9 fold higher than in the 2-month-old SHR rats and 3.7 fold higher than in the age-matched WKY rats (Figure 1). In the ventricles of the old WKY rats the ANP mRNA:18 S ratio was 3.7 fold greater than in the young ones, whereas no difference in the ANP mRNA levels was observed between the 2-month-old WKY and SHR rats. In the atria, the ANP mRNA:18 S ratio of the old WKY rats was 1.9 fold higher than that of 2-month-old WKY rats, whereas there was only a tendency for ANP mRNA levels to be slightly higher in the old vs. young SHR rats (Figure 1). The atrial ANP mRNA level of SHR did not differ significantly from the age-matched WKY rats at either age (Figure 1).

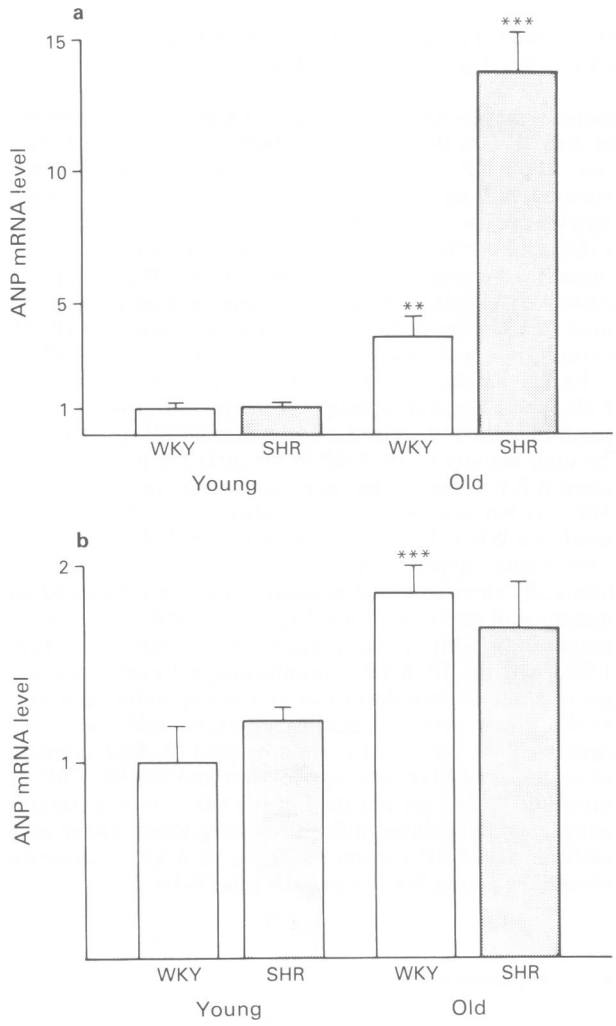


Figure 1 Atrial natriuretic peptide messenger RNA (ANP mRNA) levels in the ventricles (a) and atria (b) of Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. RNA was prepared from hearts of 2- (young) and 21-month-(old) old WKY and SHR rats and hybridized to a ^{32}P-labelled restriction fragment derived from rat ANP cDNA. ANP mRNA values were standardized by comparison to 18S levels, as assessed by dot blot hybridization to synthetic oligonucleotide probe. Arbitrary relative values of 1 were assigned to ANP mRNA:18S ratios of the young WKY rats to reflect the changes of the ANP mRNA levels in atrial and ventricular tissue. Columns show means with s.e.mean shown by vertical bars. ** $P < 0.01$ and *** $P < 0.001$ WKY vs. SHR or young vs. old (Student's t test, unpaired).

Immunohistochemistry

To characterize the topographical distribution of ANP expression in ventricular tissue, we carried out an immuno-

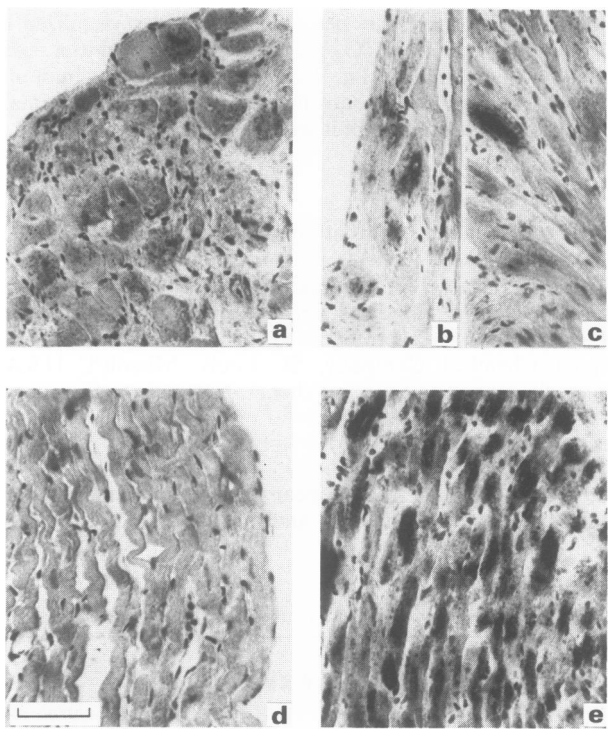


Figure 2 (a) Twenty one month old spontaneously hypertensive (SHR) rat: expression of immunoreactive atrial natriuretic peptide (IR-ANP) in the cardiocytes of the left ventricular wall. Immunoreactive granules are seen in most of the cardiocytes; (b and c) 21-month old spontaneously hypertensive (SHR) rat: strongly immunoreactive cardiocytes in the septal wall of the right atrium (b) and a binuclear cell with a high ANP content in the ventricular wall (c). (d) Normotensive Wistar-Kyoto (WKY) rat. No immunoreactivity in the cardiocytes. (e) Auricle of a spontaneously hypertensive (SHR) rat: typical strong, perinuclear localization of ANP is seen. Bar = 50 μm.

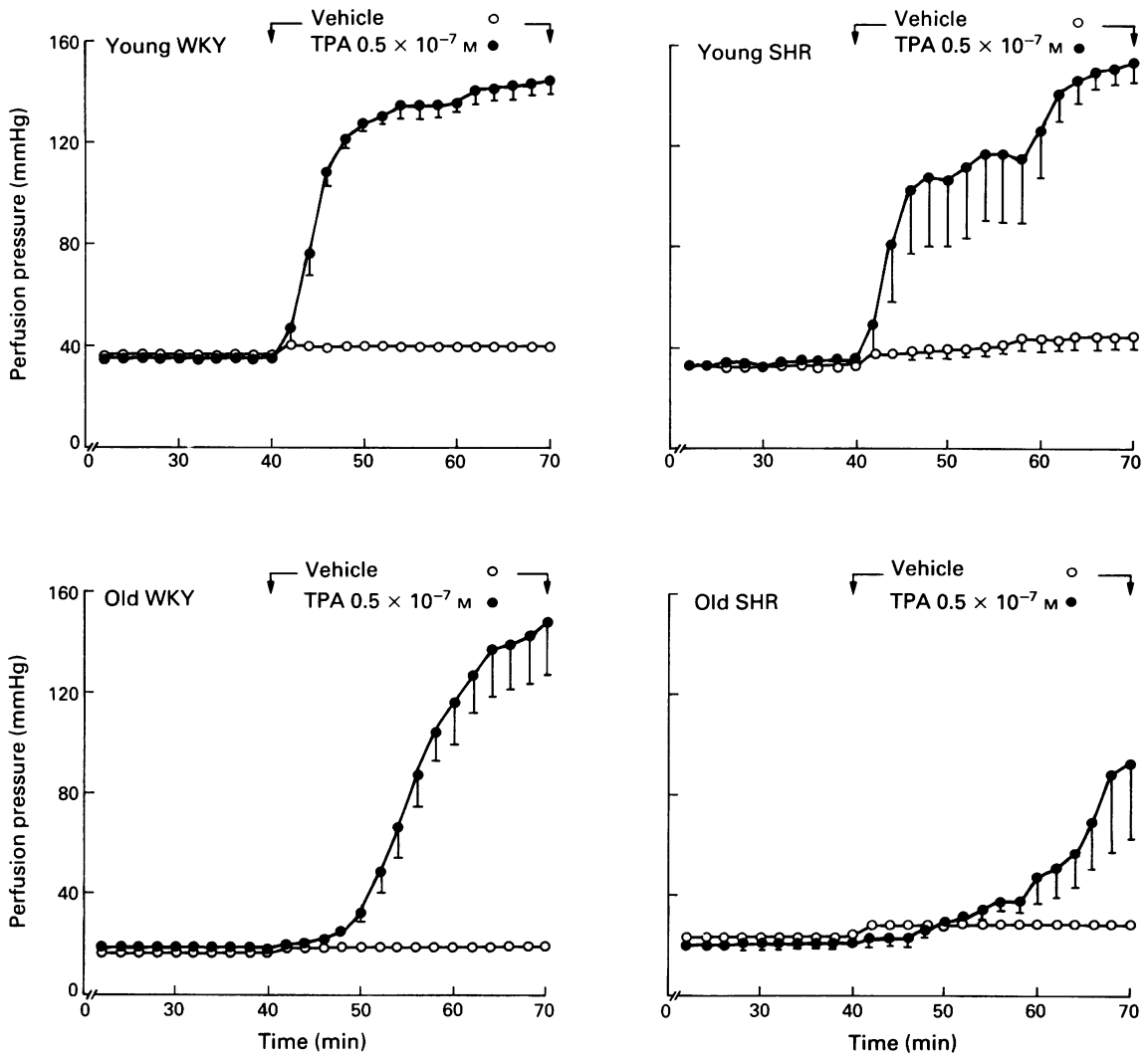


Figure 3 Effect of a phorbol ester (TPA) on perfusion pressure in isolated perfused ventricles of 2-(young) and 21-month-(old) old Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. After 20 min, atrial tissue was cut away and the remaining tissue was perfused for 20 min. Then TPA or vehicle was added to the perfusion fluid for 30 min. Each point is the mean value of four to seven separate experiments run on different isolated rat ventricles; vertical bars show s.e.mean.

histochemical analysis of heart sections. In every animal of the SHR rat strain, ANP immunoreactivity was found within the ventricular myocytes. Moderate or weak immunoreaction was seen in about 2/3 of the myocytes in all parts of the ventricular walls (Figure 2a). In some spots, especially close to the luminal surfaces of the ventricular septum (Figure 2b,c) and in the vicinity of the origin of the aorta (not shown) a few cells with a high ANP immunoreactivity were seen. Some of the most strongly stained cells were binuclear (Figure 2c). No IR-ANP was demonstrated by immunohistochemical techniques in the ventricles of a normotensive control rat (Figure 2d), while the atria of both control (not shown) and hypertensive (Figure 2e) animals expressed strong ANP immunoreaction.

Effect of phorbol ester on ANP secretion and haemodynamics in the isolated perfused hearts of WKY and SHR rats

We next evaluated the effect of the phorbol ester TPA on ANP release from normal and hypertrophic ventricular tissue after atrialectomy by using an isolated perfused heart preparation. In these experiments, the heart rate and contractile force were kept constant by pacing throughout the observation period. Atrialectomy had no effect on perfusion pressure and was (both before and after atrialectomy) 36 ± 1 mmHg ($n = 12$), 34 ± 1 mmHg ($n = 11$), 18 ± 1 mmHg ($n = 13$) and 22 ± 1 mmHg ($n = 8$) in young WKY, young SHR, old WKY and old SHR rats respectively (Figure 3). The basal concentration of IR-ANP in the perfusate was 54 ± 4 pg ml^{-1} ($n = 12$), 121 ± 3 pg ml^{-1} ($n = 11$), 374 ± 17 pg ml^{-1} ($n = 13$) and 1112 ± 21 pg ml^{-1} ($n = 8$), respectively. After atrialectomy, before the 30 min infusion of either vehicle or TPA, corresponding values were 19 ± 2 pg ml^{-1}, 25 ± 2 pg ml^{-1}, 23 ± 2 pg ml^{-1} and 394 ± 10 pg ml^{-1}, respectively (Figure 4). Thus, after atrialectomy the 21-month old WKY rats secreted 6% and SHR hearts 35% of the amount of IR-ANP secreted into perfusion fluid before atrialectomy.

Addition of 46 nM TPA to the perfusion fluid after atrialectomy and during perfusion of the ventricles at a constant flow, led to marked coronary vasoconstriction, as reflected in a rise in perfusion pressure in all the groups studied, whereas vehicle infusion had no substantial effect on coronary vascular tone (Figure 3). The maximal perfusion pressure values achieved during the TPA infusion were 145 ± 3 mmHg ($F = 159.2$, $P < 0.001$, vehicle vs. TPA, two-way analysis of variance), 152 ± 7 mmHg ($F = 22.1$, $P < 0.001$), 148 ± 22 mmHg ($F = 12.6$, $P < 0.001$) and 91 ± 30 mmHg ($F = 7.3$, $P < 0.001$) in young WKY, young SHR, old WKY and old SHR, respectively. These results agree with those published before on the effect of phorbol esters on vascular smooth muscle contraction (Rasmussen *et al.*, 1984; Ruskoaho, 1988).

The phorbol ester infusion had no effect on the perfusate IR-ANP concentration when the ventricular tissue of 2-

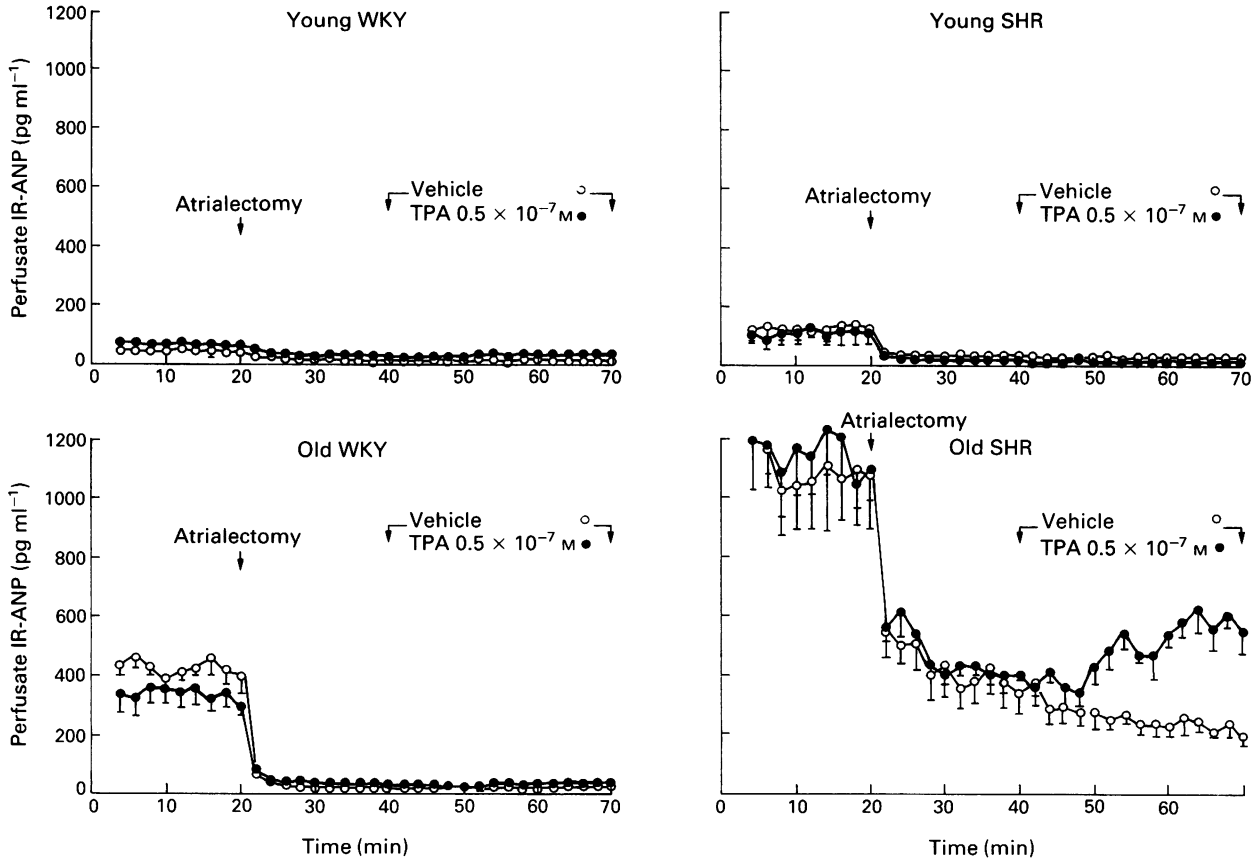


Figure 4 The effect of atrialectomy and phorbol ester on the release of immunoreactive atrial natriuretic peptide (IR-ANP) from isolated, perfused hearts of 2-(young) and 21-month-(old) old Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. After 20 min, atrial tissue was cut away and the remaining tissue was perfused for 20 min. Then, TPA or vehicle was added to the perfusion fluid for 30 min. IR-ANP secretion is expressed as pg ml^{-1}, and each point is the mean value of four to seven separate experiments run on different isolated rat hearts; s.e.mean shown by vertical bars.

month-old WKY and SHR rats was examined (Figure 4). In contrast, when the severely hypertrophied myocardium of old SHR rats was perfused with buffer containing TPA, IR-ANP secretion increased considerably from a control value of 413 ± 30 pg ml^{-1} to the maximum of 622.6 ± 75 pg ml^{-1} ($F = 10.8$, $P < 0.001$). TPA infusion had no immediate effect on IR-ANP secretion, but a gradual increase in the perfusate IR-ANP secretion was seen (Figure 4). A slight, but statistically significant increase (from 29 ± 1 pg ml^{-1} to 38.7 ± 10 pg ml^{-1}, $F = 2.2$, $P < 0.05$) in IR-ANP secretion from the hearts of very old WKY rats was also noted. Vehicle infusion had no effect on the IR-ANP secretion in any of the groups (Figure 4).

Discussion

Ventricular hypertrophy is characterized by augmentation of synthesis, storage and release of atrial natriuretic peptide from ventricular tissue, but the mechanism by which the ventricular cells secrete ANP is not yet clear. Our present results indicate that the phorbol ester TPA, known to enter cells and activate protein kinase C, increases the release of ANP from the hypertrophied but not from normal rat myocardium. The results suggest that this protein kinase C activity may be involved in the regulation of ANP secretion from ventricular cells, as has already been shown for atrial myocytes (Ruskoaho *et al.*, 1985; 1986; Matsubara *et al.*, 1988; Iida & Page, 1989; Shields & Glembotski, 1989). Further, the amount of ventricular ANP release correlated with the degree of hypertrophy, which is in good agreement with previous *in vitro* data obtained from an isolated heart preparation (Ruskoaho *et al.*, 1989a; Thibault *et al.*, 1989; Kinnunen *et al.*, 1990).

Several recent studies have demonstrated that rat (Bloch *et al.*, 1986; Wei *et al.*, 1987; Wu *et al.*, 1988) and human (Tsuchimochi *et al.*, 1988; Gardner *et al.*, 1989) ventricular myocytes can synthesize ANP during the very early neonatal or foetal periods. ANP granules, similar to those in atria, have been found within human foetal ventricles (Gardner *et al.*, 1989) and neonatal rat ventricles (Thompson *et al.*, 1986). The expression of the ANP gene decreases rapidly after birth and remains low, and often undetectable, in normal adult ventricles (Needleman *et al.*, 1989). Similarly, ANP granules disappear almost completely from the ventricles during development (Gutkowska & Nemer, 1989). The inactivation of the ANP gene is, however, reversible since reinduction of the ANP gene occurs in a variety of experimental and pathological states (e.g. pressure or volume overload) that produce ventricular hypertrophy (Lattion *et al.*, 1986; Day *et al.*, 1987; Ding *et al.*, 1987; Takayanagi *et al.*, 1987; Arai *et al.*, 1988; Ruskoaho & Leppäluoto, 1988a).

In the present study, the spontaneously hypertensive rat was used to evaluate the cellular mechanisms affecting ANP secretion from hypertrophied ventricular tissue. This rat strain is a particularly suitable experimental model for examining sequential alterations in ventricular hypertrophy. Unlike other models of cardiac hypertrophy, the condition is spontaneous and the gradual and progressive increase of cardiac mass occurs as a function of age (Trippodo & Frohlich, 1981). The old animals examined in this study were 21 months old, an age when chronic increases in arterial pressure and other possible contributing factors have resulted in a marked ventricular hypertrophy and when myocardial performance in SHR is severely impaired (Pfeffer *et al.*, 1983). By this age, immunoreactive ventricular ANP content in the hypertensive rats was 41 fold higher than in 2 month-old animals. Further, the ventricular IR-ANP levels of SHR rats were substantially higher

than in the age-matched normotensive rats, which was also seen in the emphasised role of the ventricles in secreting ANP into the perfusate in SHR rats. The contribution of the ventricles in the ANP release was 35% in old SHR and 6% in WKY rats, findings which agree with earlier studies, in which the secretion of ANP from hypertrophied ventricular tissue of SHR was greater than that from control hearts (Ruskoaho *et al.*, 1989a; Kinnunen *et al.*, 1990). In contrast, atrial ANP concentrations in the SHR rats decreased significantly with increasing age, probably because of depletion of atrial ANP stores rather than decreased synthesis of the peptide. The fact that there were no substantial differences in atrial ANP mRNA levels between the old WKY and SHR rats supports this contention.

When evaluated by immunohistochemistry, the ventricular tissue demonstrated clear evidence of ANP-containing granules in hearts of 21 month-old hypertensive rats, whereas the ventricles of their age-matched normotensive control animals showed no signs of ANP immunoreactivity. This observation supports the concept that cardiac hypertrophy results in reinduction of the ANP coding gene, efficiently expressed during the foetal and neonatal periods but dormant in normal adult ventricles. Most importantly, the formation of secretory granules in hypertrophied myocardium suggests that ANP secretion may also occur by the alternative regulatory pathway in ventricles, as is found in atria.

To test this hypothesis, we evaluted the ability of ventricular cardiomyocytes to secrete ANP in response to the administration of phorbol ester. Phorbol esters, which bind and activate protein kinase C, have been widely reported to affect the secretion rate of endocrine cells, presumably by substituting for diacylglycerol in the activation of protein kinase C (Nishizuka, 1986). Both in rat isolated perfused hearts (Ruskoaho *et al.*, 1985; 1986) and isolated cultured myocytes (Matsubara *et al.*, 1988; Iida & Page, 1989; Shields & Glembotski, 1989) protein kinase C, localized to both membrane and cytosolic fractions in the heart (Kuo *et al.*, 1984), has been shown to be activated by TPA. In perfused spontaneously beating rat hearts, TPA (15–140 nM) produced a dose-dependent increase in IR-ANP secretion (Ruskoaho *et al.*, 1985; 1986), and TPA combined with Ca^{2+} ionophore A23187 (Ruskoaho *et al.*, 1985), Ca^{2+} channel agonist Bay K 8644 (Ruskoaho *et al.*, 1986) or an α_1-adrenoceptor agonist, methoxamine (Ruskoaho & Leppäluoto, 1988b) produced a synergistic effect, thus lending support for a role of calcium-activated protein kinase C in ANP secretion. Additionally, 4α-phorbol-12,13-didecanoate, which is incapable of binding to or activating protein kinase C, was also inactive as an ANP secretagogue (Ruskoaho *et al.*, 1985). Further, in cultures of atrial myocytes, a system that allows the secretory response to be studied in the absence of extrinsic neural, hormonal or haemodynamic stimuli, TPA caused maximal ANP release into the incubation medium at a concentration of about 100 nM (Shields & Glembotski, 1989).

In the present study, we first tested the validity of the TPA concentration used by examining the effect of phorbol ester on IR-ANP secretion from perfused, spontaneously beating intact rat hearts. These studies confirmed our initial observations that TPA causes a significant ANP secretory response with accompanying marked coronary vasoconstriction (Ruskoaho *et al.*, 1985). Secondly, we demonstrated that 46 nM TPA, known at these concentrations (10–100 nM) to activate protein kinase C in this experimental model (Yuan *et al.*, 1987), caused a slowly developing increase in the IR-ANP release from ventricular tissue. A similar pattern of ANP release with respect to levels and time course has been observed before, when intact rat hearts (without atrialectomy) were perfused under these experimental conditions (Ruskoaho *et al.*, 1985; 1986). Interestingly, a marked stimulation of ventricular ANP secretion was observed only from hearts of old SHR rats with severe ventricular hypertrophy, high ANP gene expression and the presence of ANP containing granules. A slight increase in ANP secretion was also observed from the ventricles of old normotensive rats. In contrast, TPA had no effect on ANP secretion from the ventricles of the young animals. This inability of TPA to stimulate ANP release in 2-month old rats as well as the small effect in old WKY rats indicate that the ANP release observed in old SHR rats could not originate from atrial tissue, i.e. the atrial tissue had been successfully removed because if this had not been the case, phorbol ester would have increased ANP secretion similarly in all groups studied. Finally, the fact that TPA caused a marked coronary vasoconstriction both in 2- and 21-month old control and hypertrophied hearts indicates the effectiveness of the TPA concentration used in this study.

Our observation that ANP release from the severely hypertrophied ventricles of hypertensive rats can be stimulated by phorbol ester demonstrates the ability of mature ventricular tissue to secrete ANP in response to a segretagogue. Greenwald *et al.* (1989) reported that KCl produced a dose-dependent release of ANP from suspensions of neonatal rat ventricular cells in culture and suggested that, like the atrial myocyte, the ventricular myocyte possesses the cellular mechanism(s) necessary to secrete ANP by a regulated pathway. In contrast, Bloch *et al.* (1988) reported that short-term exposure to the Na^+-K^+-adenosine-triphosphatase inhibitor, ouabain, stimulates the secretion of IR-ANP from atrial, but not from ventricular, cardiomyocytes in culture. Similarly, incubation of the ventricular cell cultures with TPA does not alter the ANP secretion rate (Shields & Glembotski, 1989). Both authors subsequently conclude that ventricular cardiomyocytes utilize a constitutive secretory pathway, and a segretagogue would not be expected to enhance ANP release from this cell type. The reason for this discrepancy is not clear; however, the methods used were different (cell suspensions vs. cultured cells). Secondly, the myocytes used by Greenwald *et al.* (1989) were only 1-day-old, whereas Bloch *et al.* (1988) used 4- and Shields & Glembotski (1989) 1- to 5-day-old myocytes, which may already have differentiated in culture and lost their ability to utilize regulatory secretory pathways in a manner analogous to that of intact ventricles during development to adult tissue (Wei *et al.*, 1987). Our present data show that with development of hypertrophy and appearance of ANP containing granules, ventricular cells appear to regain the ability to secrete ANP by the regulated pathway, as documented by the stimulation of secretion from the hypertrophic ventricle in response to phorbol ester infusion.

The factors driving ANP gene expression remain to be determined. In addition to hypertrophy, ANP gene expression in the adult ventricle has also been shown to be induced by exposure to a hormonal stimulus (Gardner *et al.*, 1986; 1987; Day *et al.*, 1987; Ladenson *et al.*, 1988; Ruskoaho *et al.*, 1989b). It has been proposed that the reinduction of the ANP gene represents regression of the ventricular myocyte toward its earlier developmental form (Day *et al.*, 1987; Zeller *et al.*, 1987). Similarly, several other genes have been shown to be induced in the hypertrophied state of ventricles, including foetal type myosin heavy chain (Lompre *et al.*, 1979) and foetal myosin-light-chain-1-like protein (Hirzel *et al.*, 1985) coding genes. The fact that ventricular ANP expression is largely concentrated in the subendocardium of that chamber (Edwards *et al.*, 1988; Ruskoaho *et al.*, 1989a), a region of the ventricular wall which is subject to the highest levels of stress, suggests that transcription of the ANP gene may be responding to the increased wall tension. Of note, the distribution of β-myosin heavy chain gene expression within myocardium shows a transmural gradient (subendocardium higher) analogous to that of ANP (Gorza *et al.*, 1981). This suggests that common factor(s) such as wall stress may regulate their gene expression in the ventricular myocardium.

In conclusion, ANP gene expression is increased in the hypertrophic ventricles of genetically hypertensive rats, as reflected by elevated levels of immunoreactive ANP and ANP mRNA, the appearance of secretory granules within the ventricles, and the increased ANP secretion from the ventricular

cells. Importantly, phorbol ester stimulated ANP release from the severely hypertrophied ventricles of hypertensive animals demonstrating the ability of ventricular tissue to secrete ANP in response to a segretagogue. Thus, hypertrophied rat ventricular myocytes appear to possess the cellular mechanisms necessary to secrete ANP by a regulated pathway.

We thank Mrs Eira Kerälä, Ms Tuula Räisänen, Ms Tuula Taskinen and Ms Mirja Vahera for expert technical assistance. This study was supported by the Academy of Finland and Sigrid Juselius Foundation.

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(Received May 4, 1990
Revised September 10, 1990
Accepted September 12, 1990)

Br. J. Pharmacol. (1991), **102**, 462–466 © Macmillan Press Ltd, 1991

Effects of captopril on membrane current and contraction in single ventricular myocytes from guinea-pig

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1 Single guinea-pig ventricular myocytes were voltage-clamped and cell length was measured with a photodiode array.

2 Captopril (1×10^{-5} M) reduced both peak early current and active shortening in response to a depolarizing clamp pulse along a similar time course.

3 From a holding potential of around -45 mV peak early inward current was reduced by $37 \pm 9\%$ ($P < 0.001$) on exposure to captopril. The early current-voltage relationship was shifted outwards by captopril indicating a reduction in membrane conductance through the L-type calcium channel (I_{Ca}).

4 The amplitude of cell shortening in response to depolarizing voltage steps was reduced but the voltage-dependence of contraction after captopril was unchanged.

5 A small negative shift of the potential at which I_{Ca} was half-activated was observed after captopril. There was no change in the voltage-dependence of the inactivation variable or in the time-dependence of repriming for I_{Ca}.

6 The actions of captopril on I_{Ca} and developed shortening were dose-dependent and took place in the same proportion when I_{Ca} was increased by isoprenaline.

7 These results are discussed in relation to the effects of captopril on I_{Ca} and contraction and to its clinical usage.

Introduction

Captopril is a proline derivative whose principal mode of action is inhibition of angiotensin converting enzyme. The possibility that the drug may exert a direct action on the myocardium is of considerable interest given that captopril is extensively prescribed for treatment of heart failure and hypertension. It has recently been reported that captopril reduces developed force in isolated heart muscle of guinea-pig and man in a dose-dependent fashion (Walker *et al.*, 1988). Possible explanations for this negative inotropic effect include inhibition of endogenous angiotensin release (Lindpaintner *et al.*, 1988), interference with the action of catecholamines released by field stimulation in isolated tissue (Koch-Weser, 1965; Collis & Keddie, 1982), or alternatively a direct action of the drug on the myocytes themselves. We have set out to investigate the latter possibility using isolated single ventricular myocytes, and after confirming an effect of the drug on developed length we have sought to define the action of captopril on membrane currents using the voltage-clamp technique. A preliminary account has appeared (Bryant *et al.*, 1989).

Methods

Cells were isolated from both ventricles of female Dunkin-Hartley guinea-pigs weighing 400–600 g by an enzyme technique (Mitchell *et al.*, 1984) and were layered on a glass coverslip which formed the base of a perfusion chamber situated on the stage of an inverted microscope. Micropipettes were filled with 3 M KCl giving a resistance of 18–25 MΩ. Voltage-clamping was achieved by a single micro-electrode switch-clamp technique (Axoclamp II-A, Axon Instruments, Inc., Foster City, CA). The clamp switching frequency was 3.5–6 kHz. An agar bridge in the bath was used as the indifferent electrode. Cell length was measured with an S-series photodiode array (E.G. & G. Reticon, Wokingham, Berks.; Boyett *et al.*, 1988). Superfusate flow rate was 1–2 ml min^{-1} and solution lines were switched by solenoid valves (Lee Products Ltd., Gerrards Cross, Bucks.) just before entry to the bath. The temperature was maintained at 32 ± 1°C by a heating coil surrounding the input line which was controlled by a feed-back circuit using a sensing thermocouple positioned between the heating coil and the chamber.

Voltage, current and length signals were recorded on magnetic tape (Racal Store 7 recorder, Hythe, Southampton) for later analysis. The signals were played back through a 6-pole Bessel filter and sampled at 1 kHz by an A to D converter (CED 1401, Cambridge Electronic Design Ltd., Cambridge). Analysis of the digitized records was performed on a Compaq Deskpro 286 computer using software provided by Dr J. Dempster, Strathclyde University.

Superfusate composition was as follows (mM): Na^+ 136, K^+ 5.4, Mg^{2+} 1.2, Ca^{2+} 1.8, glucose 11.1, HEPES 5.0, titrated with NaOH to pH 7.4. Captopril was dissolved in water before being added to the superfusate in appropriate dilution.

Results were compared by Student's *t* test for paired samples and are expressed as mean ± standard error of the mean (s.e.mean).

Results

Time-dependence of captopril action

The holding potential was set between -40 and -45 mV in order to inactivate the sodium channel and the T-type calcium channel. Depolarizing pulses of 500 ms duration were applied from this potential to 0 mV at a frequency of 0.2 Hz. Figure 1 shows the effects of captopril 1×10^{-5} M on the change in cell length associated with contraction (a) and on peak inward current (b) using this protocol. Captopril reduced the amplitude of inward current and active shortening which both decreased along a similar time course. The drug has a rapid onset of action and the changes were complete within 90 s. On removal of captopril there was recovery of both current and contraction in some cells but this was not a consistent finding at the higher concentrations used.

Peak inward current at this potential is composed chiefly of Ca^{2+} current (Mitchell *et al.*, 1987) and it seems possible that

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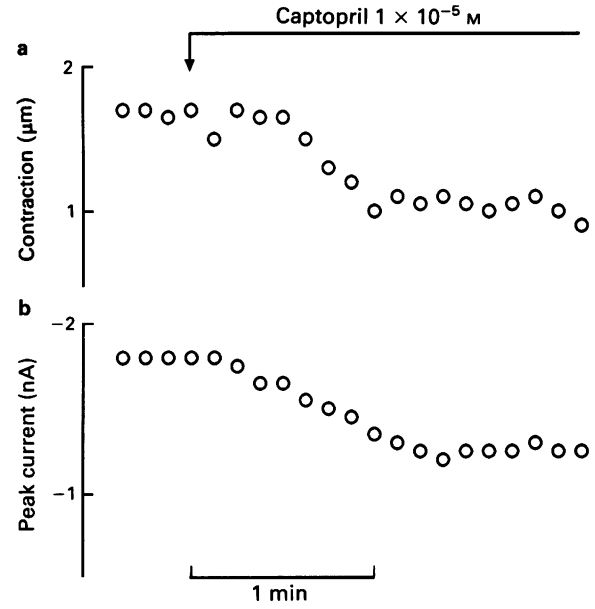


Figure 1 Effects of captopril on the reduction in cell length (a) and on peak inward current (b) in a single cardiac myocyte. Step depolarizations to 0 mV from a holding potential of −40 mV were elicited at a frequency of 0.2 Hz. Application of the drug is indicated by the arrow. The changes are complete in approximately 90 s.

a reduction in this current could account for the fall in developed length after captopril. We therefore set out to look at the effects of the drug on the L-type Ca^{2+} current in more detail.

Current-voltage relationships

Figure 2 shows current records from a single cell obtained by depolarizing from a holding potential of −45 mV to the potentials shown. There is an inward peak of membrane current at the onset of the depolarizing step as L-type calcium current (I_{Ca}) activates rapidly (Fox *et al.*, 1987). Inactivation of this current follows a much slower time course and at the more positive potentials shown an increasing outward current,

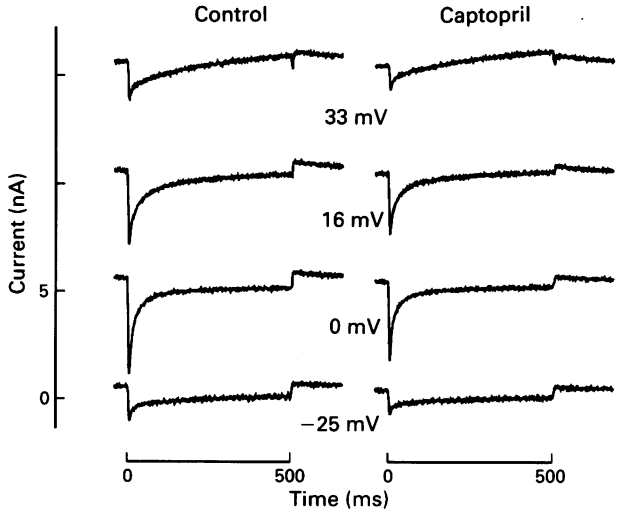


Figure 2 Membrane current records showing the effects of depolarizations lasting 500 ms from a holding potential of −45 mV to the potentials shown by the records. The left-hand records were obtained in control solution, the right-hand after captopril 1×10^{-5} M. Identical clamp steps were used for each sequence of depolarizations. The labelled tickmarks on the abscissa scale refer to the lowest pair of records. For clarity the remaining records have been shifted outwards by 5 nA and the unlabelled tickmarks indicate the zero current level for these records.

I_K, develops during the later part of the clamp step. The right-hand records were obtained from the same cell a few minutes after exposure to captopril (1×10^{-5} M) and it can be seen that captopril has reduced the peak early current at each potential.

Current-voltage relationships for this cell are shown in Figure 3. The circles represent peak early current at increments of approximately 10 mV from the holding potential and show that I_{Ca} has been reduced by the drug (cf., for example, Kameyama *et al.*, 1986). The potential at which inward current was maximal did not change in the presence of captopril. In 18 cells, a concentration of 1×10^{-5} M-captopril reduced mean maximum inward current from 2.3 ± 0.3 to 1.5 ± 0.2 nA ($P < 0.001$). The reversal potential for I_{Ca} showed no significant change after captopril. There was also no significant change in the holding current or in the current at the end of the 500 ms depolarizing clamp steps (shown as squares in the graph), indicating that there is no significant action of the drug on the magnitude of the delayed rectifier I_K.

Length-voltage relationships

The changes in length associated with depolarizing voltage-clamp steps in this cell are illustrated in Figure 4. It can be seen from the original length records in Figure 4a that the amplitude of the phasic component of contraction, produced by the depolarization, increases with voltage and then declines as the potential becomes more positive. Figure 4b shows that after captopril the voltage-dependence of this U-shaped relationship is maintained but the amplitude of contraction at each potential is reduced. The peak shortening has been reduced from 6.4 to 3.9 μm, amounting to a 40% reduction in developed length. In 5 cells the peak reduction in developed length seen at this concentration of captopril (1×10^{-5} M) was $47 \pm 3\%$ ($P < 0.01$).

Kinetic parameters for I_{Ca}

In order to find out whether the reduction in I_{Ca} is attributable to a reduction in conductance for I_{Ca} or to a shift in the kinetics of this channel we have measured the voltage-dependence of the kinetic variables for the current. The activation variable for the current was calculated from the chord conductance for peak early current at each potential step, expressed as a fraction of peak conductance. Figure 5 shows a typical result from one cell. The voltage at which I_{Ca} was half-activated moved -3.8 ± 0.6 mV after exposure to captopril 10^{-5} M ($P < 0.01$, $n = 5$). The inactivation variable was measured by stepping the membrane potential from approx-

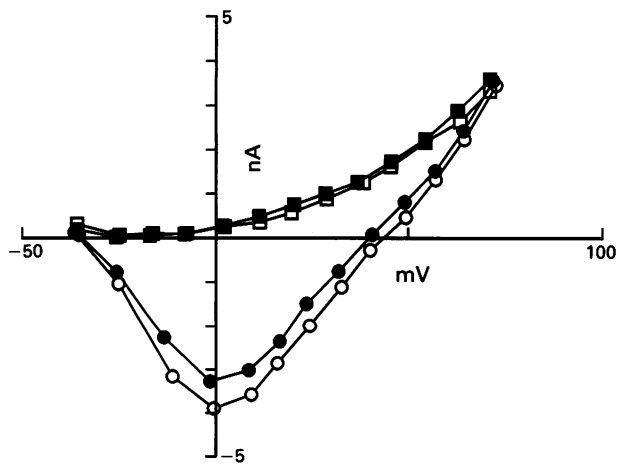


Figure 3 Current-voltage relationships for peak early current (circles) and current at the end of 500 ms depolarizing steps (squares) obtained from a holding potential of −45 mV. Open symbols represent control, closed symbols, captopril, 1×10^{-5} M.

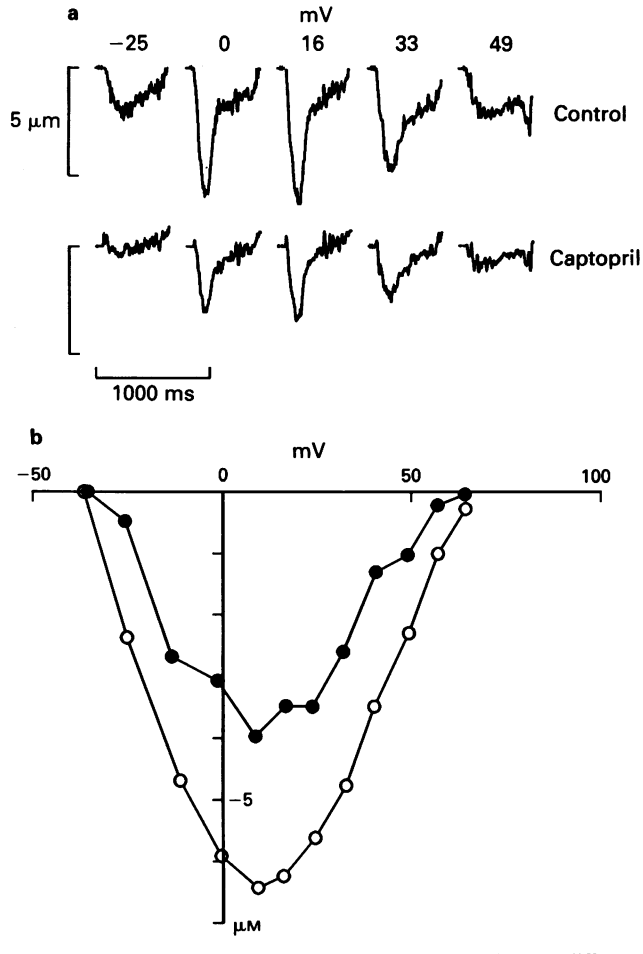


Figure 4 (a) Changes in cell length on depolarization to different potentials. The upper records in this panel are controls; the lower set were recorded after captopril. The potentials to which the cells were depolarized are shown at the top of each vertical pair of records. The top of each length calibration bar represents resting cell length (129 μm). (b) Relationships between the change in cell length on depolarization and clamp potential before (○) and after (●) captopril (1×10^{-5} M), plotted from the data used to illustrate the upper panel. The experimental protocol was the same as in Figure 3.

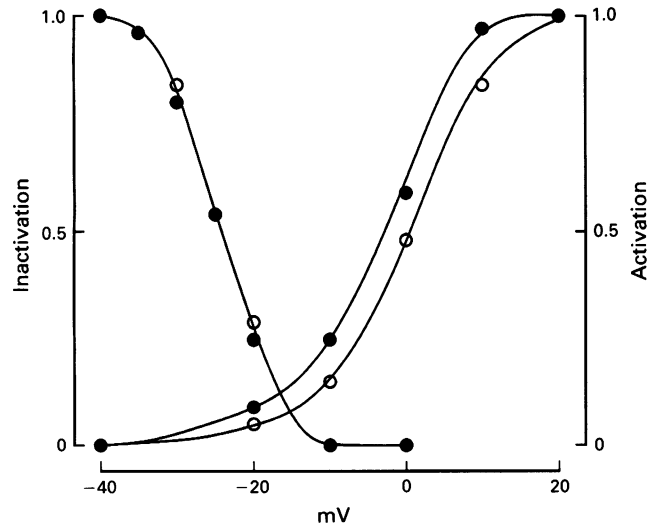


Figure 5 Voltage-dependence of the steady-state activation (d_∞) and inactivation (f_∞) variables from a representative myocyte before (○) and after captopril 1×10^{-5} M (●). Curves drawn by eye. The inactivation curve is the same after captopril; the activation curve is shifted to the left by a small amount. Note that the inactivation curve was constructed from a holding potential of −40 mV so that by default $f_\infty = 1$ at this potential and the bottom end of this curve may therefore have been abbreviated (Fox *et al.*, 1987).

imately −40 mV to different conditioning potentials for 200 ms followed by a test pulse to 0 mV for 100 ms. There was no significant shift in the voltage dependence of the inactivation variable (mean of differences -1.7 ± 1.1 mV, $P < 0.5$).

The recovery of I_{Ca} from inactivation was studied by use of a two-pulse protocol. Both pulses were to 0 mV and the interpulse interval was increased from 10 to 500 ms. The ratio of currents produced by the two pulses as a function of interpulse interval is shown for the mean data in Figure 6. The time for 50% recovery from inactivation was 81 ± 13 ms before and 83 ± 11 ms after captopril (NS, $n = 4$).

Dose-response curve for captopril

In order to show that the changes in I_{Ca} and contraction which we have described are attributable to a pharmacological effect of the drug it is also necessary to define the concentration-dependence of these actions. Figure 7 shows the

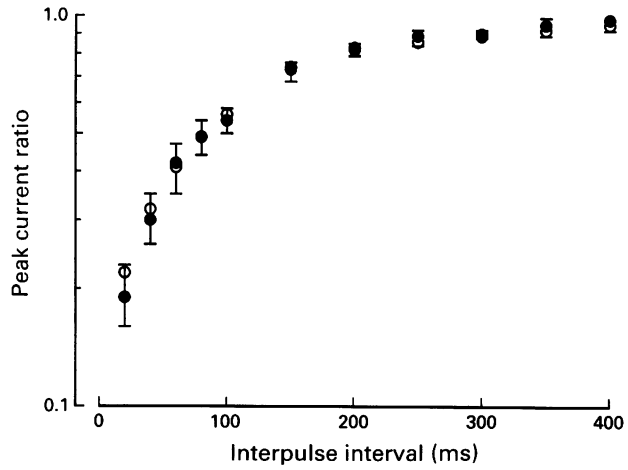


Figure 6 Time-dependence of restitution of I_{Ca} in 4 cells before (○) and after (●) captopril 5×10^{-5} M. Recovery of early peak current is expressed as a fraction of the current seen in the absence of a prepulse, as a function of interpulse interval. There was no significant shift in the time for 50% recovery after captopril.

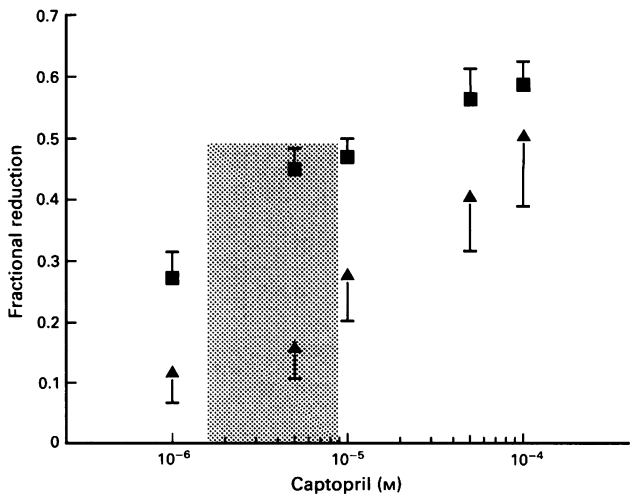


Figure 7 Incremental dose-response curves for change in developed length (■) and peak early inward current (▲) expressed as fractional reduction of control values for each parameter, plotted against captopril concentration. The voltage-clamp protocol involved depolarizing steps from a holding potential of −40 mV to 0 mV lasting 500 ms, applied every 5 s. Standard error bars are shown for $n = 4$ cells. The stippled area represents the therapeutic range of plasma concentrations of the drug in man.

effects of incremental concentrations of captopril on the mean change in length associated with step depolarization to 0 mV and on mean I_{Ca} recorded simultaneously, from completed protocols in 4 cells. There was a significant reduction in both contraction and I_{Ca} at the lowest concentration of captopril used (10^{-6} M). It appears that the decrease in contraction is relatively greater than the decrease in I_{Ca} at each concentration tested. Maximal inhibition does not appear to have been achieved. Further increases in concentration of the drug resulted in transient inward currents and cell death followed rapidly.

Further experiments on the mechanism of captopril action on I_{Ca}

The effect of captopril on the early current-voltage relationship (Figure 3) strongly suggests an action on the L-type calcium channel, either directly or by a secondary action on a mechanism controlling I_{Ca} amplitude. When I_{Ca} was blocked with gallopamil (D600), subsequent addition of captopril (1×10^{-5} M) had no effect on the remaining membrane currents and current-voltage relationship. In order to confirm that the effect is specific to the calcium channel we increased the calcium current with isoprenaline and then exposed the myocytes to captopril in the presence of isoprenaline. Isoprenaline 1×10^{-7} M increased I_{Ca} seen on depolarization from −45 mV to 0 mV by 173 ± 17%. Exposure to captopril, 1×10^{-5} M in the presence of isoprenaline reduced I_{Ca} by 21 ± 4% ($n = 4$). The drug therefore reduced I_{Ca} amplitude by a similar extent to that seen in the absence of the β-adrenoceptor agonist.

Discussion

These experiments show that captopril reduces developed length in isolated mammalian ventricular myocytes in a time-dependent and concentration-dependent fashion and that this negative inotropic effect appears to be linked to a reduction in L-type calcium current. The change in I_{Ca} is attributable to a reduction in membrane conductance rather than to a shift in the kinetic variables. The basis for this action may be a reduction in open-channel probability after captopril which would be detectable in patch-clamp studies of this channel. We have carried out some provisional patch-clamp experiments which indicate an effect of the drug directly on the outer surface of the channel, rather than via intracellular second messenger systems. A small negative shift of the activation variable *per se* would be expected to give rise to an increase in current at potentials between the holding potential and the top of the activation curve. Clearly this effect is offset by the fall in I_{Ca} conductance produced by the drug.

Possible mechanisms of action of the drug on the L-type calcium channel may be considered. The shift of the d_∞ variable is small and is in the opposite direction to that reported to occur on exposure to the phenylalkylamine calcium antagonists. For example Cohen & Lederer (1987) found that D600 produced a positive shift of this variable in neonatal rat ventricular myocytes. On the other hand these workers and Fox *et al.* (1987) found that the dihydropyridine agonist Bay K 8644 gives rise to a negative shift of the activation curve. We may rule out the possibility that inhibition of neuronal catecholamine release is involved since isolated myocytes are effectively denervated (Dow *et al.*, 1981) and the effects of the drug on the activation variable are not those which would be expected from reversal of β-adrenoceptor stimulation (Cohen & Lederer, 1987). Angiotensin II increases the calcium current in neonatal rat ventricular myocytes (Allen *et al.*, 1987). Could the drug be inhibiting endogenous angiotensin II synthesis, which has been demonstrated to occur in cardiac muscle (Nakashima *et al.*, 1982)? In isolated hearts, no angiotensin II synthesis has been demonstrated without the presence of angiotensin I in the perfusate (De Graeff *et al.*, 1986; Lindpaintner *et al.*, 1988). This explanation is therefore unlikely for the *in vitro* effects here described since there was no angiotensin I in the perfusate. That the effect of captopril on I_{Ca} is proportionately similar when this current has been increased by catecholamines suggests that the drug is having a fairly direct effect on the channel rather than acting via some less specific mechanism.

We have consistently observed a good temporal relationship between the onset of negative inotropy and the decline in I_{Ca}. Even though the amplitude is reduced, the time course and voltage-dependence of the developed shortening response are unchanged after captopril. Nevertheless it is unlikely that a decrease in I_{Ca} is the sole mechanism of the reduced contractile response to captopril. From Figure 3 it appears that the magnitude of I_{Ca} at −10 mV is close to that at 0 mV in the presence of captopril. However the length changes accompanying these currents (Figure 4) are different, the shortening at 0 mV after captopril being nearly 2 μm less than that at −10 mV in control solution. Terrar & Victory (1988) have shown, in guinea-pig ventricular myocytes under switch-clamp conditions, that on exposure to verapamil or nifedipine, a 60% fall in I_{Ca} gives a reduction in contraction of about 35%. From the data in Figure 7 we may estimate that a 35% reduction in developed shortening after captopril would be accompanied by a decrease in I_{Ca} of only 13%. The relationship between I_{Ca} and the contractile response is not linear, but even allowing for this it is necessary to postulate an action of captopril on excitation-contraction coupling distal to I_{Ca}, which may involve a reduction in Ca^{2+} release from the sarcoplasmic reticulum in response to a given increment in intracellular Ca^{2+}. Further work will be necessary to explore these additional effects.

We may consider briefly possible implications of this work which are relevant to the clinical use of the drug. Plasma levels of captopril in human subjects following therapeutic administration of the drug have been in the range 1.7–8.9 × 10^{-6} M, at which concentrations there is a significant effect of the drug in reducing developed shortening and I_{Ca} in guinea-pig single ventricular myocytes. When treatment with captopril is started in patients with heart failure it is usual to prescribe a small first dose because of the risk of hypotension. The mechanism of this 'first-dose' effect is likely to involve enzyme inhibition as the magnitude of the fall in arterial pressure is related to the resting pretreatment plasma renin activity (Dzau *et al.*, 1980; Faxon *et al.*, 1982). However, the finding that captopril has a direct negative inotropic effect *in vitro*, at concentrations similar to those found in plasma after routine administration of the drug, raises the possibility that a reduction of myocardial calcium current *in vivo* may play a part in the hypotensive effect of the drug, particularly in patients with impaired left ventricular function.

There is good evidence that the vasodilator action of captopril cannot be fully explained in terms of reduction of circulating angiotensin II (Gavras *et al.*, 1978). Inhibition of central and peripheral tissue renin conversion has been demonstrated and may be involved in the hypotensive action of the drug (e.g. Jin *et al.*, 1988). Aaronson *et al.* (1988) have described a dihydropyridine-sensitive calcium current in vascular smooth muscle cells from rabbit ear artery. L-type Ca^{2+} current has been shown to be the predominant Ca^{2+} channel in coronary artery smooth muscle cells from guinea-pig (Ganitkevich & Isenberg, 1990). Given this homology between L-type calcium channels in heart and in vascular smooth muscle it is possible that a reduction of I_{Ca} in vascular smooth muscle is contributing to the vasodilatation produced by captopril and so to the reduction of arterial pressure and afterload which follows its therapeutic administration.

We conclude that captopril reduces I_{Ca} and developed length in guinea-pig single ventricular myocytes.

This work was supported by BHF grant no. 87/63. K.O.R. is an MRC Training Fellow. Captopril was obtained as a gift from E.R. Squibb & Sons Ltd. We are indebted to Dr T. Powell for help with single cell isolation and to Mr A.J. Spindler for making equipment. The preliminary patch-clamp experiments were carried out in collaboration with Dr T.J.A. Allen.

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(Received July 23, 1990
Revised October 10, 1990
Accepted October 12, 1990)

Br. J. Pharmacol. (1991), **102**, 467–477 © Macmillan Press Ltd, 1991

Characterization in rat aorta of the binding sites responsible for blockade of noradrenaline-evoked calcium entry by nisoldipine

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1 The effectiveness of the calcium antagonist, 1,4-dihydropyridine nisoldipine, as an inhibitor of contraction and ^{45}Ca entry evoked by noradrenaline in rat aorta has been investigated and correlated with binding characteristics in intact artery.

2 Contractions evoked by noradrenaline were concentration-dependently depressed by nisoldipine (0.3–300 nM). About 60% of the response was resistant to inhibition, while KCl-induced contractions could be completely blocked. Noradrenaline-induced contractions were also less sensitive to nisoldipine inhibition than were KCl-induced contractions.

3 Preincubation of the aorta with nisoldipine in high KCl depolarizing solution increased the inhibition of the contraction evoked by a short application of noradrenaline or KCl to a similar extent.

4 The inhibition by nisoldipine of ^{45}Ca influx evoked either by KCl depolarizing solution or by noradrenaline correlated well with the inhibition of the contractile responses. However, while KCl-stimulated ^{45}Ca influx was totally abolished by nisoldipine (300 nM), 38% of the noradrenaline-stimulated ^{45}Ca influx was resistant to inhibition by nisoldipine (300 nM).

5 The study of [^{3}H]-(+)-PN 200-100 ([^{3}H]-(+)-isradipine) binding in intact aorta showed the presence of a homogeneous population of specific binding sites. K_D values were dependent on the KCl concentration in the bath while B_{max} was unaffected. Binding of [^{3}H]-(+)-isradipine was also increased in tissue exposed to noradrenaline; in the presence of 10^{-5} M noradrenaline, binding parameters of [^{3}H]-(+)-isradipine were close to the values obtained in aorta bathed in 20 mM KCl solution.

6 Displacement of [^{3}H]-(+)-isradipine specific binding by nisoldipine was determined in segments of mesenteric artery and of aorta. The potency of nisoldipine was dependent on the incubation conditions applied to the vessel, as follows: KCl (100 mM) depolarizing solution > noradrenaline (10^{-5} M) = KCl (25 mM) solution > physiological solution. The K_i value measured in aorta exposed to noradrenaline (10^{-5} M) was close to the IC_{50} value of nisoldipine on the noradrenaline-evoked contraction.

7 The membrane potential value of rat aorta was estimated by the distribution of [^{3}H]-tetraphenylphosphonium bromide ([^{3}H]-TPP$^+$). [^{3}H]-TPP$^+$ uptake concentration-dependently decreased when the KCl concentration in the bath was increased from 5.9 to 130 mM. Noradrenaline also concentration-dependently decreased [^{3}H]-TPP$^+$ uptake; the maximum effect (1–10 μM noradrenaline) was comparable in amplitude to the effect of 25 mM KCl solution.

8 It is concluded that in rat aorta, noradrenaline activates voltage-operated calcium channels that contain the specific, voltage-sensitive binding sites for calcium antagonistic dihydropyridines. The existence of a fraction of noradrenaline-stimulated ^{45}Ca entry that is resistant to nisoldipine blockade suggests that another Ca^{2+} entry pathway is also opened by the agonist.

Introduction

In rat aorta, stimulation of α_1-adrenoceptors by noradrenaline increases cytosolic free Ca^{2+} and produces contraction (Godfraind, 1976; Cauvin & Malik, 1984; Minneman, 1988). It has been shown that the early fast component of the contraction evoked by a high concentration of noradrenaline persisted in the absence of extracellular calcium, implying that noradrenaline releases Ca^{2+} from intracellular stores. It is now well established that activation of α_1-adrenoceptors causes the hydrolysis of inositol phospholipids, through the coupling of the adrenoceptor with phospholipase C. Inositol 1,4,5-trisphosphate released into the cytosol then induces the release of Ca^{2+} from internal stores, which is responsible for the initial phase of the contraction (Minneman, 1988). The other part of the contraction, which constitutes the tonic, sustained component of the contraction, is dependent on the presence of Ca^{2+} in the bathing solution (Godfraind & Kaba, 1969). Noradrenaline has indeed been reported to enhance $^{45}Ca^{2+}$ influx (Godfraind, 1976). This observation indicates that the stimulation of α_1-receptors opens channels in the membrane, allowing Ca^{2+} to enter the cell. Dihydropyridine calcium antagonists are known to prevent the increased Ca^{2+} entry produced by stimulation in vascular smooth muscle, without affecting resting Ca^{2+} fluxes or intracellular Ca^{2+} release (Godfraind *et al.*, 1986). However, in rat aorta as in most vascular smooth muscles, there are important differences in the potency of calcium antagonists against Ca^{2+} entry stimulated by receptors or by high K^+ depolarization, implying that separate Ca^{2+} entry pathways are called into play by the two types of stimulation (for review see Spedding & Cavero, 1984; Cauvin & van Breemen, 1985). This observation led to the hypothesis of the existence of Ca^{2+} channels gated by receptor activation (ROCs) and of Ca^{2+} channels gated by potential (POCs) (Bolton, 1979). If the existence of POCs is well documented, there are few reports demonstrating the existence of ROCs (see Rink, 1988), and the events coupling α_1-adrenoceptor stimulation and Ca^{2+} entry have not been elucidated. There is some evidence that, at least in some tissues, noradrenaline evokes a depolarization, although results are conflicting as far as the degree of depolarization and the relation between depolarization and tension generation are concerned (see Bolton & Large, 1986; Haeusler & De Peyer, 1989). On the other hand, Nelson *et al.* (1988) recently reported that at a concentration that did not depolarize, noradrenaline shifted the relationship between Vm and contraction to more negative potentials, so that noradrenaline contracts arteries by interacting with POCs, rather than by

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opening ROCs, leaving the question of the existence of ROCs open. The aim of the present work was to investigate the mode of interaction of the calcium antagonistic dihydropyridines nisoldipine and (+)-PN 200-110 ((+)-isradipine), with the putative ROCs and POCs in rat aorta. A comparative study of binding behaviour, inhibitory action on $^{45}Ca^{2+}$ influx and on contraction induced by noradrenaline and KCl-depolarization, allowed us to characterize the existence of voltage-dependent binding sites for dihydropyridine calcium antagonists and to show that noradrenaline-stimulated Ca^{2+} entry in rat aorta is partly carried out through POCs. The lower sensitivity to calcium antagonists of the action of noradrenaline compared to responses induced by KCl-depolarization may be accounted for by the sensitivity of dihydropyridines binding site to membrane potential.

Some of these results have been described previously (Godfraind *et al.*, 1989; Morel & Godfraind, 1990).

Methods

Wistar rats (weighing 250–300 g) were used. They were killed by decapitation, the thoracic aorta and the superior mesenteric artery were removed and immersed in physiological solution (mM: NaCl 122, KCl 5.9, $NaHCO_3$ 15, $MgCl_2$ 1.25, $CaCl_2$ 1.25 and glucose 11) maintained at 37°C and aerated with a gas mixture of 95% O_2, 5% CO_2. Tissues were cleaned of adherent connective and fat tissue and cut in rings. When necessary endothelium was stripped off by mechanical rubbing.

Measurement of contractile responses

Aortic rings, about 2 mm wide, were suspended in 50 ml organ baths filled with physiological solution under a resting tension of 2 g. Rings were connected to an isometric level with two strain gauges as part of a balanced bridge. The output was fed into an analog/digital converter, which was connected to a microcomputer. The programme allowed input from up to 8 force transducers simultaneously. After suitable calibration, tension was displayed on the screen of the microcomputer, recorded on a printer, and stored on disk at predetermined time intervals. After mounting in organ chambers, rings of arteries were allowed to equilibrate for 90 min in physiological solution. Contraction was evoked either by changing the physiological solution in the bath to a K^+-rich depolarizing solution containing (mM): NaCl 22, KCl 100, $NaHCO_3$ 15, $MgCl_2$ 1.25, $CaCl_2$ 1.25 and glucose 11, or by addition of noradrenaline to the bath. The first stimulation was used to check the sensitivity of the preparation to the relaxing effect of acetylcholine (1 μM). After wash out for 30 min in physiological solution, a control stimulation was recorded for 35 min. After wash out and relaxation, the vessels were incubated for 90 min in physiological solution with or without various concentrations of nisoldipine. A third contraction was then induced in the continuous presence of the antagonist. Results obtained in the presence of the antagonist were compared to controls that were treated simultaneously as described above but without incubation with the antagonist.

Measurement of ^{45}Ca influx

Calcium influx was estimated by measuring changes in the specific activity of the calcium fraction resistant to displacement by lanthanum (Godfraind, 1976). Noradrenaline-dependent ^{45}Ca influx was estimated after an equilibration period of 60 min in physiological solution maintained at 37°C and aerated with a gas mixture of 95% O_2 and 5% CO_2. After preincubation of 90 min in the same solution with or without nisoldipine, the artery segments were incubated for 5 min in physiological solution containing $^{45}Ca^{2+}$ and the antagonist and then for a further 2 min in the same solution with noradrenaline 10^{-5} M. A similar procedure was followed with the $^{45}Ca^{2+}$-K-depolarizing solution, except that prazosin (10^{-8} M) was added with nisoldipine (Godfraind, 1983). Thereafter the preparations were washed for 5 min in 500 ml of La^{3+} solution (mM: NaCl 122, KCl 5.9, $MgCl_2$ 1.25, $LaCl_3$ 50, glucose 11 and Tris-maleate buffer 15; pH 6.8) to remove extracellular Ca^{2+}. Arterial segments were then dried between two sheets of filter paper, weighed, dissolved in 0.1 ml of a mixture of perchloric acid: H_2O_2 (1:1). The radioactivity of the samples was counted in a liquid scintillation counter as usual with appropriate controls.

Measurement of [^3H]-(+)-isradipine binding

Binding studies were performed following the same protocol as described for the contraction experiments. Rings of mesenteric arteries or of aorta (1–2 mg wet weight) were incubated for 90 min with [^{3}H]-(+)-isradipine (New England Nuclear, 76 Ci mmol^{-1}) in physiological solution or for 60 min in physiological solution followed by 30 min in depolarizing solution containing different concentrations of KCl, maintained at 37°C and aerated with a mixture of 95% O_2:5% CO_2. When the effect of noradrenaline was tested, noradrenaline was added to the physiological solution during the last 30 min of incubation. [^{3}H]-(+)-isradipine concentration ranged from 40 to 100 pM. At the end of the incubation time, each ring was dried on filter paper, weighed and dissolved in 0.1 ml of a mixture of perchloric acid: H_2O_2 (1:1). Radioactivity of the tissue was counted by liquid scintillation with an efficiency of 42%. Non-specific binding was determined in the presence of 10^{-6} M nifedipine, and subtracted from the total binding to give the specific binding.

Membrane potential determination

The membrane permeant lipophilic cation [^{3}H]-tetraphenylphosphonium ([^{3}H]-TPP^+, Amersham, 26 Ci mmol^{-1}) was used to estimate the membrane potential in rat aortic segments. This compound has been shown to accumulate intracellularly as a function of membrane potential (Lichtshtein *et al.*, 1979). Aortic rings were incubated for 60 min with [^{3}H]-TPP^+ (10^{-5} M). At the end of the incubation time, each ring was dried on filter paper, weighed and dissolved in 0.1 ml of a mixture of perchloric acid: H_2O_2 (1:1). Radioactivity of the tissue was counted by liquid scintillation.

[^{3}H]-TPP^+ uptake due to the electrical potential across the membrane was estimated by subtracting the uptake measured in high KCl-solution (mM: KCl 130, NaCl 0, other constituents as in normal physiological solution), which was assumed to abolish the membrane potential. Intracellular concentration of TPP^+ ($[TPP^+]_{in}$) was calculated as shown in the following equation:

$$[TPP^+]_{in} = (TPP^+_{total} - TPP^+_{130\ mM\ K^+})/V_i$$

where TPP^+_{total} is the total uptake of TPP^+ in physiological buffer, $TPP^+_{130\ mM\ K^+}$ is the total uptake in a solution containing 130 mM KCl, and V_i the intracellular volume (Kamp & Miller, 1987). Extracellular volume was determined by the diffusion space of [^{14}C]-inulin and was 375 ml kg^{-1} wet weight. Intracellular volume, obtained by subtracting the extracellular volume from the total tissular volume (calculated to be equal to 910 ml kg^{-1} on the basis of a density of 1.1), was 535 ml kg^{-1}. This intracellular volume is larger than the intracellular water volume and includes the volume of soluble and non-soluble material. It partially takes into account the dissolution of [^{3}H]-TPP^+ inside internal membranes. [^{3}H]-TPP^+ also accumulates inside intracellular organelles such as mitochondria that have a negative membrane potential. The uptake of [^{3}H]-TPP^+ in mitochondria is proportional to the cytoplasmic concentration of [^{3}H]-TPP^+, and will be higher in low external KCl concentration than in high external KCl concentration. To abolish the participation of mitochondrial uptake in the total uptake of [^{3}H]-TPP^+, uptake of [^{3}H]-TPP^+ was measured in physiological solution and in 130 mM

KCl-solution in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (0.3 μM), an uncoupler of phosphorylation in mitochondria which has been reported to abolish the mitochondrial membrane potential without affecting the cytoplasmic membrane potential of intact synaptosomes (Scott & Nicholls, 1980).

Average membrane potential of the preparations was calculated from Nernst's equation:

$$E_{TPP+} = RT/F \ln [TPP^+]_{out}/[TPP^+]_{in}$$

where $[TPP^+]_{out}$ is the concentration of TPP^+ in the medium and $[TPP^+]_{in}$ is the intracellular TPP^+ concentration measured in the presence of the mitochondrial uncoupler.

Drugs

(+)-PN 200-110 (isradipine; isopropyl 4-(2,1,3-benzoxadiozol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate) (Sandoz), nisoldipine and nifedipine (Bayer) were dissolved in ethanol as stock solutions at 10 mM and further diluted in water as required prior to use. [^{3}H]-(+)-isradipine was obtained from New England Nuclear (specific activity 76 Ci mmol^{-1}); [^{3}H]-tetraphenylphosphonium ([^{3}H]-TPP^+) was obtained from Amersham (specific activity 26 Ci mmol^{-1}). All experiments with dihydropyridine were done under illumination of a sodium lamp.

Statistical analysis

Results of the experiments are expressed as means ± s.e.mean. Drug concentration producing 50% inhibition of the responses (IC_{50}) and their confidence limits (p = 95%) were estimated by linear regression from log concentration-effect curves. In binding experiments, binding parameters were obtained by linear regression of Scatchard plot of the data. K_i values were obtained by the method of Cheng & Prusoff (1973). Tests of significance have been made using Student's *t* test, *P* values smaller than 0.05 being considered significant.

Results

Nisoldipine effect on contractile responses of rat aorta to noradrenaline and KCl

The presence of a functional endothelium is known to influence the contractile response of rat aorta to agonists (Eglème *et al.*, 1984). For this reason, the sensitivity to nisoldipine of the contractile response to noradrenaline was investigated in intact aorta and after removal of endothelial cells. The contraction produced by the application of noradrenaline (10 μM) was significantly higher in aortic rings without endothelium compared to intact aorta (3376 ± 100 mg and 2717 ± 109 mg (n = 16) respectively, $P < 0.01$), in agreement with previous observations (Godfraind *et al.*, 1985). The presence of endothelium also affected the pattern of the response: contractions were more sustained after endothelium removal while tension tended to decrease slowly with time in intact preparations. Nisoldipine (30 pM–300 nM) produced a concentration-dependent depression of the contraction evoked by 10^{-5} M noradrenaline in rat aorta with intact endothelium as well as after endothelium removal. An important part of the contraction was resistant to nisoldipine. In the presence of the endothelium, the nisoldipine-resistant fraction, measured in the presence of 100 nM nisoldipine, was equal to 60 ± 2.8% of the control contraction (n = 14). It was significantly higher after endothelium removal (71 ± 2%, n = 14). However, the presence of endothelium did not influence the IC_{50} value of nisoldipine, as shown in Figure 1a. In aortic rings with or without endothelium, nisoldipine potency increased with the duration of exposure of the tissue to noradrenaline, as indicated by a leftward shift of the dose-effect curves measured after 35 min exposure to noradrenaline; IC_{50} values were 570 pM and 170 pM in intact tissue and 645 pM and 181 pM in rubbed preparations, after 2 and 35 min exposure to noradrenaline, respectively (Table 1). As the endothelium did not appear to influence the IC_{50} value of nisoldipine as an inhibitor of noradrenaline-induced contractions, further experiments were performed in intact aortic rings.

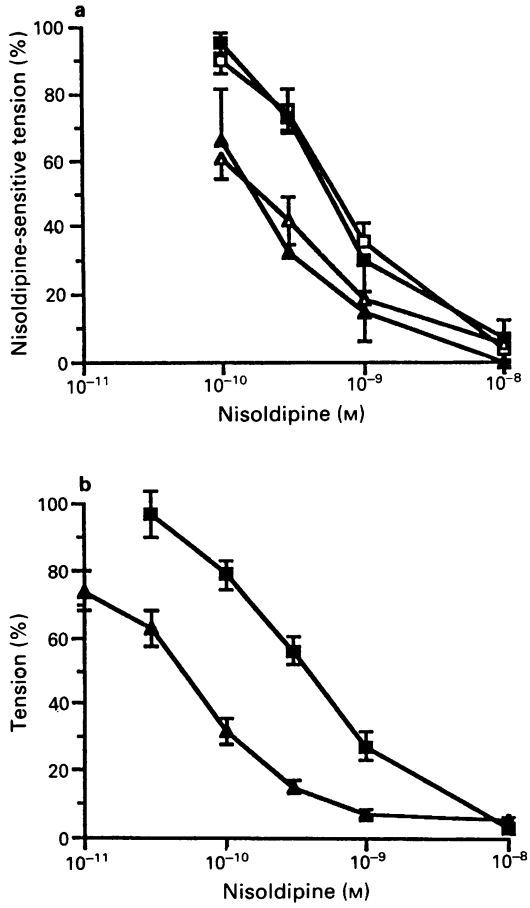


Figure 1 Effect of nisoldipine on the contraction of aortic rings exposed to noradrenaline (10^{-5} M) (a) or to 100 mM KCl depolarizing solution (b). Aortic rings with intact endothelium (closed symbols) or without endothelium (open symbols) were preincubated for 90 min in the presence of different concentrations of nisoldipine. Responses were measured 2 min (□, ■) or 35 min (△, ▲) after commencement of the contraction and are expressed as a percentage of the contraction measured in the absence of nisoldipine. Nisoldipine-sensitive contraction of each aortic ring was obtained after subtraction of the nisodipine-resistant contraction, measured in the same ring in the presence of 100 nM nisoldipine. Data are means from 4–9 determinations; vertical bars show s.e.mean.

Contractions evoked by the 100 mM KCl-depolarizing solution are not affected by the presence of the endothelium (Godfraind, 1986). Their sensitivity to nisoldipine was investigated in intact preparations. The contractile response to 100 mM KCl was concentration-dependently inhibited by nisoldipine (10 pM–10 nM); 10 nM nisoldipine completely abolished the response (Figure 1b). Contractions measured in the presence of nisoldipine were not sustained; the degree of inhibition markedly increased with the duration of depolarization, as already reported by Wibo *et al.* (1988). IC_{50} values measured 2 and 35 min after initiation of contraction were 374 and 49 pM, respectively (Table 1). As shown in Table 1, the potency of nisoldipine was significantly lower on noradrenaline-induced contraction than on KCl-induced contraction at 35 min, while the difference was not significant at 2 min.

In another series of experiments, the inhibition of the contractile response to a short test pulse of KCl or of noradrenaline was tested after incubation of aortic rings in the presence of a fixed concentration of nisoldipine (100 pM) for 60 min in

Table 1 Effect of nisoldipine on contraction, $^{45}Ca^{2+}$ influx (IC_{50}) and [^{3}H]-(+)-isradipine binding (K_i) in aorta and mesenteric artery exposed to 100 mM KCl depolarizing solution or to noradrenaline (NA, 10 μM)

		Contraction IC_{50} (pM)	*$^{45}Ca^{2+}$ influx* IC_{50} (pM)	K_i (pM)
Aorta				
Endothelium present	KCl 2 min	374 (332–421)	743 (560–986)	
	35 min	49 (44–56)	42 (32–55)	67 (57–78)
	NA 2 min	570 (488–821)	617 (464–821)	
	35 min	170 (124–233)		160 (131–196)
Endothelium removed	NA 2 min	645 (566–735)		
	35 min	181 (149–220)		
Mesenteric artery	Physiol solution			263 (204–339)
	KCl 2 min	287 (244–237)		
	35 min	49 (45–53)		58 (45–75)

IC_{50} values and their 95% confidence limits (in parentheses) in the aorta were calculated from the data shown in Figure 1. K_i values in aortic segments were obtained by the method of Cheng & Prusoff (1973) from IC_{50} values calculated from data shown in Figure 8.

normal physiological solution followed by 30 min either in normal physiological (KCl, 5.9 mM) solution or in 40 mM KCl-depolarizing solution. The duration of the 100 mM KCl test pulse was short (1 min) to ensure that the effect of this high concentration of KCl could be neglected. Ca^{2+} was omitted from all bathing solutions during the last 30 min of incubation with nisoldipine to avoid the development of tension in the presence of high KCl concentration. Figure 2 shows that the effectiveness of nisoldipine as an inhibitor of the contraction induced by KCl 100 mM solution or by exposure to noradrenaline (10 μM) was similarly enhanced after preincubation of the tissue in 40 mM KCl depolarizing solution (run 2). A third run was then performed to test the reversibility of the increase of inhibition induced by depolarization. In this experiment, after the second run, arteries were further incubated in the presence of nisoldipine (100 pM) in normal, Ca-free physiological solution. Determination of the contractile responses to noradrenaline (10 μM) and to KCl (100 mM) clearly indicated that the increased inhibition observed after preincubation in high KCl solution was reversed when arteries were further equilibrated in normal physiological solution. The nisoldipine-resistant fraction of the tension evoked by noradrenaline, measured in the presence of nisoldipine 100 nM, was not significantly modified by the preincubation conditions (not shown).

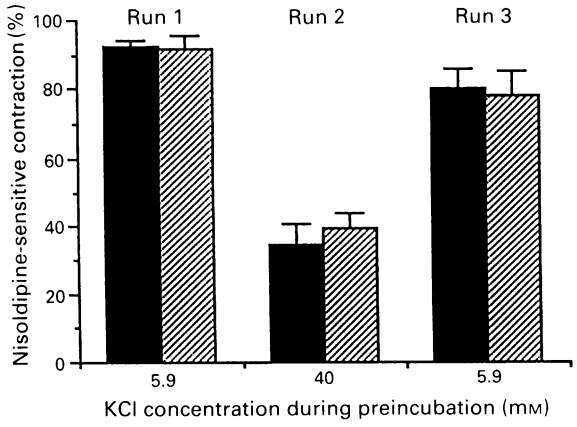


Figure 2 Inhibition produced by nisoldipine on noradrenaline-induced contraction (hatched columns) and on KCl-induced contraction (solid columns) after preincubation in physiological solution or in 40 mM KCl depolarizing solution. After recording of control contractions, aortic rings were preincubated for 60 min in the presence of nisoldipine (0.1 nM) in physiological solution. They were then incubated for 30 min in zero Ca^{2+} physiological solution with nisoldipine (0.1 nM) before contraction was elicited by exposure to 10^{-5} M noradrenaline or to 100 mM KCl-solution for 1 min, in the continuous presence of nisoldipine (run 1). Aortic rings were thereafter incubated for 30 min in zero Ca^{2+}, 40 mM KCl-depolarizing solution containing the same concentration of nisoldipine (0.1 nM) before a second run of contraction was recorded. At the end of this contraction, aortic rings were further incubated in zero Ca^{2+} physiological solution with nisoldipine (0.1 nM) and a third run of contraction was recorded. Nisoldipine-resistant contraction was determined in the presence of nisoldipine 100 nM in the same conditions and subtracted from the total response to give the nisoldipine-sensitive contraction. All contractions were corrected for control responses recorded in the same conditions. Data are means from 4 determinations with s.e.mean shown by vertical bars.

Nisoldipine effect on $^{45}Ca^{2+}$ influx stimulated by noradrenaline and KCl in rat aorta

Exposure of aortic segments to noradrenaline (10 μM) for 2 min increased the $^{45}Ca^{2+}$ influx in the lanthanum-resistant compartment by 77.7 $\pm$ 3.08 μmol Ca^{2+} kg^{-1} wet weight (n = 15). Noradrenaline-stimulated $^{45}Ca^{2+}$ influx was only partly blocked by nisoldipine; 38% of the noradrenaline stimulated $^{45}Ca^{2+}$ influx was unaffected by nisoldipine (29.8 $\pm$ 3.2 μmol Ca^{2+} kg^{-1}) (Figure 3a). Figure 3b shows the concentration-effect curve of the inhibition by nisoldipine (30 pM–300 nM) of the $^{45}Ca^{2+}$ influx stimulated by 2 min exposure to 10^{-5} M noradrenaline. The IC_{50} value for nisoldipine of the nisoldipine-sensitive part of the influx correlated well with the inhibition of the contraction, measured at the same time (Table 1).

Figure 3 also illustrates the effect of nisoldipine on the $^{45}Ca^{2+}$ uptake stimulated by 100 mM KCl. K^+-stimulated $^{45}Ca^{2+}$ influx was dose-dependently depressed by nisoldipine (30 pM–100 nM) and completely abolished by 100 nM of the antagonist. The degree of inhibition of the KCl-stimulated $^{45}Ca^{2+}$ influx was enhanced when the muscles were preincubated in the presence of nisoldipine in the depolarizing solution for 33 min before exposure to the KCl (100 mM)-depolarizing solution containing $^{45}Ca^{2+}$ for 2 min, so that dose-effect curves were shifted to the left after a total of 35 min of depolarization compared to 2 min of depolarization (Figure 3b). IC_{50} values measured after 2 and 35 min in the depolarizing solution were 743 pM and 42 pM, respectively. These values correlated well with the inhibition of the contraction induced by 100 mM KCl, measured at the same time intervals (Table 1).

[^{3}H]-(+)-isradipine binding in segments of arteries exposed to different KCl concentrations

In aorta bathed in physiological solution (KCl 5.9 mM), the level of specific binding of [^{3}H]-(+)-isradipine was very low, and reached only 10–15% of the total binding. When aortic segments were incubated in 100 mM KCl depolarizing solution, [^{3}H]-(+)-isradipine specific binding increased with the duration of incubation. At 100 pM [^{3}H]-(+)-isradipine binding reached a steady state after 45 min (not shown). At this ligand concentration, specific binding was about 60% of the total binding. When tissues were thereafter transferred

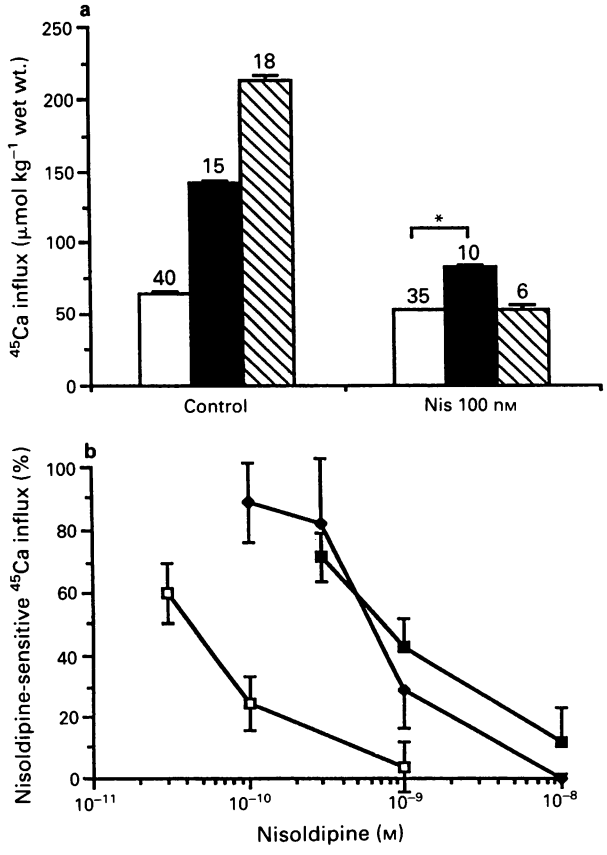


Figure 3 Effect of nisoldipine on $^{45}Ca^{2+}$ influx. (a) ^{45}Ca influx was measured after 7 min incubation in ^{45}Ca-containing physiological solution (unstimulated arteries open columns) or after 5 min incubation in ^{45}Ca-containing physiological solution followed by 2 min incubation in ^{45}Ca-containing 100 mM KCl solution (hatched columns) or ^{45}Ca-containing physiological solution with 10 μM of noradrenaline (solid columns). Aortic rings were preincubated for 90 min in physiological solution with (Nis) or without (control) nisoldipine (100 nM). Data are means from *n* determinations; s.e.mean shown by vertical bars. * indicates significant difference. (b) Nisoldipine dose-effect curves on ^{45}Ca influx stimulated by KCl or noradrenaline. KCl-stimulated ^{45}Ca influx was measured in aortic rings incubated for 2 min in ^{45}Ca-containing 100 mM KCl solution either after 90 min preincubation in physiological solution (■) or after 60 min preincubation in physiological solution and 30 min in 100 mM KCl solution (□) with different concentrations of nisoldipine. Noradrenaline-stimulated ^{45}Ca influx (◆) was measured after 7 min incubation in ^{45}Ca-containing physiological solution, noradrenaline being added to the solution during the last 2 min. Aorta rings were preincubated for 90 min in the presence of different concentrations of nisoldipine. Control ^{45}Ca influx was measured in aortic rings incubated for 7 min in ^{45}Ca-containing physiological solution; this value was subtracted from total ^{45}Ca content of stimulated aortic rings. Data are means from 6–12 determinations; vertical bars show s.e.mean.

from the 100 mM KCl solution into physiological solution, the level of specific binding decreased and after 45 min in the physiological solution it was similar to that measured in arteries exposed to the physiological solution for 90 min (Table 2).

To test the effect of the KCl concentration on [^{3}H]-(+)-isradipine binding, specific binding was determined in the presence of a fixed concentration of [^{3}H]-(+)-isradipine in solutions containing various concentrations of KCl. The NaCl concentration was correspondingly modified. As illustrated in Figure 4, binding at 100 pM [^{3}H]-(+)-isradipine increased when KCl concentration was enhanced from 5.9 mM (physiological solution) to 100 mM.

Saturation studies of the specific binding of [^{3}H]-(+)-isradipine were determined at equilibrium in solutions containing

Table 2 Influence of KCl concentration on [^{3}H]-(+)-isradipine specific binding in rat aorta

Incubation conditions	[^{3}H]-(+)-*isradipine specific binding* (fmol mg^{-1} wet wt.)	
KCl 5.9 mM 45 min	0.58 ± 0.14	$P < 0.01$
KCl 100 mM 45 min	2.42 ± 0.26	
KCl 100 mM 45 min + KCl 5.9 mM 45 min	0.39 ± 0.22	NS
KCl 5.9 mM 90 min	0.42 ± 0.25	

All aortic segments were first incubated with [^{3}H]-(+)-isradipine (50 pM) for 60 min. Incubation was further carried out as indicated, in the presence of [^{3}H]-(+)-isradipine (50 pM). Data are means ± s.e.mean from 6 determinations.

15, 20 or 100 mM KCl. Figure 5 shows the results of these experiments. Scatchard plots of saturation curves were linear; binding parameters calculated from these data are given in Table 3. The B_{max} value was not significantly affected by the concentration of KCl in the medium. On the contrary, K_D values decreased when arteries were exposed to increasing concentrations of KCl. K_D values determined from saturation studies in aortic segments bathed in 100 mM KCl were close to K_D values measured in rat aortic microsomes (0.085 (0.065–0.112) nM; Wibo *et al.*, 1988).

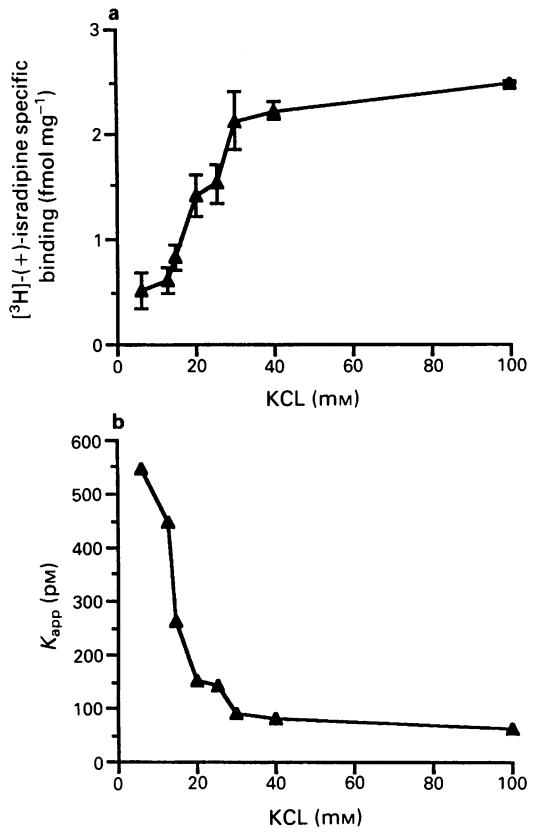


Figure 4 Influence of the KCl concentration on [^{3}H]-(+)-isradipine specific binding. (a) [^{3}H]-(+)-isradipine specific binding was measured after 60 min of incubation in physiological solution containing [^{3}H]-(+)-isradipine at 50 pM followed by 30 min incubation in solutions containing different concentrations of KCl and the same concentration and specific activity of [^{3}H]-(+)-isradipine. Data are means from 5–20 determinations; s.e.mean shown by vertical bars. (b) Influence of the KCl concentration on the value of K_{app}, the apparent dissociation constant of [^{3}H]-(+)-isradipine. K_{app} value was calculated from the data of Figure 4a according to Michealis Menten's equation, $K_{app} = L(B_{max} - b)/b$ with L = [^{3}H]-(+)-isradipine concentration (50 pM), b the specific binding of [^{3}H]-(+)-isradipine and a B_{max} value of 5.5 fmol mg^{-1} wet wt.

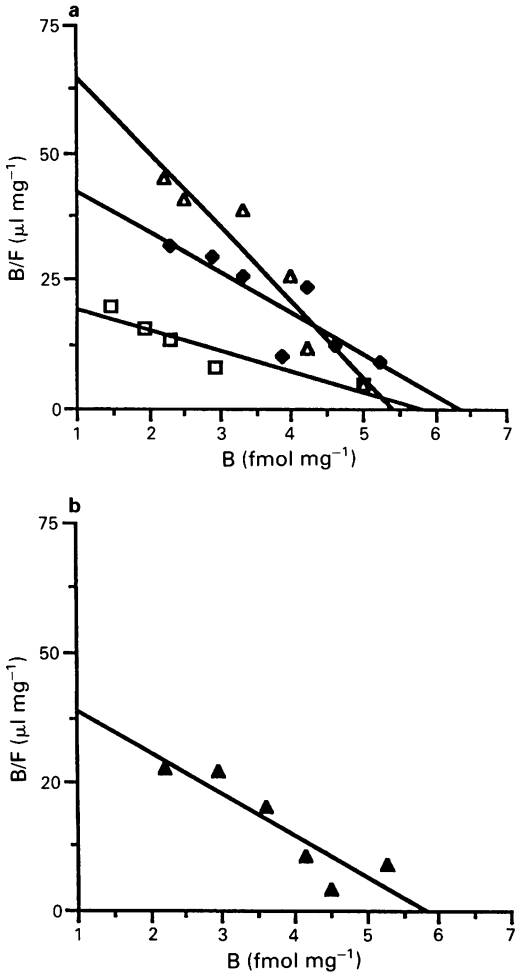


Figure 5 Scatchard plot of the [^{3}H]-(+)-isradipine specific binding as a function of [^{3}H]-(+)-isradipine concentration in aortic segments incubated in various KCl concentrations (a) or exposed to noradrenaline (10^{-5} M) (b). Aortic segments were incubated for 60 min in physiological solution containing different concentrations of [^{3}H]-(+)-isradipine and further incubated for 30 min in solutions containing KCl at 15 mM (□), 20 mM (◆) or 100 mM (△) or in physiological solution containing noradrenaline 10 μM (▲) containing the same concentrations and specific activities of [^{3}H]-(+)-isradipine. Data are means from 10–12 determinations.

On the basis of the observation that varying the KCl concentration changed only the apparent dissociation constant of [^{3}H]-(+)-isradipine (K_{app}), we calculated the value of K_{app} at different KCl concentrations from Michaelis-Menten's equation applied to the binding data shown in Figure 4a, with unique value of B_{max} of 5.5 fmol mg^{-1} wet wt. These values are shown in Figure 4b: a small variation of the KCl concentration between 5.9 and 20 mM induced a large modification of the K_{app} value. A further increase of the KCl concentration only slightly decreased the value of K_{app}.

Table 3 Binding parameters of [^{3}H]-(+)-isradipine in aortic segments incubated in different KCl concentrations or exposed to noradrenaline 10 μM (NA)

	K_D (pM)	B_{max} (fmol mg^{-1} wet wt.)
KCl 15 mM	250	5.8 ± 0.8
KCl 20 mM	125	6.3 ± 0.7
KCl 100 mM	68	5.4 ± 0.3
KCl 5.9 mM + NA 10^{-5} M	126	5.8 ± 1.6

Binding parameters of [^{3}H]-(+)-isradipine were calculated by linear regression of data shown in Figure 5.

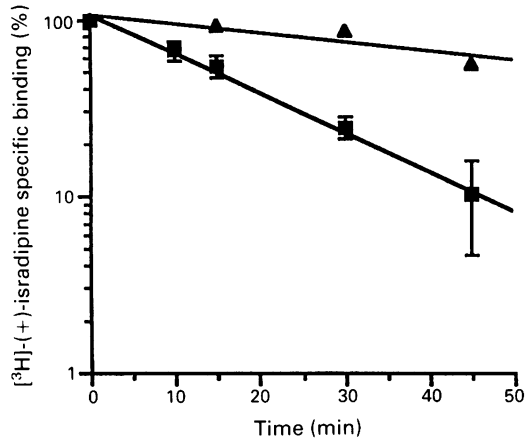


Figure 6 Time-course of [^{3}H]-(+)-isradipine dissociation in aortic segments. Aortic segments were first incubated with [^{3}H]-(+)-isradipine (80 pM) in KCl-depolarizing solution for 60 min. Dissociation was induced by transferring the aortic segments to various solutions containing 1 μM nifedipine: physiological solution (KCl, 5.9 mM) (■); 100 mM KCl-solution (▲). Semi-logarithmic plot demonstrating the existence of a single process of dissociation. Data are means from 3–6 experiments performed in triplicate; vertical bars show s.e.mean.

Electrophysiological and binding studies in cardiac cells have shown that dissociation kinetics of dihydropyridine calcium antagonists are markedly influenced by the membrane potential of the cells (Triggle *et al.*, 1989). To examine whether this could be applied to vascular smooth muscle, dissociation was studied either in physiological solution, or in 100 mM KCl solution. Dissociation was produced by the addition of an excess of unlabelled competitor ((+)-isradipine or nifedipine 10^{-6} M), after the tissue had been equilibrated with [^{3}H]-(+)-isradipine in 100 mM KCl solution. The tritiated ligand concentration was maintained unchanged during the dissociation process so that non specific binding was unchanged. Figure 6 shows that membrane potential influenced the rate of dissociation of [^{3}H]-(+)-isradipine. A monophasic dissociation curve indicated the existence of a single process of dissociation. The dissociation rate measured in physiological solution gave a value of K_{-1} equal to 0.050 ± 0.007 min^{-1} ($n = 6$). The dissociation rate was lower in 100 mM KCl-solution (k_{-1} 0.012 ± 0.002 min^{-1}, $n = 6$). The value of k_{-1} in depolarized aortic segments was close to the value measured by Triggle *et al.* (1989) in depolarized cardiac myocytes (0.018 ± 0.0012 min^{-1}) but not in physiological solution since a markedly higher k_{-1} value was found by those authors in cardiac myocytes in physiological solution (0.53 ± 0.06 min^{-1}).

Binding of [^{3}H]-(+)-isradipine in tissues exposed to noradrenaline

[^{3}H]-(+)-isradipine binding was determined in aorta bathed in physiological solution containing different concentrations of noradrenaline. In the presence of noradrenaline (10^{-7}–10^{-5} M) the specific binding of 80 pM [^{3}H]-(+)-isradipine was significantly increased without change of the non-specific binding (Figure 7).

Equilibrium binding studies in the presence of 10^{-5} M noradrenaline indicated that this agonist did not affect the linear pattern of the Scatchard plot of binding data (Figure 5b). Maximum binding capacity was similar to that measured in other conditions, while the K_D value was close to the value measured in 20 mM KCl solution (Table 3).

Nisoldipine effect on [^{3}H]-isradipine binding

The effect of nisoldipine on [^{3}H]-(+)-isradipine specific binding was determined in rat aorta bathed in KCl (25 and

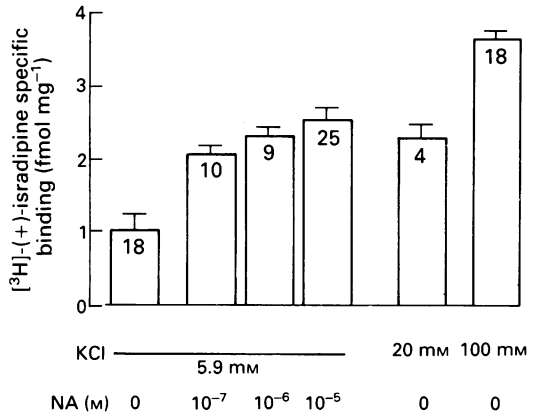


Figure 7 Effect of noradrenaline on [^{3}H]-(+)-isradipine specific binding measured in aortic segments incubated for 60 min in physiological solution containing [^{3}H]-(+)-isradipine at 80 pM, and further incubated for 30 min in solutions containing 5.9 mM (physiological solution), 20 mM or 100 mM of KCl or in physiological solution containing different concentrations of noradrenaline, in the presence of the same concentration and specific activity of [^{3}H]-(+)-isradipine. Data are means of n determinations; vertical bars show s.e.mean.

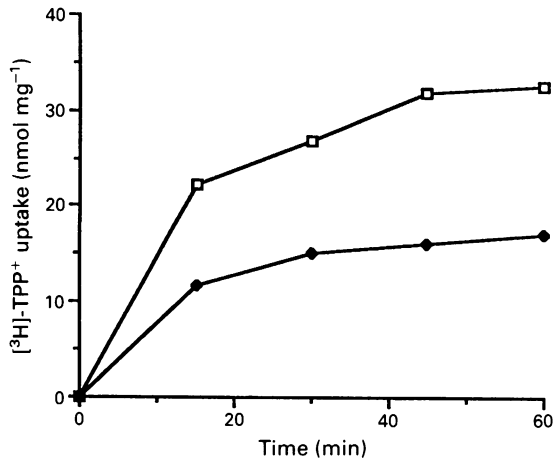


Figure 9 Time-course of the uptake of [^{3}H]-tetraphenylphosphonium bromide ([^{3}H]-TPP$^+$) in aortic segments. Aortic rings were incubated for different times in physiological solution (□) or in solution containing 130 mM KCl (◆) in the presence of [^{3}H]-TPP$^+$ (10 μM). Data are means from 4 determinations, s.e.means were smaller than symbols.

100 mM) solutions (Figure 8), and in rat mesenteric artery bathed in KCl (100 mM) solution or in normal physiological solution (KCl 5.9 mM), as [^{3}H]-(+)-isradipine binding can be determined in the latter tissue in resting conditions (Morel & Godfraind, 1987). Nisoldipine was able to displace completely [^{3}H]-(+)-isradipine specific binding in both tissues and in all cases, nisoldipine potency was higher in 100 mM KCl solution. K_i values obtained in KCl 100 mM solution were in good agreement with IC_{50} values on KCl (100 mM)-induced contraction and ^{45}Ca influx measured after 35 min exposure of the tissues to the depolarizing solution (Table 1). On the other hand, the K_i value in the mesenteric artery bathed in physiological solution was close to the IC_{50} value on the KCl contraction of the same tissue exposed for only 2 min to the depolarizing solution.

Nisoldipine also displaced [^{3}H]-(+)-isradipine binding in aorta exposed to 10^{-5} M noradrenaline, in a dose-dependent manner and the displacement curve was parallel to that obtained in aortic segments exposed to the depolarizing solution (KCl 100 mM) and to KCl (25 mM) solution (Figure 8).

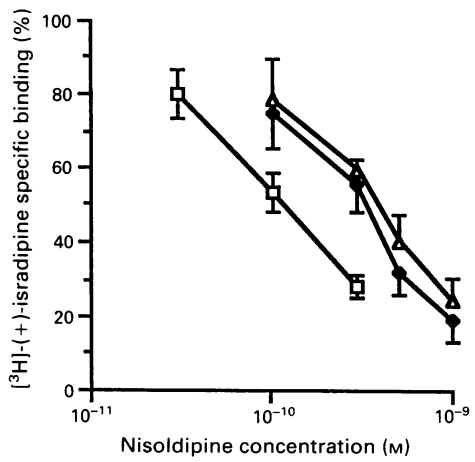


Figure 8 Effect of nisoldipine on [^{3}H]-(+)-isradipine specific binding in aortic segments: binding was measured in aortic segments incubated in the presence of different concentrations of nisoldipine for 60 min in physiological solution containing [^{3}H]-(+)-isradipine (40–60 pM) and further incubated for 30 min either in physiological solution with noradrenaline (10^{-5} M) (◆), in 25 mM KCl solution (△), or in 100 mM KCl solution (□), containing [^{3}H]-(+)-isradipine at the same concentration. Data are means from 10 determinations; vertical bars show s.e.mean.

The IC_{50} value of nisoldipine in the aorta exposed to noradrenaline (10^{-5} M) was close to the value obtained in the aorta bathed in KCl (25 mM) solution. The K_i value in the presence of noradrenaline (10^{-5} M) corresponded to the IC_{50} value of nisoldipine on contraction evoked by 10^{-5} M noradrenaline when measured after 35 min of stimulation (Table 1).

[^3H]-tetraphenylphosphonium bromide uptake

[^{3}H]-TPP$^+$ uptake was measured in aortic segments with intact endothelium to estimate the membrane potential of the preparations. The uptake of [^{3}H]-TPP$^+$ increased with the duration of the incubation and reached a steady state level after 45 min (Figure 9). Increasing the KCl concentration in the bathing solution from 5.9 mM to 130 mM (with simultaneous decrease of the NaCl concentration) dose-dependently decreased [^{3}H]-TPP$^+$ uptake. At equilibrium, the [^{3}H]-TPP$^+$ content of the tissues bathed in 130 mM KCl solution was 51% of the [^{3}H]-TPP$^+$ content of arteries bathed in physiological solution. Further increase of the KCl concentration up to 150 mM did not significantly modify the uptake of [^{3}H]-TPP$^+$ (not shown). At these high KCl concentrations, [^{3}H]-TPP$^+$ uptake presumably represents KCl-insensitive accumulation in membranes and inside internally negative compartments, as membrane potential may be assumed to be abolished. This value was subtracted from [^{3}H]-TPP$^+$ uptake measured in other conditions to evaluate the value of the KCl-sensitive component of [^{3}H]-TPP$^+$ uptake. The participation of mitochondria in the [^{3}H]-TPP$^+$ uptake was estimated by the use of a mitochondrial uncoupler (carbonyl cyanide *m*-chlorophenylhydrazone) (Scott & Nicholls, 1980). In the presence of 0.3 μM of the mitochondria uncoupler, [^{3}H]-TPP$^+$ uptake was reduced by 53 ± 2.1% (n = 38); the degree of inhibition was unaffected by the composition of the incubation medium. When [^{3}H]-TPP$^+$ uptake corrected for the mitochondrial uptake, was inserted into the Nernst's equation, a membrane potential value of −63.3 ± 0.67 mV (n = 40) was obtained for aorta incubated in physiological solution.

The influence of increasing concentrations of KCl in the bath on the K$^+$-sensitive [^{3}H]-TPP$^+$ uptake is represented in Figure 10, where it is compared to the effect of noradrenaline. Exposure of the aortic segments to noradrenaline (10^{-8} M–10^{-5} M) in physiological solution decreased the KCl-sensitive [^{3}H]-TPP$^+$ uptake without changing the KCl-insensitive uptake; the effect of the agonist was dose-dependent (Figure 10b). Reduction of [^{3}H]-TPP$^+$ uptake plateaued at noradrenaline concentrations higher than 10^{-6} M; [^{3}H]-TPP$^+$

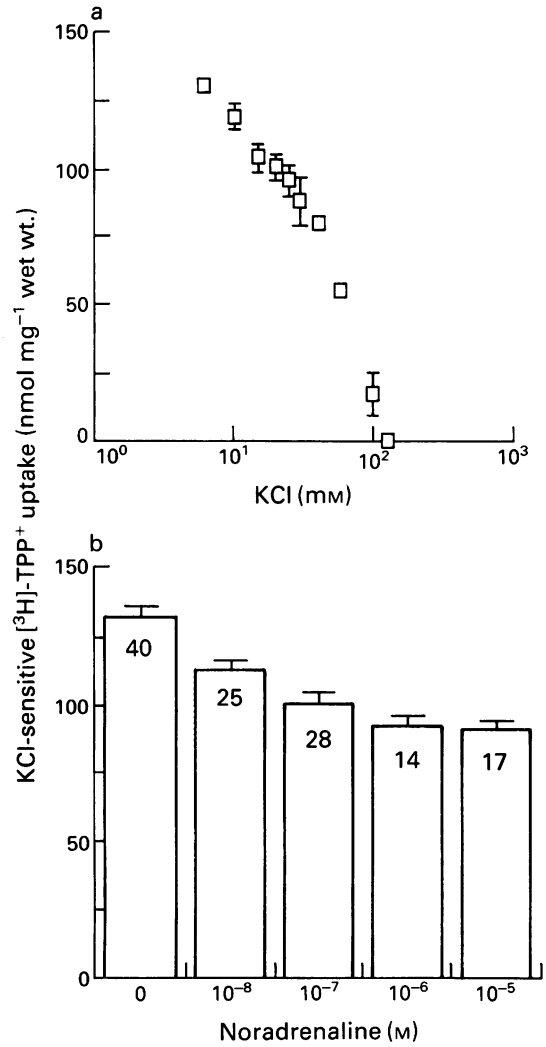


Figure 10 Effect of KCl and noradrenaline on the KCl-sensitive [^{3}H]-tetraphenylphosphonium bromide ([^{3}H]-TPP$^+$) uptake in aortic segments: uptake was measured in aortic segments incubated with [^{3}H]-TPP$^+$ (10 μM) for 60 min in solutions containing different concentrations of KCl (a) or in physiological solution containing different concentrations of noradrenaline (b). KCl-sensitive [^{3}H]-TPP$^+$ uptake was obtained after subtraction of the uptake of [^{3}H]-TPP$^+$ in a solution containing 130 mM KCl and the same concentration of [^{3}H]-TPP$^+$. Data are means from at least 12 determinations; s.e.mean shown by vertical bars.

content of tissues exposed to noradrenaline 10^{-6}–10^{-5} M was close to the values measured in aorta incubated in solution containing KCl concentration near 25 mM. This effect of noradrenaline was not affected by nisoldipine (10^{-7} M) but was inhibited by the α-adrenoceptor antagonists phentolamine (10^{-6} M) and prazosin (10^{-6} M) (Table 4). The uptake of [^{3}H]-TPP$^+$ measured in the presence of 0.3 μM of the mitochondrial uncoupler in aorta exposed to noradrenaline (10^{-5} M) or to 25 mM KCl solution is shown in Table 4; membrane potential values estimated by applying the Nernst equation to these data are −55.4 mV and −56 mV, in aortic segments exposed to noradrenaline 10^{-5} M and KCl 25 mM, respectively.

Discussion

By use of segments of intact vessels, this study shows the existence of voltage-dependent specific binding sites for calcium antagonistic dihydropyridines in rat aorta. Voltage-dependence of the binding was related to a modification of binding affinity without change of maximum binding capacity. These results are in agreement with previous reports describing voltage-sensitive binding of dihydropyridines in cardiac cells (Kokubun *et al.*, 1986; Wei *et al.*, 1989), in mesenteric artery (Morel & Godfraind, 1987), coronary artery (Sumimoto *et al.*, 1988) and cerebral microvessels (Morel & Godfraind, 1989).

The relationship between the apparent dissociation constant (K_{app}) of [^{3}H]-(+)-isradipine, and KCl concentration in the bath indicates a modulation of binding affinity as a function of the membrane potential imposed to the cell. These results may be interpreted according to the modulated receptor model: binding sites coexist in two different conformations, one with high affinity, associated with inactivated Ca^{2+} channels in depolarized preparations, and the other with low affinity for the ligand, associated with resting Ca^{2+} channels. K_{app} has been estimated for various KCl concentrations which clamped smooth muscle membrane potential at various levels. Assuming that K_{app} is a function of the proportion of high and low affinity sites, its value can be calculated from the equation:

$$1/K_{app} = L/K_L + (1 - L)/K_H \quad (1)$$

with L representing the proportion of low affinity binding sites, K_L the low affinity dissociation constant and K_H the high affinity dissociation constant. At KCl concentrations higher than 30 mM, the value of K_{app} of [^{3}H]-(+)-isradipine in rat aorta was not markedly affected by variations in the KCl concentration. The K_{app} value at 100 mM KCl may be assumed to correspond to the K_D value for the high affinity binding sites. The high affinity dissociation constant measured in intact depolarized tissue is close to the K_D value found in membrane preparation (Wibo *et al.*, 1988) indicating that prolonged depolarization and tissue homogenization favoured a similar conformation of calcium channels, which is most likely, according to the modulated receptor model, the inactivated form of the calcium channel (see Bean, 1984; Sanguinetti & Kass, 1984). At lower KCl concentrations, the K_{app} value was very sensitive to small changes of KCl concentration and the absolute value of the K_D for the low affinity binding site could be much higher than that measured in

Table 4 [^{3}H]-Tetraphenylphosphonium bromide ([^{3}H]-TPP$^+$) distribution in segments of rat aorta

	K^+-sensitive [^{3}H]-TPP$^+$ content (nmol mg^{-1} wet wt.)			
Drug added	*KCl* (5.9 mM)	*NA* (10^{-5} M)	*KCl* (20 mM)	*KCl* (25 mM)
None	138.7 ± 4.8 (25)	98.7 ± 3.7 (25)[a]	100.3 ± 4.9 (16)[a]	95.8 ± 6.1 (5)
Nisoldipine (10^{-7} M)	146.6 ± 3.8 (6)	97.1 ± 7.4 (6)		
Phentolamine (10^{-6} M)	137.8 ± 13 (5)	120.1 ± 9.6 (5)[b]		
Prazosin (10^{-6} M)	136.5 ± 4.4 (6)	125.6 ± 6.2 (6)[b]		
Carbonyl cyanide *m*-chloro phenylhydrazone (5×10^{-7} M)	64.4 ± 2.8 (10)[b]	44.9 ± 1.9 (6)[a, b]		44.5 ± 2.3 (6)[a, b]

[^{3}H]-TPP$^+$ content of rat aortic segments was measured after 60 min incubation in the indicated solution. Aortic segments were preincubated for 30 min in the presence of phentolamine or prazosin or for 60 min in the presence of nisoldipine. Data are means from (*n*) determinations ± s.e.mean. [a]Significantly different from corresponding control in KCl 5.9 mM; [b]significantly different from [^{3}H]-TPP$^+$ content in segments incubated in the same solution without any addition.

physiological solution. This marked sensitivity of binding affinity to small variations of KCl concentration implies that any situation causing a small variation of the membrane potential near the resting level may induce important change in the affinity and in the potency of (+)-isradipine and other voltage-sensitive dihydropyridines such as nisoldipine.

As a consequence of the modulated receptor model, and because those drugs are characterized by slow association kinetics, inhibition of KCl-induced contractile responses by voltage-sensitive calcium antagonists is markedly dependent on the duration of contact of the drug with the depolarized tissue (Wibo *et al.*, 1988). The IC_{50} value of nisoldipine on KCl-induced contraction measured after a short depolarization was close to the value of K_i obtained in aorta incubated in physiological solution and reflected the binding to a low proportion of high affinity sites in resting tissue. The potency of nisoldipine as an inhibitor of KCl-induced contraction and ^{45}Ca entry was increased after prolonged depolarization. This resulted either in a time-dependent effect on 100 mM KCl-induced contraction or in an increase of the inhibition of short contractions after preincubation of the tissue with the antagonist in depolarizing solution. In agreement with our previous observations (Morel & Godfraind, 1987), Nelson *et al.* (1988) using rabbit mesenteric artery, and Triggle *et al.* (1988), using guinea-pig longitudinal smooth muscle, have shown that the potency of nitrendipine and related 1,4-dihydropyridines as inhibitors of KCl-induced tension is a function of membrane potential. Nisoldipine IC_{50} values on KCl-stimulated $^{45}Ca^{2+}$ entry or contraction measured after 35 min of exposure of the tissue to the high KCl-depolarizing solution were very close to the K_i value measured in 100 mM KCl solution, which is consistent with equation 1.

In many tissues, calcium entry blockers have been reported to be less potent inhibitors of the contraction induced by agonist stimulation than by depolarization (Cauvin *et al.*, 1983; Marriott, 1988; Godfraind *et al.*, 1986). This observation led to the hypothesis of the existence of two different types of calcium channels, one controlled by the occupation of specific receptors and the other controlled by membrane potential (Cauvin & van Breemen, 1985). However, analysis of the effect of nisoldipine in rat aorta shows that although agonist- and depolarization-induced responses have different sensitivities to calcium entry blocking drugs, this does not necessarily mean that responses are mediated by different Ca^{2+} channels. It is worth noting that, while the potency of nisoldipine was higher against the 100 mM KCl-induced contraction than against the noradrenaline-induced contraction when determination of the inhibition was performed after prolonged contraction, there was no significant difference between IC_{50} values of nisoldipine on $^{45}Ca^{2+}$ entry or contraction induced by 100 mM KCl-solution or by noradrenaline, when the responses were measured after a short duration of stimulation (2 min). The inhibitory potency of nisoldipine on the response evoked by a short application of noradrenaline could be increased by preincubation of the tissue with the antagonist in depolarizing solution, just as the contractile response induced by short exposure to KCl-depolarizing solution was, suggesting that noradrenaline-evoked contraction depends at least partly on the same nisoldipine-sensitive Ca^{2+} entry pathway as depolarization-induced contraction. Modulation of the voltage-dependent binding sites of dihydropyridines by noradrenaline was confirmed by binding studies. Noradrenaline produced an increase in the affinity of both nisoldipine and (+)-isradipine, which can be attributed to an increase in the proportion of high affinity binding sites for dihydropyrines. As this effect of noradrenaline was comparable to that of 20 mM KCl, it may be suggested that it is the result of a small depolarization produced by noradrenaline.

Despite the fact that data concerning the effect of noradrenaline on membrane potential are quite variable, it generally appears that high concentrations of noradrenaline produce a small depolarization while low concentrations may induce a contractile response without change of the membrane potential (see Bolton & Large, 1986). $[^3H]$-TPP^+ was used in the present work to estimate and compare the membrane potential of aorta exposed to KCl and noradrenaline. TPP^+ is a membrane permeant lipophilic cation that is distributed across the membranes as a function of membrane potential (Lichtshtein *et al.*, 1979). It has been successfully used in different cell lines to measure membrane potential (Tan & Tashjian, 1984; Greenberg *et al.*, 1986; Wei *et al.*, 1989). The membrane potential value estimated from the TPP^+ distribution is an average value of the potential of all the cells of the preparation and is then directly comparable with any other data like those from $[^3H]$-(+)-isradipine binding. The main disadvantages of the TPP^+ method are that (1) it is an indirect method and transformation of uptake data in potential values requires the estimation of a cytosolic concentration of TPP^+, which depends on the value of the intracellular volume, (2) it does not allow detection of rapid or transient changes of potential, and (3) the existence of an uptake of TPP^+ in internally negative organelles like mitochondria may give overestimated values of potential. The uptake of TPP^+ into mitochondria can be suppressed by a mitochondrial uncoupler, which has been reported to depolarize selectively the mitochondrial membrane without affecting the cellular membrane potential in intact synaptosomes (Scott & Nicholls, 1980). In rat aortic segments, $[^3H]$-TPP^+ uptake was dose-dependently reduced when KCl concentration was increased up to 130 mM and remained unchanged when KCl concentration was further increased to 150 mM. A concentration of KCl of 130 mM was assumed to abolish the membrane potential and the tissue content of TPP^+ measured in these conditions was considered to represent KCl-insensitive uptake. When $[^3H]$-TPP^+ uptake data, corrected for KCl-insensitive uptake and for mitochondrial uptake, were translated into membrane potential value, the membrane potential value that was obtained (−63.3 mV) was similar to the data obtained with the classical microelectrode technique in rat aorta (−53.8 mV, Chen & Suzuki, 1989; −57 mV, Taylor *et al.*, 1988) and in other vascular tissues (−63.3 mV in rabbit ear artery, Droogmans *et al.*, 1977; −59.2 mV in rat small mesenteric arteries, Mulvany *et al.*, 1982; −70 mV in guinea-pig mesenteric artery, Kuriyama & Suzuki, 1981), indicating that the $[^3H]$-TPP^+ distribution may be a fairly good indicator of membrane potential. Noradrenaline produced a dose-dependent decrease of the KCl-sensitive $[^3H]$-TPP^+ uptake, with a maximum effect close to that of 30 mM KCl. In rat aorta exposed to 0.1 μM of noradrenaline, Chen & Suzuki (1989) measured a depolarization of 7 mV. For this noradrenaline concentration, the uptake of $[^3H]$-TPP^+ gave a value between the values measured in aorta bathed in 10 and 15 mM KCl, corresponding to a mean value of depolarization of about 5 mV which corresponds with Chen & Suzuki's result. This effect of noradrenaline also corresponds to observations reported in other vessels (Casteels *et al.*, 1977; Haeusler, 1983; Mulvany *et al.*, 1982). The mechanisms underlying the depolarizing effect of noradrenaline are still conflicting. Recently however, evidence has been provided, which suggests that the noradrenaline-produced depolarization is mediated by an increase in chloride and cation membrane conductance (Amédée & Large, 1989; Pacaud *et al.*, 1989).

The effects of noradrenaline on $[^3H]$-TPP^+ distribution and on $[^3H]$-(+)-isradipine do correspond very well, and are in agreement with the hypothesis that the effect of noradrenaline on $[^3H]$-(+)-isradipine and nisoldipine binding is related to the depolarization produced by the agonist. These data suggest that the noradrenaline-evoked contraction is caused by the opening of voltage-dependent Ca channels as a result of the depolarization produced by noradrenaline. Comparison of noradrenaline and KCl effects on membrane potential shows that depolarization produced by higher concentrations of noradrenaline is large enough to cause Ca^{2+} entry and contraction, while the depolarization produced by lower concentrations of the agonist ($<10^{-7}$ M) is probably too small to open voltage-dependent Ca^{2+} channels. We postulate an

additional effect of noradrenaline on the activation of the Ca^{2+} channel. Noradrenaline at a concentration that does not depolarize cells has been shown to increase the open-state probability of Ca^{2+} channels in rabbit mesenteric artery (Nelson *et al.*, 1988). An increase of the L-type Ca^{2+} current mediated by an adrenoceptor different from the α- or β-adrenoceptor has been reported in rabbit ear artery (Benham & Tsien, 1988). The occurrence of this type of adrenoceptor in rat aorta is less probable, as all responses to noradrenaline were abolished by pre-treatment with phentolamine or prazosin (Alosachie & Godfraind, 1988).

Our observations indicate that opening of voltage-operated calcium channels as a result of noradrenaline-induced depolarization was apparently not the only mechanism of increase of intracellular calcium leading to contraction. Beside the component of the contraction that is known to be related to the release of intracellular Ca^{2+} (Godfraind & Kaba, 1969), a fraction of noradrenaline-stimulated $^{45}Ca^{2+}$ entry was resistant to nisoldipine blockade, suggesting that another Ca^{2+} entry pathway was opened by the agonist. Further experiments are required in order to characterize the nature of this pathway.

The authors wish to thank N. Bussy for her technical assistance. This work was supported by the Fonds National de la Recherche Scientifique Médicale (grant FRSM No. 3.9006.87) and by the Ministère de l'Education et de la Recherche Scientifique (grant Action Concertée n° 89/95-135).

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(Received June 11, 1990
Revised September 6, 1990
Accepted September 19, 1990)

Br. J. Pharmacol. (1991), **102**, 478–482 © Macmillan Press Ltd, 1991

Diabetes-induced changes in cardiac β-adrenoceptor responsiveness: effects of aldose reductase inhibition with ponalrestat

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1 The responses of isolated left atria and papillary muscles to isoprenaline, forskolin and calcium have been examined in 3 week streptozotocin-diabetic rats and the effects of oral ponalrestat administration (25 mg kg^{-1} daily) on diabetes-induced changes in cardiac responsiveness investigated.

2 Three weeks after animals were made diabetic, cardiac responses to isoprenaline were enhanced and this was accompanied by an increase in the density of ventricular [^{3}H]-dihydroalprenolol binding sites. Treatment of animals with ponalrestat prevented the increase in cardiac β-adrenoceptor responsiveness and receptor number.

3 Diabetes also enhanced the sensitivity of cardiac tissues to forskolin, an effect that was not prevented by the treatment of animals with ponalrestat.

4 Ponalrestat treatment increased the resting and maximum tensions developed by cardiac tissues from diabetic animals and increased the maximum tensions developed by tissues from control animals. Diabetes alone had no effect on resting or maximum developed tensions.

5 Ponalrestat therefore prevents the changes in β-adrenoceptor density and responsiveness induced by short-term diabetes in the rat and also increases the tension developed by cardiac muscle, an effect observed in diabetic and normal animals.

Introduction

Tissues in which glucose transport is not insulin-dependent have been found to be particularly susceptible to the pathological changes which occur in diabetes. In such tissues, a hyperglycaemic state markedly increases the intracellular concentration of glucose with a subsequent activation of the polyol pathway which converts glucose to sorbitol and then fructose. The initial enzyme in this pathway, aldose reductase, has a poor affinity for its substrate and glucose is only metabolised via the polyol pathway when the intracellular concentration of glucose is high. In diabetes, the rate of sorbitol production via this pathway exceeds the capacity of sorbitol dehydrogenase to convert it to fructose and sorbitol accumulates intracellularly.

Some of the complications often observed in diabetes have been associated with the intracellular accumulation of sorbitol and may be prevented by aldose reductase inhibitors. In experimental diabetes, aldose reductase inhibition has been shown to prevent or reverse the development of cataracts (Fukushi *et al.*, 1980; Stribling *et al.*, 1985), glomerular abnormalities (Goldfarb *et al.*, 1986) and defective nerve conduction in peripheral nerves (Tomlinson *et al.*, 1984). Furthermore, the effect of diabetes on the rate of contraction and relaxation of cardiac muscle can be attenuated by pretreatment with the aldose reductase inhibitor, ponalrestat (Cameron *et al.*, 1989).

We have previously reported that in the short-term diabetic rat, cardiac β-adrenoceptor responsiveness and receptor number are significantly increased (Austin *et al.*, 1987). The present study examines whether these changes in β-adrenoceptor responsiveness can be prevented by pretreatment with the aldose reductase inhibitor ponalrestat.

Methods

Pretreatment of animals

Female Wistar rats (200–250 g) were made diabetic by injection of streptozotocin (50 mg kg^{-1}) via the tail vein. The drug was administered in 0.1 M citrate buffer (pH 4.5); control rats received citrate buffer alone. One day after streptozotocin injection, blood glucose levels were measured with a Glucochek S.C. meter and BM.1-44 Glycaemie test strips. Streptozotocin-injected rats with a blood glucose greater than 15 mmol l^{-1} were considered diabetic and used in the study; those with a lower blood glucose were discarded. Half the diabetic and half the control rats were then treated orally with 25 mg kg^{-1} ponalrestat (Statil, I.C.I.; Prodiax, Merck & Co.) daily for 3 weeks.

Isolated cardiac tissues

Three weeks after streptozotocin or control injection, animals were killed by a blow to the head and exsanguinated. Left atria and left ventricular papillary muscles were rapidly removed and set up in a Krebs-bicarbonate solution (composition in mM: NaCl 118.4, KCl 4.7, $NaHCO_3$ 25.0, glucose 11.7, $MgSO_4$ 1.2, $KHPO_4$ 1.2 and $CaCl_2$ 1.9) gassed with 5% CO_2 in oxygen at 37°C. Tissues were suspended under 0.8 g tension and paced at 1 Hz by square-wave pulses (5 ms duration; threshold voltage +50%), delivered via bipolar electrodes, from Grass S48 stimulators. Isometric developed tension was recorded via Lectromed UF1 (57 g sensitivity range) force transducers, on a Devices M19 polygraph.

Tissues were equilibrated for 30 min with several changes of bathing medium. In each tissue, a cumulative concentration-response curve to isoprenaline was constructed, followed after washout by one to forskolin and then calcium chloride. All experiments were performed in the presence of metanephrine (10 μM) and desipramine (1 μM) to inhibit extraneuronal and neuronal uptake respectively. Propranolol (1 μM) was also present when forskolin responses were obtained.

Radioligand binding

After removing the papillary muscles, the remaining ventricular tissue was quickly immersed in ice-cold Tris buffer (50 mM) and a crude membrane fraction was prepared from the ventricular tissue by homogenizing in ice-cold Tris (50 mM) buffer for 15 s in an Ultra-Turrax homogenizer, followed by 4 times

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4 strokes of a Teflon homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min in an M.S.E. Chillspin centrifuge. The supernatant was then recentrifuged at 30,000 *g* for 20 min in an M.S.E.18 centrifuge. The pellet was finally resuspended in 3 ml of Tris buffer and used in a receptor binding assay. These procedures were carried out at 4°C.

The crude membrane fraction was assayed for β-adrenoceptor binding sites by use of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA): 100 μl samples of the membrane suspension were incubated at 37°C for 30 min with a range of radioligand concentrations (0.5–16.0 nM) in a final volume of 250 μl. Non-specific binding was determined by displacement with 200 μM isoprenaline. Incubations were terminated by the addition of 1 ml ice-cold buffer, followed by rapid filtration through Whatman GF/B glass fibre filters. The filters were rapidly washed 3 times with 5 ml ice-cold buffer. Radioactivity remaining on the filters was measured by standard scintillation counting techniques. Specific binding as a percentage of the total binding was approximately 65%. Protein content of the membrane fraction was determined by the method of Lowry *et al.* (1951).

Statistical analysis

Mean blood glucose levels were calculated with s.e.mean and differences examined by use of Student's *t* test.

Isolated tissues: increases in developed tension were plotted as a percentage of the maximum increase for each drug. Individual EC_{50} values were determined and geometric mean EC_{50} values with 95% confidence limits calculated. Student's *t* test was performed on logarithmic EC_{50} values. Mean maximum responses (with s.e.mean) were calculated and Student's *t* test performed on these values to test for differences between control and diabetic animals.

Binding experiments: dissociation constants (K_d) and the maximum number of binding sites (*B*m) for specific binding were calculated by Scatchard analysis of saturation curves performed in duplicate. Mean values (with s.e.mean) were calculated and unpaired Student's *t* test employed to examine differences between groups of animals.

Drugs used

[^{3}H]-dihydroalprenolol (110 Ci mmol^{-1}) was obtained from the Radiochemical Centre, Amersham, Bucks. Ponalrestat (ICI 128436, Statil; MK538, Prodiax) was kindly supplied as a gift by ICI pharmaceuticals, Macclesfield, Cheshire. All other drugs were obtained commercially as the hydrochloride salts from Sigma (St. Louis, MO, U.S.A.). All reagents were of Analar grade.

Results

At the time of death, 3-week diabetic animals had a mean (±s.e.mean) blood glucose concentration of 19.3 ± 1.3 mmol l^{-1} ($n = 8$), which was significantly ($P < 0.001$) elevated with respect to the mean value of 4.5 ± 1.0 mmol l^{-1} for control animals ($n = 6$). Ponalrestat treatment of animals did not affect blood glucose levels, the mean values for ponalrestat-treated diabetic and ponalrestat-treated non-diabetic rats being 17.7 ± 1.2 mmol l^{-1} ($n = 6$) and 4.9 ± 0.6 mmol l^{-1} ($n = 6$) respectively.

Isolated tissues

The resting tensions developed by isolated left atria and papillary muscles from non-diabetic rats were similar to those for tissues from diabetic animals (Table 1). Ponalrestat treatment of animals did not significantly alter the resting tensions developed by tissues from non-diabetic rats, but the treatment of diabetic animals with ponalrestat significantly increased the resting tensions developed by both left atria and papillary muscles (Table 1).

Cardiac responses to isoprenaline

Both left atria and papillary muscles from diabetic animals were supersensitive to the positive inotropic effects of isoprenaline when compared with tissues from non-diabetic rats (Figure 1). This was indicated by a reduction of isoprenaline EC_{50} values (4.7 fold for left atria; 10.7 fold for papillary muscles) in tissues from the streptozotocin-injected animals (Table 2).

Treatment of non-diabetic rats with ponalrestat did not significantly alter the sensitivity of tissues to isoprenaline. Treat-

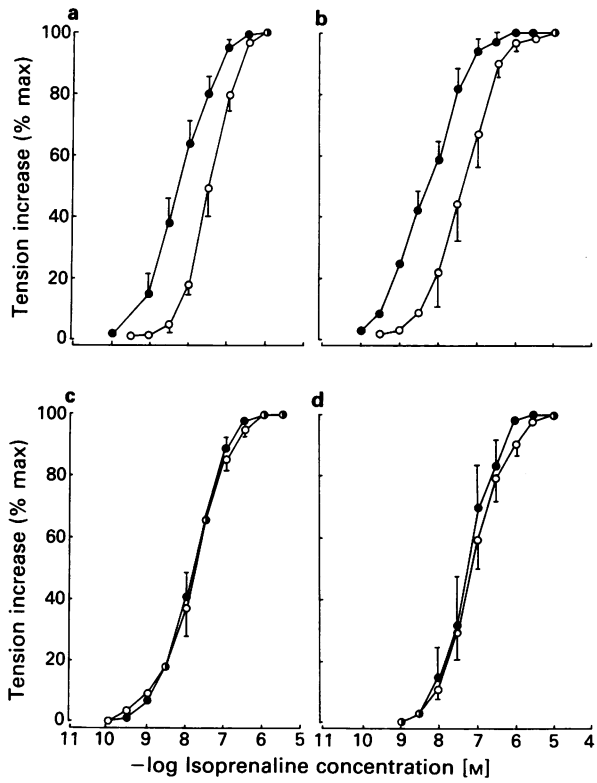


Figure 1 Mean concentration-response curves to isoprenaline of left atria (a and c) and papillary muscles (b and d) from untreated (a and b) and ponalrestat-treated (c and d) diabetic (●) and non-diabetic (○) animals.

Table 1 Mean tensions (g) developed by tissues of diabetic and non-diabetic rats before the addition of any drugs

	Left atria		*Papillary muscles*	
Treatment	*Non-diabetic*	*Diabetic*	*Non-diabetic*	*Diabetic*
Untreated rats	0.33 ± 0.12 (5)	0.28 ± 0.04 (8)	0.29 ± 0.05 (6)	0.29 ± 0.04 (7)
Ponalrestat-treated rats	0.43 ± 0.09 (6)	0.54 ± 0.06* (6)	0.20 ± 0.09 (5)	0.73 ± 0.09*** (4)

(n) is the number of experiments. * $P < 0.005$, *** $P < 0.001$ compared with tissues from untreated diabetic rats.

Table 2 Geometric mean EC_{50} values and maximum increases in developed tension to isoprenaline of left atria and papillary muscles from non-diabetic (non-diab), diabetic, ponalrestat-treated non-diabetic (PTND), and ponalrestat-treated diabetic (PTD) rats

	Left atria		*Papillary muscle*	
Treatment	EC_{50} (nM)	*Maximum* (g)	EC_{50} (nM)	*Maximum* (g)
Non-diab.	39.7 (29.1–50.3)	0.19 ± 0.01	75.8 (50.0–101.6)	0.30 ± 0.11
Diabetic	8.4 (6.3–10.5)*	0.14 ± 0.04	7.1 (5.2–8.9)*	0.14 ± 0.05
PTND	19.3 (8.1–46.1)	0.49 ± 0.06**	85.8 (33.0–223.0)	0.71 ± 0.25
PTD	15.4 (6.9–34.3)	0.47 ± 0.07††	59.0 (13.5–258.0)††	0.43 ± 0.07†

* $P < 0.05$, ** $P < 0.01$ compared with non-diabetic rats.
† $P < 0.05$, †† $P < 0.01$ compared with diabetic rats.
Each value is the mean of 4–8 experiments.

ment of diabetic rats with the aldose reductase inhibitor however, prevented the enhancement of β-adrenoceptor-mediated responses induced by diabetes (Figure 1), the EC_{50} values for isoprenaline being similar in diabetic and non-diabetic animals when both groups were treated with ponalrestat (Table 2). As previously stated, the resting tensions developed by tissues from diabetic animals were increased by ponalrestat treatment. The maximum increases in tension induced by isoprenaline were also greater in tissues from animals pretreated with ponalrestat. This occurred in all tissues examined whether from non-diabetic or diabetic animals, although the change failed to reach a level of significance for papillary muscles from non-diabetic rats (Table 2).

Responses to forskolin

Untreated diabetes resulted in a leftward shift of concentration-response curves to forskolin (Figure 2) with a significant reduction in EC_{50} values for both left atria and papillary muscles (Table 3). In ponalrestat-treated animals the supersensitivity to forskolin induced by diabetes was enhanced, ponalrestat tending to reduce EC_{50} values in tissues from diabetic animals and increase EC_{50} values in tissues from non-diabetic animals (Table 3).

The maximum increases in developed tension induced for forskolin were also greater in tissues from animals treated with ponalrestat, although this was only significant for left atria from diabetic animals and papillary muscles from non-diabetic animals (Table 3).

Responses to calcium

The sensitivity of tissues to calcium was similar in all groups of animals studied, neither diabetes nor ponalrestat significantly altering EC_{50} values (Table 4). However, the treatment of animals with ponalrestat increased the maximum tensions developed by tissues in response to calcium, although this did not reach a level of statistical significance for papillary muscles from non-diabetic animals (Table 4).

[^{3}H]-dihydroalprenolol binding

The density of [^{3}H]-DHA binding sites was greater ($P < 0.05$) in membranes prepared from diabetic animals (73.6 ± 11.8 fmol mg^{-1} protein, $n = 7$) than in membranes prepared from normal animals (38.4 ± 2.1 fmol mg^{-1} protein, $n = 6$). The dissociation constants for binding were similar in the two groups (1.2 ± 0.3 nM and 1.2 ± 0.4 nM in diabetic and non-diabetic experiments respectively). The increase in the density of [^{3}H]-DHA binding sites induced by diabetes was prevented by ponalrestat treatment, the density being 45.5 ± 5.6 fmol mg^{-1} protein ($n = 7$) in ponalrestat-treated non-diabetic rats and 58.3 ± 12.5 fmol mg^{-1} protein ($n = 5$) in ponalrestat-treated diabetic rats. Dissociation constants were also similar in the two groups being 1.3 ± 0.3 nM and

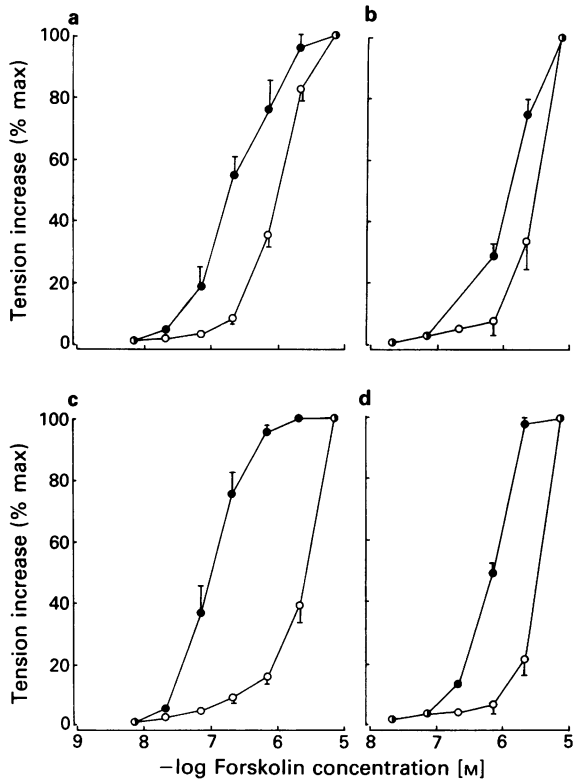


Figure 2 Mean concentration-response curves to forskolin of left atria (a and c) and papillary muscles (b and d) from untreated (a and b) and ponalrestat-treated (c and d) diabetic (●) and non-diabetic (○) animals.

Table 3 Geometric mean ($n > 4$) EC_{50} values and maximum increases in developed tension induced by forskolin on cardiac tissues from non-diabetic (non-diab), diabetic, ponalrestat-treated non-diabetic (PTND) and ponalrestat-treated diabetic (PTD) rats

	Left atria		*Papillary muscle*	
Treatment	EC_{50} (μM)	*Maximum* (g)	EC_{50} (μM)	*Maximum* (g)
Non-diab	1.02 (0.82–1.26)	0.23 ± 0.10	2.72 (1.81–4.10)	0.25 ± 0.04
Diabetic	0.24 (0.09–0.57)*	0.16 ± 0.05	1.33 (1.09–1.63)*	0.23 ± 0.07
PTND	2.6 (1.95–3.48)	0.45 ± 0.08	3.68 (3.28–4.13)	0.64 ± 0.18*
PTD	0.10 (0.06–0.18)‡	0.60 ± 0.09†	0.73 (0.59–89)‡	0.46 ± 0.09

* $P < 0.05$ compared with non-diabetics.
† $P < 0.01$ compared with diabetics.
‡ $P < 0.01$ compared with PTND.

Table 4 Geometric mean ($n > 4$) EC_{50} values and maximum increases in developed tension induced by calcium on cardiac tissues from non-diabetic (non-diab), diabetic, ponalrestat-treated non-diabetic (PTND) and ponalrestat-treated diabetic (PTD) rats

	Left atria		*Papillary muscle*	
Treatment	EC_{50} (mM)	*Maximum* (g)	EC_{50} (mM)	*Maximum* (g)
Non-diab	2.2 (0.2–28.0)	0.13 ± 0.04	5.1 (4.2–5.8)	0.33 ± 0.16
Diabetic	4.5 (2.9–7.0)	0.19 ± 0.07	3.1 (1.7–5.7)	0.31 ± 0.05
PTND	4.2 (3.0–5.8)	0.51 ± 0.11*	4.5 (2.3–8.9)	0.64 ± 0.21
PTD	7.1 (4.4–11.0)	0.44 ± 0.6††	3.9 (2.5–6.2)	0.84 ± 0.13††

* $P < 0.05$ compared with non-diabetics.
†† $P < 0.01$ compared with untreated diabetics.

0.8 ± 0.2 nM in ponalrestat-treated non-diabetic and ponalrestat-treated diabetic rats respectively.

Discussion

The results obtained in the present study confirm the previously reported cardiac β-adrenoceptor supersensitivity observed in rats with streptozotocin-induced diabetes of two and four weeks duration (Austin *et al.*, 1987). The increase in sensitivity to isoprenaline was not accompanied by a change in sensitivity to calcium and we have previously shown that responses to phenylephrine in the presence of propranolol, are also unaffected by diabetes of less than four weeks duration (Austin *et al.*, 1987). The increase in sensitivity to β-adrenoceptor agonists however, was accompanied by an increase in sensitivity to forskolin, an agent that elevates intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels by direct activation of adenylyl cyclase (Seamon & Daley, 1983; Daley, 1984). The enhanced cardiac sensitivity to isoprenaline was also accompanied by an increase in the number of cardiac β-adrenoceptors, as indicated by an increase in the density of [^{3}H]-DHA binding sites to ventricular membranes. These results suggest that more than one mechanism is involved in the enhancement of responses to inotropic agents acting via the β-adrenoceptor-cyclic AMP pathway observed in diabetes.

Treatment of animals with ponalrestat prevented the increase in cardiac responsiveness to isoprenaline induced by diabetes, without altering the sensitivity of tissues from non-diabetic animals to this agonist. Ponalrestat also prevented the increase in the density of [^{3}H]-DHA binding sites induced by diabetes but did not prevent the change in cardiac sensitivity to forskolin observed in diabetic animals. These results suggest that the diabetes-induced increase in responsiveness to isoprenaline is not linked to the post-receptor mediated change in cardiac sensitivity that is detected by use of forskolin. This result may be explained if diabetes induces a change in a pool of adenylyl cyclase not associated with the β-adrenoceptor. Stimulation of adenylyl cyclase by forskolin can occur in the absence of the regulatory G_s protein associated with the β-adrenoceptor (Seamon & Daley, 1983; Daley, 1984) and a number of intracellular pools of adenylyl cyclase may be activated. Evidence for separate pools of adenylyl cyclase can be found in the heart and adipose tissue, where cyclic AMP accumulation to forskolin needs to be ten fold greater than that to isoprenaline, to produce equivalent inotropic (Roger & Shahid, 1984) or lipolytic responses (Schimmel, 1984). If such a separate pool of adenylyl cyclase is activated by forskolin, the present results would indicate that the diabetes-induced increase in activity in this pool is not prevented, but may actually be enhanced by aldose reductase inhibition. Alternatively, several cyclic AMP-independent effects of forskolin on glucose transport and ion channels have recently been demonstrated in a variety of tissues (Laurenza *et al.*, 1989) and could be involved in the diabetes induced changes in sensitivity to forskolin.

One of the major complications of long-term diabetes is a defect in nerve function, which occurs in somatic and autonomic nerves (Clark *et al.*, 1979; Harati, 1987). Although Akiyama *et al.* (1989) could find no change in noradenaline content in the hearts of rats diabetic for 2–4 weeks, it has been reported that noradrenaline turnover is depressed in 2–3 week diabetic rats, indicating a reduction in sympathetic nerve activity (Yoshida *et al.*, 1985; 1987). It has also been shown that chemical sympathectomy with 6-hydroxydopamine (Chess-Williams *et al.*, 1987a) or reserpine (Chess-Williams *et al.*, 1987b) can increase cardiac β-adrenoceptor number and responsiveness. Diabetes-induced changes in sympathetic nerve function may therefore be responsible for the post-junctional adrenoceptor changes identified in the present study. Although Hawthorn *et al.* (1985) failed to demonstrate any change in cardiac responsiveness to forskolin following the pretreatment of guinea-pigs with reserpine, this may reflect the shorter duration (seven days) of pretreatment. The prevention by ponalrestat of the changes in cardiac adrenoceptor number and responsiveness may also be due to an effect on nerve function, a hypothesis supported by the finding that the reduced sympathetic nerve activity reported by Yoshida *et al.* (1987) could be prevented by aldose reductase inhibitors.

In addition to an effect on cardiac β-adrenoceptor responses in diabetic animals, ponalrestat also increased both the resting and maximum tensions developed by tissues from diabetic animals and increased the maximum tensions developed by tissues from non-diabetic animals. It has been shown that in more long-term diabetes in the rat (3 months duration) cardiac muscle has a greater dependency on extracellular calcium for contraction (Bielefeld *et al.*, 1983) and this finding may be linked to a defective calcium handling by the sarcoplasmic reticulum of diabetic animals (Ganguly *et al.*, 1983; Heygler *et al.*, 1987). Ponalrestat-treatment corrects this defect in contractile function in long-term diabetic animals (Cameron *et al.*, 1989). In normal and short-term diabetic rats no such defect exists (Bielefeld *et al.*, 1983), but the present results demonstrate that ponalrestat can still increase the tension developed by cardiac tissue.

The present study has therefore established that the treatment of diabetic rats with the aldose reductase inhibitor, ponalrestat prevents the increase in cardiac β-adrenoceptor responsiveness and receptor number induced by diabetes of three weeks duration. Furthermore, ponalrestat-treatment increases tension development in cardiac tissue, the mechanism of which has yet to be determined.

This work was supported by the British Heart Foundation and the Mersey Regional Health Authority. R.C.W. was a Bristol-Myers lecturer.

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(Received February 19, 1990
Revised July 23, 1990
Accepted September 26, 1990)

Br. J. Pharmacol. (1991), **102**, 483–491 © Macmillan Press Ltd, 1991

Effects of aluminium on electrical and mechanical properties of frog atrial muscle

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1 The effects of aluminium on membrane ionic currents were studied in single cardiac myocytes. Most of the work was done on frog atrial cells, but some experiments were also carried out on single cells isolated from rabbit ventricles and atria.

2 The effects of aluminium on the force of contraction of frog atrial trabeculae were also investigated.

3 Aluminium was prepared from $AlCl_3$ as a stock 0.5 M solution which has a pH of 3.5. Before each experiment, this solution was added to the control solution, to give a final concentration of 20–100 $\mu g\,ml^{-1}$ aluminium (0.75–3.75 mM $AlCl_3$). The solutions were brought to a pH of 7.4 or 7.6, at which they consist of a mixture of amorphous aluminium hydroxides and a very small amount of soluble ionic aluminium complexes: free aluminium cations (less than 10 pM), aluminohydroxide anions (less than 8 μM). The addition of this suspension reduced the peak inward calcium currents in single rabbit atrial and ventricular cells and in frog atrial cells. In the latter, the peak current was reduced (at +10 mV) to 45% of control (mean of 9 cells). This effect was reversible upon washout, and was obtained at all membrane potentials, with no shift of the calcium current voltage relationship along the voltage axis.

4 Aluminium also reduced the time-dependent potassium current I_K. This reduction was observed at all membrane potentials. For example, at +10 mV, the mean reduction of I_K ($n = 9$) was to 69% of the control amplitude. This effect, which was very difficult to reverse, was not due to I_K rundown. The fully activated current-voltage relationships (obtained by standard 'tail' analysis) showed that the effect of aluminium was due mainly to a decrease in conductance and not to a shift in the activation range of I_K. The mean voltage of half activation was shifted by 8 mV in the depolarizing direction ($n = 5$).

5 The background potassium current IK_1 was also slightly but consistently changed in a complex fashion, with an outward shift at membrane potentials positive to −60 mV. For example, at a membrane potential of −40 mV, the mean shift was by 22 ± 4 pA. At more negative potentials, there was an inward shift in the current amplitudes. For example, for steps to −100 mV the current elicited was larger (more inward) by 53 pA (mean value, $n = 10$). The reversal potential was slightly shifted (< 10 mV) in the hyperpolarizing direction.

6 The force of contraction of frog atrial trabeculae was altered by aluminium in a complex manner, which showed marked seasonal variation. During most of the year, 50–100 $\mu g\,ml^{-1}$ aluminium caused a biphasic change, with an early small and consistent decrease, followed by a large increase in twitch amplitude. For a short period corresponding to the (local) winter months the sensitivity to aluminium was greatly enhanced. Aluminium 100 $\mu g\,ml^{-1}$ totally abolished contraction ($n = 5$), while a lower concentration (20 $\mu g\,ml^{-1}$) produced a sustained reduction in the force of contraction. Similar biphasic and seasonal responses have been reported to be induced by lanthanum.

7 The biphasic changes in twitch amplitude were independent of the transmembrane sodium gradient. Aluminium produced the same effects when 90% of the extracellular sodium was replaced by lithium. Caffeine (5 mM) attenuated or even inverted the positive inotropic effect of aluminium. These results imply that aluminium alters the release of calcium from intracellular, caffeine-sensitive stores. This could be effected either by augmenting the amount released during each activation, and/or by increasing the loading of stores prior to release.

8 These results are discussed in light of several of the previously reported actions of aluminium.

Introduction

In recent years, interest in aluminium toxicity has been stimulated by two major observations: (1) Aluminium has been implicated in a neurological disorder developing in patients undergoing long-term dialysis (Alfrey *et al.*, 1976; Arieff *et al.*, 1979; Altmann *et al.*, 1988). Abnormal accumulation of aluminium has also been detected within the brain tissue of patients with Alzheimer's disease and the Parkinsonian dementia complex of Guam (Crapper *et al.*, 1973; Perl *et al.*, 1982; Candy *et al.*, 1986). Aluminium salts also induce memory impairment in susceptible experimental animals, accompanied by the formation of neuronal cytoskeletal abnormalities (Klatzo *et al.*, 1965; Crapper & Dalton, 1973). (2) The growing acidification of surface waters has led to augmented aluminium absorption (Cronan *et al.*, 1986; Slanina *et al.*, 1986). This has been linked to an increased incidence of Alzheimer's disease (Martyn *et al.*, 1989).

However, surprisingly little work has been devoted to the study of the physiological implications of exposure to aluminium. No reports exist on the effects of aluminium on the heart, even though recent results demonstrate a massive accumulation in this tissue (Bertholf *et al.*, 1989). In other excitable tissues, very little work has been done. Electrophysiological studies of aluminium-injected rabbits and cats revealed a reduced frequency of neuronal spikes and an impaired long-term potentiation in brain cells (Crapper & Tomko, 1975; Farnell *et al.*, 1982). Earlier investigations of the effects of aluminium on the excitable properties of isolated neurones and on neurotransmitter release presented seemingly contradictory results: aluminium blocked the sodium action potential in the lobster axon and diminished the voltage dependent influx of calcium into synaptosomes and smooth muscle cells (Blaustein & Goldman, 1968; Hava & Hurvitz, 1974; Koening & Jope, 1987). On the other hand, aluminium enhanced the release of

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acetylcholine at the frog motor synapse (Banin & Meiri, 1987). The sodium-calcium spike of neuroblastoma cells was unaffected by aluminium even after several hours of exposure (Roll *et al.*, 1989).

Several of these observations suggest that aluminium might exert its neurotoxic action without directly affecting the excitable properties, but rather by altering the intracellular calcium level. Thus, the inhibition of the neuronal sodium-potassium-activated and magnesium-activated ATPases by aluminium (Lai *et al.*, 1980) and its interference with calcium binding to calmodulin (Siegal & Haug, 1983) may cause an elevated concentration of calcium in the brains of aluminium-treated animals (Farnell *et al.*, 1985).

In the present study we investigated the influence of aluminium on the physiological activity of cardiac cells. We examined aluminium effects on several membrane ionic currents and on contractile properties, as indicators of the intracellular concentration of calcium ions. The study of the cellular actions of aluminium, which relate to a possible impairment of cardiac function may be of importance in view of the use of aluminium containing medications as well as the growing concern over the progressive acidification of surface waters, with its consequent increase in the level of free aluminium ions (Lione, 1985; Ganrot, 1986; Cronan *et al.*, 1986; MacDonald & Martin, 1988). We have found that aluminium changes the force of contraction of atrial trabeculae in a complex fashion: an initial decrease was followed by a pronounced increase. The voltage-sensitive calcium current and a time-dependent potassium current were both attenuated in the presence of aluminium. The background potassium current was also altered. Possible molecular mechanisms for these effects of aluminium are discussed.

Methods

Preparations

The experiments were divided into two sections. The first part of the work involved measurements of ionic currents from enzymatically isolated single cardiac cells. These were obtained from bullfrog (*Rana catesbeiana*) atria. An additional short series of experiments was done with myocytes obtained from rabbit (New Zealand white) atria and ventricles. The second part of the work consisted of measurements of twitch amplitude in frog (*Rana ridibunda*) atrial trabeculae. These experiments were performed during a whole year, in the course of which seasonal variations were found (see below).

Single cell experiments

Single frog atrial cells were isolated by a method described in Giles & Shibata (1985): 1 × 1 mm atrial pieces were cut into a Ca-free Ringer solution. Two enzyme solutions were then added sequentially. Initially, the cells were incubated for 45 min, at room temperature (22–24°C), in a Ca-free Ringer solution containing collagenase 240 u ml^{-1} (Yakult Co., Japan), with trypsin 3600 u ml^{-1} (Sigma type III). The solution was then pipetted off and washed first with Ca-free Ringer solution containing 0.1% bovine albumin (Sigma) and then with Ca-free Ringer solution. The pieces were then re-incubated in 5 ml Ca-free Ringer solution containing collagenase 120 u ml^{-1} for a further 45–60 min. Finally, the cells were suspended in Ca-free Ringer solution and gently triturated. Electrophysiological experiments were performed on cells taken from this suspension.

In a small number of experiments the effects of aluminium on calcium currents were studied, using rabbit atrial and ventricular cells. These were prepared by the methods of Giles & Shibata (1985). Briefly, rabbit hearts were perfused retrogradely through an aortic cannula on a Langendorff apparatus. After a brief wash with normal calcium-containing Tyrode solution, the heart was perfused for 10 min with a calcium-free Tyrode solution, and then for 10 min with a collagenase solution (Yakult Co., Japan, 12.5 u ml^{-1}). This solution also contained protease (Sigma, type P5147) 12.5 u ml^{-1} and taurine 20 mM. Thereafter, atrial or ventricular chunks were cut into a beaker containing (in Tyrode) collagenase 250 u ml^{-1}, protease 250 u ml^{-1}, taurine 20 mM, albumin 10 mg ml^{-1} and $CaCl_2$ 0.1 mM. The beaker was gently agitated and samples of dissociated cells were then removed at different times (5–20 min), so that the most viable ones were obtained for the experiments. The cells were stored in Tyrode solution containing taurine 20 mM, albumin 10 mg ml^{-1} and $CaCl_2$ 0.15 mM.

For the measurement of contraction, single trabeculae were isolated from frog atria. The trabeculae measured less than 0.5 mm in diameter, and were 3–5 mm in length.

In all the single cell experiments, the cells were placed in a small Petri dish with a flow system enabling constant perfusion at room temperature (20–22°C). The compositions of the control solutions were the following (in mM): for the rabbit cells: NaCl 150, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, glucose 5.5 and HEPES 5.0. For the frog cells: NaCl 110, KCl 2.0, $CaCl_2$ 1.1, $MgCl_2$ 1.0, glucose 5.5 and HEPES 5.0. The pH was adjusted with NaOH to 7.4 or 7.6 in the rabbit and frog cells, respectively. The aluminium containing solutions were titrated to the appropriate pH.

Activity recording

The cells were voltage-clamped by a whole cell gigaseal suction microelectrode technique, slightly modified from Giles & Shibata (1985). The electrodes (resistances 2–5 MΩ), contained solutions with the following composition (in mM): K-aspartate 120, KCl 30, Na_2ATP 4.0, HEPES 5.0, pH adjusted with KOH to 7.2. Current and voltage records were digitized (at 2 kHz, using an analog to digital conversion board, Data Translation DT 2801-A) and stored on an IBM-AT for subsequent analysis on a Dec Vax 11-750. Since partial dialysis of the cells occurs with this method, with some 'rundown' of ionic currents, only cells with stable current records before and after application of aluminium were analyzed, to avoid complications of interpretation due to current rundown.

In a second series of experiments the force of contraction of frog atrial trabeculae was measured. One end of the trabecula was pinned to the bottom of the chamber, and the other end was connected by a pin to a force transducer (Akers 800) and stimulated at 0.5 Hz (at 2 times threshold intensity), during constant perfusion with the control Ringer solution. The trabeculae were gently stretched to give the maximal amplitude of force. The trabeculae were stimulated with a microprocessor-driven stimulator (AMP Instruments, Jerusalem), through silver-silver chloride wires embedded in the Sylgard-coated bottom of the chamber. The stimulation pulses were 2–5 ms in duration, and 2–3 times threshold in intensity. Great care was taken to ensure that all solution changes were carried out only after the force amplitude was extremely stable. This was monitored continuously on a chart recorder and storage oscilloscope.

Aluminium containing solutions

$AlCl_3$ was dissolved in water to obtained a concentrated stock solution (0.5 M, pH = 3.5). Appropriate amounts of the stock medium were added to the control solution before each experiment and the pH was adjusted to 7.4 or 7.6 with NaOH, in the mammalian and frog experiments respectively. During the titration process, the majority of aluminium assumed the form of an amorphous aluminium-hydroxide precipitate $Al(OH)_3$. Saturation values of soluble aluminium hydroxides in a neutral aqueous solution are presumably very low: 8×10^{-6} M for $Al(OH)_4^-$ and 10^{-11} M for $Al(H_2O)_6^{3+}$ (MacDonald & Martin, 1988). Several recent publications indicate that the trivalent cation is the biotoxic form of this substance (Martin, 1986; Birchall & Chappell, 1988). Never-

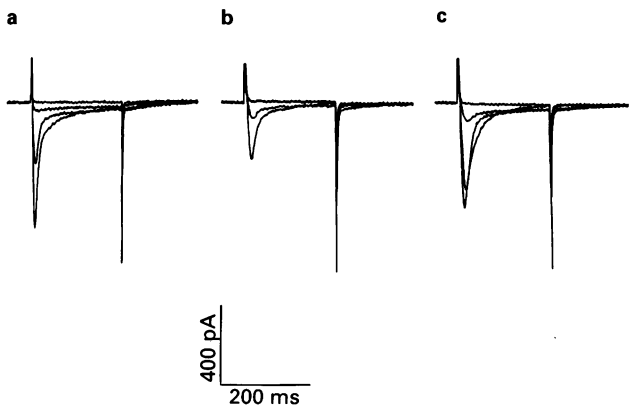


Figure 1 The effects of aluminium 50 μg ml^{-1} on calcium currents in a rabbit atrial cell. The cell was held at −40 mV, and 200 ms depolarizing pulses were given at a rate of 0.2 Hz. Current traces are shown in response to steps to −30, −20, −10 and 0 mV. In (a) are the superimposed traces in the control Tyrode medium, with the traces in (b) obtained 3–4 min after addition of aluminium to the perfusate. The traces in (c) were obtained 7–8 min after washout.

theless, in earlier experiments we observed that after filtering the suspension we could no longer obtain the prominent effects which had been seen prior to the removal of the amorphous substance (Banin & Meiri, unpublished results). In the present experiments, we therefore perfused our preparations with the entire aluminum hydroxide suspension. Permanent stirring ensured homogeneous distribution of the amorphous $Al(OH)_3$. Presumably, no significant transition of the aluminium hydroxide to one of its crystalline forms took place during the few hours' long experiments (Morefield *et al.*, 1986).

The dose of aluminium used in these experiments, 20–100 μg ml^{-1}, was chosen for the following two reasons. (1) Our previous experiments showed that this dose has a prominent, reproducible and reversible physiological effect (Banin & Meiri, 1987). (2) In man and experimental animals, accumulations of aluminium have been found in the brain, bone, skeletal and cardiac muscle tissues. In dialyzed patients who show the dialysis encephalopathy syndrome the level of aluminium in skeletal muscle reaches 80 μg g^{-1}, even though plasma levels equal only 5–125 ng ml^{-1} (Alfrey *et al.*, 1976; Altman *et al.*, 1988). In aluminium-injected rabbits, accumulation of 18 μg g^{-1} tissue of aluminium was found in the heart, with only 2.2 ng ml^{-1} in the serum (Bertholf *et al.*, 1989).

Results

Transmembrane ionic currents

Calcium currents The effects of aluminium on the calcium current were investigated mainly in frog atrial cells. However, in view of the possible toxicological relevance of aluminium, we also did a short series of experiments with mammalian cells, using rabbit atrial and ventricular myocytes. In neither frog nor rabbit cells was any attempt made to distinguish between different calcium currents (Bean, 1985).

In 6 out of 6 single rabbit myocytes (3 atrial and 3 ventricular), a change from normal Tyrode solution to one containing aluminium 50 μg ml^{-1} diminished the peak calcium current obtained by depolarizing steps from a holding potential of −40 mV. Such an effect is shown in Figure 1, which also demonstrates the fact that the effect of aluminium on these cells was at least partially reversible. Note, however, that the currents after washout are not completely identical, indicating that aluminium may have had additional more subtle effects. This issue was studied in greater detail in frog atrial cells.

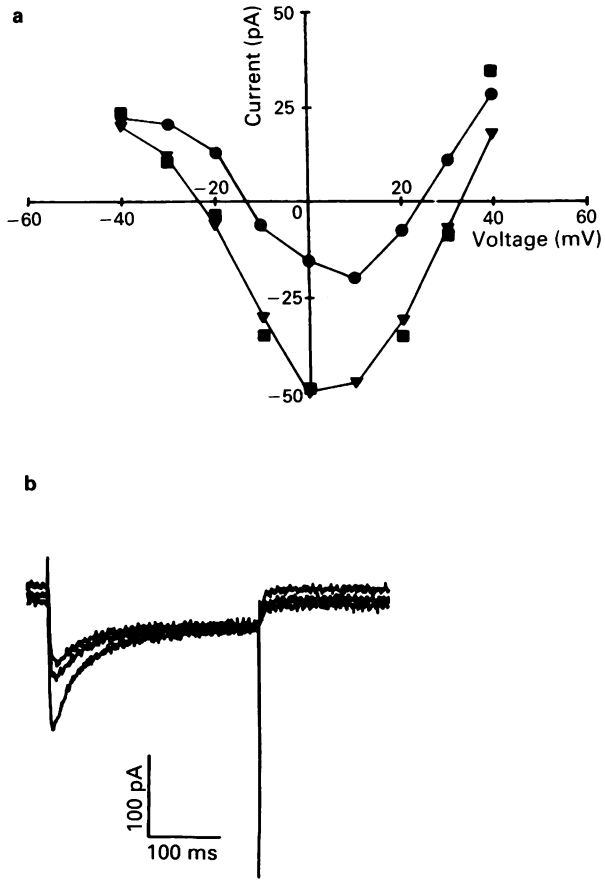


Figure 2 The effects of aluminium 50 μg ml^{-1} on the peak calcium current in a frog atrial cell. The cell was held at −50 mV, and stimulated at 0.2 Hz. (a) The currents were measured as the difference between the peak amplitude and the zero current level. The peak currents are plotted as a function of membrane potential in the control Ringer solution (▼), after 5–6 min in aluminium (●) and after 20 min washout (■). (b) Superimposed current traces (from another cell) in control (largest current amplitude) and 7 and 15 min after addition of aluminium. This panel illustrates the outward shift that occurred in the holding current, with the lowest (most inward) level in the control medium.

The effects of aluminium on calcium currents were studied in 9 frog atrial cells. In all cases, aluminium 50 μg ml^{-1} caused a substantial reduction in the peak amplitude of the inward current. The reduction was uniform at all membrane potentials, as illustrated in Figure 2, which shows the complete current-voltage relationship.

There are two accepted ways of measuring the magnitude of calcium currents – as the difference between the peak inward level and the zero current level, or as the difference between the peak inward level and the level at the end of the depolarizing pulse. In Figure 2 the first method was used. Since aluminium reduced the current at most membrane potentials so that it was no longer net inward, for a quantitative assessment, the second method of measurement was used. In this way the mean reduction of the calcium current at +10 mV was to 44.7 ± 8.1% (s.e.mean) of the control value (n = 9).

It will be noted that the changes in the current traces following the addition of aluminium are slightly different in the rabbit atrial cells (Figure 1) as compared to the frog atrial cells (Figure 2). The difference lies in the well established fact that whereas frog atrial cells possess a substantial amount of the inwardly rectifying, background potassium current IK_1 (Hume & Giles, 1983), rabbit atrium does not have this current at −40 or −50 mV (Giles & Imazumi, 1988). The shift in holding current in Figure 2 implies that aluminium also affects the potassium current, when present. These results are presented in the next section.

Background time-independent potassium current (IK_1) In frog atrial cells, in addition to changes in calcium currents, aluminium consistently altered the background, 'time-independent' potassium current, IK_1. This effect was studied by constructing isochronal current-voltage relationships (CVRs), by plotting current amplitudes at the end of the 200 ms voltage steps, as a function of membrane potential. These 'isochronal' current-voltage relationships give a good estimate of the inward rectifier current IK_1, since the delayed rectifier current in these cells, I_K, has a much slower onset (Hume *et al.*, 1986). This assumption is borne out by the fact that the plots of the CVRs that we obtained showed a prominent inward rectification, as is typical of this current in frog atrial cells (Giles & Shibata, 1985). It was found that aluminium caused small but consistent outward shifts in the current level at membrane potentials positive to −60 or −50 mV. For example, at −40 mV the holding current was shifted in an outward direction by a mean value of 22 ± 4 pA ($n = 8$). At more negative membrane potentials small shifts in the inward direction were observed. For example, in response to 100 ms hyperpolarizing steps to −80, −100 and −120 mV, the currents in aluminium were larger by a mean of 23, 53 and 65 pA, respectively. No significant change was seen in the reversal potential in this current, with a small shift (less than 10 mV) in the hyperpolarizing direction. A typical example can be seen in Figure 3.

Such complex effects cannot be explained by a simple block of IK_1 by aluminium, as obtained for example with barium (Giles & Shibata, 1985), but probably reflect multiple actions. Such effects may be indirect, and not necessarily at the channel level itself (see Discussion).

Time-dependent potassium current (I_K) In frog atrium, the dominant outward current is the time-dependent potassium current, I_K (Hume & Giles, 1983). Since aluminium was considered likely to have multiple effects, we examined this current as well. The exposure of single frog atrial cells to aluminimum, 50 $\mu g\,ml^{-1}$, was found to reduce I_K very rapidly (within 3 min). This was found in all 12 cells which were studied. Figure 4a shows a typical example of this reduction in one of the trials. In these experiments, calcium current blockers were not used. However, the reduction in I_K was independent of the effects of aluminium on I_{Ca}, since in these cells the activation of I_K is considered to be independent of I_{Ca} activation (Hume *et al.*, 1986). In support of this was the finding that at very strong depolarizations, to membrane potentials close to the reversal potential for the calcium current, I_K was still substantially reduced by aluminium, even when no calcium current was evoked. Interestingly, in contrast to the calcium current, which was relatively easy to reverse after a short (10–20 min) exposure to aluminium (as in the mammalian cells, cf. Figure 1), the effects of aluminium on I_K were practically irreversible. In only one case was the reduction of I_K by aluminium substantially reversed after a 30 min washout. It was therefore extremely important to exclude the possibility that the reduction of I_K was due to a current 'rundown'. Such a possibility was ruled out by monitoring the stability of I_K for several minutes, before exposing to aluminium. Figure 4b shows superimposed current traces obtained over several minutes before, and a trace 3 min after aluminium was added. It is clear that the amplitude of I_K is stable before the addition of aluminium, and is depressed immediately following its addition. Figure 4b also illustrates the effects of aluminium on I_K in response to depolarizations to +60 mV, in which no I_{Ca} was elicited.

The reduction of I_K occurred over the entire range of membrane potentials as illustrated in Figure 5a. I_K amplitudes were highly variable, as seen in the scatter in Figure 5. The steps to the more positive potentials, such as illustrated in Figure 4b, were only attempted in a small number of cells, and are thus not included in Figure 5. We further examined whether the reduction of I_K was due to a shift in its activation voltage range. By use of a standard 'tail current analysis' (e.g.

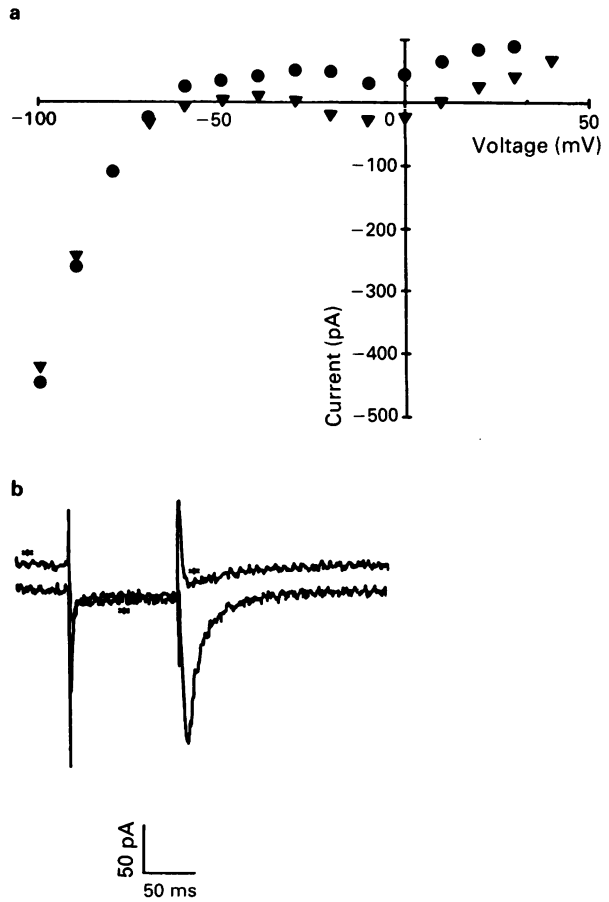


Figure 3 The effects of aluminium 50 $\mu g\,ml^{-1}$ on the background potassium current IK_1. This cell was held at −40 mV, and 100 ms voltage steps were given to different membrane potentials, at a rate of 0.2 Hz. The current level at the end of the step, relative to zero current level, is plotted against membrane potential. (a) The current-voltage relationships obtained with this protocol in control (▼) and 4–5 min after the addition of aluminium (●) illustrate the typical response. (b) Superimposed current traces for a hyperpolarizing step from −40 to −80 mV in control and 12 min after adding aluminium (indicated by asterisks). These indicate the outward shift in the current level at −40 mV as well as the slight increase in the current amplitude at −80 mV. Note also that the return to −40 mV elicits a large inward current (probably a mixed calcium and sodium current) in the control medium. This current is nearly absent in the presence of aluminium.

Giles & Shibata, 1985), a fully activated current-voltage relationship was constructed. Figure 5b shows an example of one cell with a plot of the (non-normalized) tail current amplitudes against membrane potential before and after addition of aluminium. There is obviously no large shift in the activation range of I_K following exposure to aluminium. This was verified by plotting the normalized values (not shown). Superposition of the normalized tail amplitudes vs membrane potential curves showed that neither the threshold nor the potential at which the current was 50% activated were significantly altered by aluminium. Aluminium shifted the mean half activation potential by 8 mV in the depolarizing direction. This could only partly account for the decrease in I_K, since in some cells there were very large decreases in I_K with very little shift in the activation range. Thus, the main effect of aluminium is due to a decrease in the membrane conductance to I_K, rather than to a shift in its activation range.

The effects of aluminium on contraction

The changes produced by aluminium on the force of contraction proved to be very complex, in several different ways. The first aspect of this complexity stemmed from the fact that we

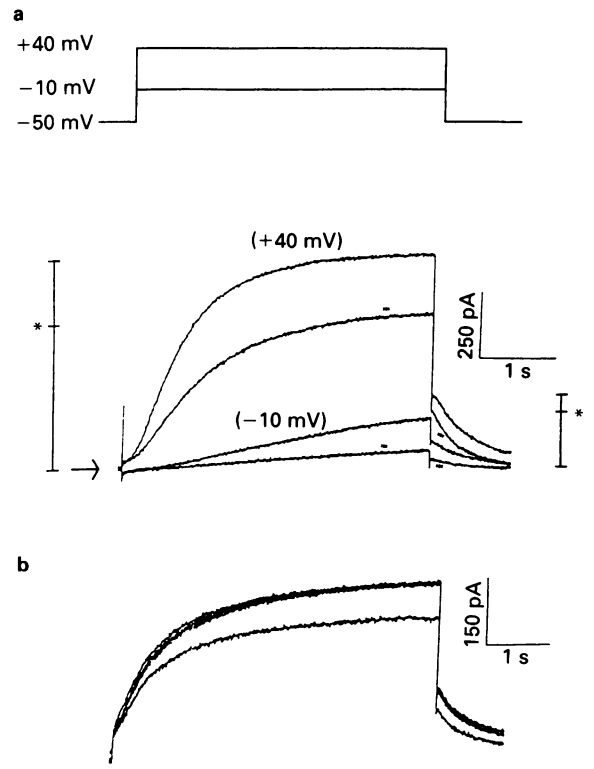


Figure 4 The effects of aluminium 50 $\mu g\,ml^{-1}$ on the time-dependent potassium current I_K. (a) This cell was held at −50 mV, and 4 s pulses were given at a rate of 0.1 Hz. The superimposed current traces shown are the ones in response to steps to −10 and to +40 mV. The traces marked by the asterisks were obtained after 9–10 min in aluminium. The vertical bars show how the measurements of the currents were made. On the left are the steady state current magnitudes for this pulse, and on the right the tail current magnitudes. The arrow indicates the holding current level and the asterisks denote the aluminium current traces. The voltage step is described on the top. (b) Superimposed current traces (from another cell), held at −40 mV: 5 depolarizing pulses to +60 mV were given every 10 s. The top traces are superimposed traces obtained over a 3–4 min period just before the addition of aluminium, indicating no rundown of the current. The smaller current was obtained 3 min after the addition of aluminium. Note the small outward shift in the holding current.

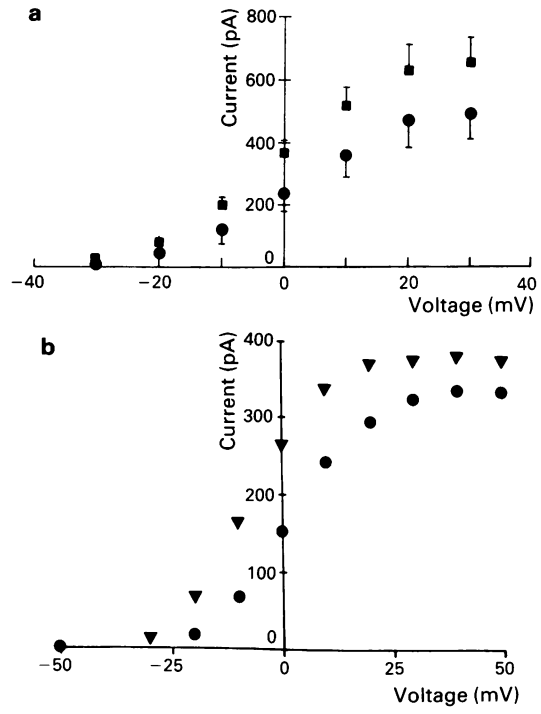


Figure 5 Changes in the time-dependent I_K current-voltage relationships caused by aluminium: (a) is a plot of the current amplitudes (mean with s.e.mean shown by vertical bars) as a function of membrane potential, before (■) and after (●) the addition of aluminium 50 $\mu g\,ml^{-1}$. The currents were measured from the holding current at −40 mV. (b) is a plot of the 'fully activated' current-voltage relationship. This was obtained by the standard 'tail analysis' method (e.g. Hume *et al.*, 1988). The cell was held at −40 mV, and 5 s voltage steps were applied at a rate of 0.1 Hz. The plot shows the (non-normalized) amplitude of the 'tail' currents obtained on returning to −40 mV, as a function of the membrane potential during the preceding step. (▼) Control; (●) 7–8 min after aluminium 50 $\mu g\,ml^{-1}$ was added. This current-voltage relationship illustrates that the shift of the activation range is by no more than a few mV. Neither the threshold for activation nor the maximal activation potential are much affected, and the main efficacy of aluminium is to depress the tail amplitudes at all membrane potentials. This implies that the reduction in amplitude results from a decrease in the channel conductance.

found a very profound seasonal variation in the effects of aluminium on contraction. A straightforward diminution of the force of contraction, which might be expected to accompany the reduction in the transmembrane calcium influx, was only observed in 'winter' frogs (see below). The 'standard' response in most cases, during most months of the year, was quite different.

The initial series of experiments was performed in the months of October–December. In most cases, the typical response elicited by the addition of aluminium 100 $\mu g\,ml^{-1}$ was as follows: within 2–3 min there was a slight but very consistent reduction in the force of contraction. This was followed after several minutes by a marked increase in the force of contraction. By normalizing the control amplitudes (as 100%), we constructed a plot of the averaged values of force, as a function of time after the addition of aluminium. This is illustrated in Figure 6, which also shows examples of the traces obtained in control, and during the two phases of response to aluminium.

Using exactly the same experimental protocol during the months of February and March we obtained quite different results. The sensitivity of the trabeculae to aluminium was greatly enhanced. In addition the secondary increase in force of contraction was virtually absent. The same standard concentration of aluminium (100 $\mu g\,ml^{-1}$) caused a very rapid and complete abolition of contraction ($n = 5$). By use of lower concentrations (20 $\mu g\,ml^{-1}$) it was possible to measure the extent of the sustained reduction in the force of contraction. This reduction was highly variable, ranging from a complete inhibition to a 24% reduction, with a mean amplitude which was 34% the control value ($n = 9$). This reduction was reversible after a long washout period (over 30 min). In 3 cases, after the initial reduction, there was a very small secondary increase of the twitch amplitude, which never approached the control amplitudes.

In the final stages of this project, the changes in contractile force following exposure to aluminium were again examined during the months of May-June. The sensitivity of the atrial trabeculae was again very similar to the initial one, with aluminium 100 $\mu g\,ml^{-1}$ giving biphasic effects in 5 cases. In 7 other trabeculae, aluminium 100 $\mu g\,ml^{-1}$ either increased the force of contraction with no obvious sign of an early decrease ($n = 4$), or had no effect on the force of contraction ($n = 3$). It thus seems that during the latter months there was a transition towards the initial biphasic pattern of responses, with a clear return to the initial, lower sensitivity.

A very preliminary probe was attempted in an effort to establish the possible mechanism(s) leading to the positive inotropic effects of aluminium. It was assumed that the initial decrease in the force of contraction and the maintained decrease (or complete abolition) which was observed during the 'winter' months were due to the reduction in the transmembrane calcium currents, as found in the single cell voltage

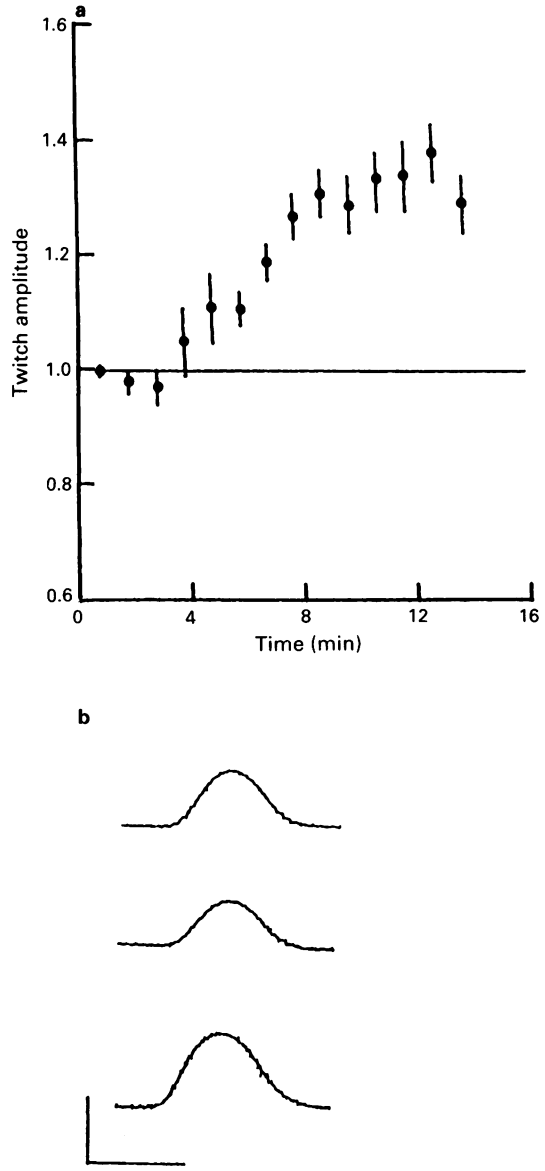


Figure 6 The changes in twitch amplitude produced by aluminium $100\,\mu g\,ml^{-1}$. Atrial trabeculae were stimulated at 0.5 Hz in normal Ringer solution until a stable amplitude was obtained. At time 0, aluminium was added. The plot shows the mean normalized values of twitch amplitude, obtained from 13 trabeculae exposed to aluminium. An early drop in amplitude is followed by a large increase in the force of contraction. Longer exposures were not usually tried. In (b) are sample tension traces from the control medium (top), 3 min in aluminium (middle) and 10 min in aluminium (bottom). The calibration bars denote 10 mg (vertical) and 500 ms (horizontal).

clamp experiments. However, there was obviously an additional effect of aluminium, which was superimposed upon the reduced calcium current and, which during most months of the year, led to a secondary increase in the force of contraction. There are a variety of mechanisms which can increase the twitch amplitude. Several of these possibilities were examined.

Frog atrium has a very potent sodium/calcium exchanger system for the extrusion of calcium (e.g. Horackova & Vassort, 1979). An inhibition of this system by aluminium would rapidly increase the force of contraction due to the impaired extrusion of calcium. In order to test this possibility, we exposed trabeculae to aluminium after replacing 90% of the extracellular sodium with lithium ions. This replacement eliminates calcium extrusion which is dependent on transmembrane sodium gradients, since lithium does not replace sodium on the exchanger (e.g. Kimura *et al.*, 1987). In our experiments, we waited until the complex changes in twitch amplitude caused by the changes in the sodium gradient were complete and a new stable twitch amplitude was attained. In 5 trabeculae, the addition of aluminium $100\,\mu g\,ml^{-1}$ produced a marked increase in the force of contraction (an early 'dip' was seen in 3 of these). The maximal increase in the force of contraction a mean of $46 \pm 8\%$ (s.e.mean), which is closer (and possibly larger) than the increase induced by aluminium in normal Ringer solution (see Figure 6). The effect of aluminium was again reversible after washout. Thus, the positive inotropic effect of aluminium is probably not largely dependent on the normal sodium gradient. It is thus likely that the major mechanism for positive inotropy would not be an inhibition of the sodium/calcium exchanger. These experiments have wider implications, by ruling out additional potential positive inotropic mechamisms which depend on the sodium gradient, such as an inhibition of the Na^+/K^+ pump (see Discussion).

The most likely mechanism for an increase in the force of contraction (with partially blocked calcium channels, and by a mechanism which is independent of changes in sodium gradients) is by an alteration in the release of calcium from intracellular stores. Positive inotropic effects could be obtained by increasing the amount of calcium released from the stores during each action potential, and/or by increasing the calcium loading of these stores prior to activation. An overall 'dumping' of calcium from various stores, such as membrane-associated sources (Langer, 1984) or from the sarcoplasmic reticulum (SR) is unlikely, since this would have led to a rise in tonic, baseline tension. This was not observed.

We investigated a possible effect of aluminium on internal stores by using caffeine, which, although having a variety of known effects on cardiac muscle (e.g. Hess & Wier, 1984), is also known to release calcium from intracellular stores in frog atrium (Niedergerke & Page, 1983). It was assumed that a depletion of the SR calcium stores by caffeine might attenuate the effects of aluminium. This was found to be the case in 6 trabeculae, which were exposed to caffeine 5 mM. After the addition of caffeine, and the equilibration of the twitch amplitudes at a new level, aluminium $100\,\mu g\,ml^{-1}$ was added (still in caffeine). In 4 cases, this led to a reduction in the force of contraction, while in 2 cases aluminium did not affect the contraction. In 2 cases, a complete series was done, in the following manner. A positive inotropic effect was obtained by addition of aluminium $100\,\mu g\,ml^{-1}$ to normal Ringer solution. After washout, caffeine was added and contraction was monitored until a new steady state amplitude was achieved. Aluminium was then added again (still in the presence of caffeine). This produced a negative inotropic effect. After a long washout (over 60 min) of caffeine and aluminium, aluminium was again added to normal Ringers solution. The positive inotropic effect was restored as illustrated in Figure 7.

Discussion

The results presented here are the first description (known to us) of the effects of aluminium on ionic currents and contractile properties of cardiac cells. Several complications relating to the chemistry of aluminium compounds may have hampered earlier work. The dissolution of $AlCl_3$ in water gives very acidic solutions. At physiological pH values, aluminium in water undergoes complexation into several hydrated species. The free concentration of aluminium hydroxide anions (as $Al[OH]_4^-$) cannot exceed several micromolars, whereas as cations ($Al[H_2O]_6^{+3}$) the concentration is less than 10^{-11} M (Birchall & Chappell, 1988). The solutions we used were therefore a mixture of several species, some of which were insoluble.

Furthermore, earlier work pointed to a multitude of biochemical actions wrought by aluminium. We will examine our results in light of some of these previously reported effects. Although we have no direct evidence, we may assume that

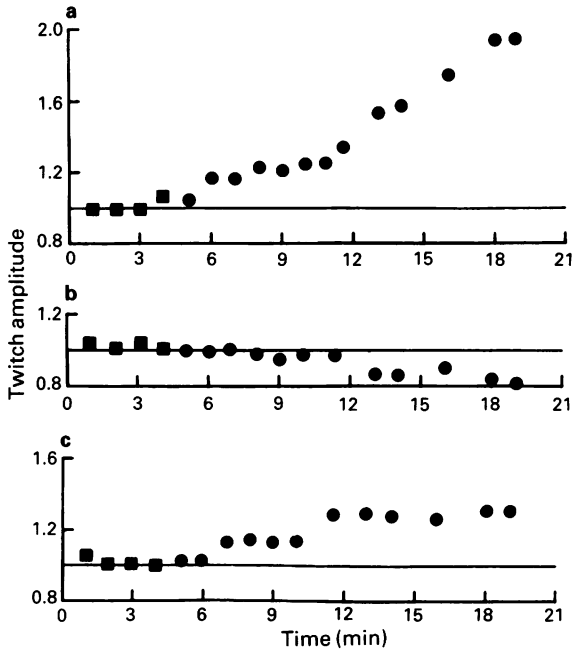


Figure 7 The attenuation of the positive inotropic effect of aluminium by caffeine. Normalized twitch amplitudes are plotted for a trabeculum stimulated at 0.5 Hz. (a) Amplitudes in control (■) and following the addition of aluminium 100 $\mu g\,ml^{-1}$ (●); (b) shows the normalized twitch amplitudes after equilibration in caffeine 5 mM (■) and following the addition of aluminium (●). Following the pre-exposure to caffeine, aluminium has a negative inotropic effect. Both caffeine and aluminium were washed out for 60 min and aluminium was again added to normal Ringer solution. Panel (c) illustrates that a positive inotropic effect (although smaller than initially) could again be elicited by aluminium.

aluminium exerts some of its actions after entering the cells (MacDonald & Martin, 1988). Indirect support of this, by analogy, is the recent detection (by indo-1 fluorescence) of an influx of another trivalent ion, lanthanum, into ventricular cells (Peeters *et al.*, 1989).

We found that both the calcium and the time-dependent potassium currents in frog atrial cells were markedly diminished by aluminium. Both of these currents have been found to be regulated to some extent by an adenosine 3',5'-cyclic monophosphate (cyclic AMP)-dependent phosphorylation of the respective channels (e.g. Bean *et al.*, 1984; Kameyama *et al.*, 1985; Giles *et al.*, 1989). The regulation of the two channels is probably independent, since selective manipulations have been reported (Hume *et al.*, 1986; Walsh *et al.*, 1988). The effect of aluminium on these two currents may be related to earlier findings of Martin (1986) who suggested that Al^{3+} attaches to ATP molecules 10^7 more strongly than Mg^{2+}, forming inactive ATP-Al, which disrupts the activity of yeast and brain hexokinases (Womack & Colowick, 1979). We may thus speculate that aluminium interferes with the action of protein kinases which regulate the two channels. The fact that the inhibition of the two currents is only partial may tie in with the fact that there is probably a basal channel conductance which is independent of phosphorylation (Kameyama *et al.*, 1986).

A reduction in I_K can result from a reduction in the conductance in K^+ ions through the membrane channels, and/or by a shift in the membrane potential range in which this current is activated. The measurement of the activation voltage range is done by standard 'tail analysis'. This requires giving pulses which fully activate the I_K current, to different potentials, and measuring the tail currents following the pulses. The problem with I_K is that even very long pulses do not always fully activate all of I_K, at all potentials (see Duchatelle-Gourdon *et al.*, 1989; Giles *et al.*, 1989). There can be thus underestimation of steady state I_K at some potentials, leading to some error in the activation curve. Giving very long pulses to positive membrane potentials can be harmful to single myocytes, so that this problem is difficult to overcome. However, in our experiments, the activation of I_K in frog atrial myocytes is mono-exponential (Hume *et al.*, 1986). We can therefore conclude from the results shown in Figure 4a (pulse to +40 mV) and 4b, that most of the reduction in I_K is caused by a decreased conductance, since the current is nearly fully activated at the end of the pulse. Some of the reduction could be caused by a small shift in the voltage range of I_K activation. Figure 5 shows that the threshold for the appearance of I_K is not changed by aluminium. Thus, any shift in the voltage range of current activation, would probably be secondary to the reduction in the conductance of I_K.

The changes in the background current IK_1 may also be partly related to the strong competition of aluminium with magnesium. Since magnesium ions are now believed to be responsible for the inwardly directed rectification properties of IK_1 channels (Vandenberg, 1987), the reduced degree of rectification (Figure 3) may be due to a reduced blocking efficacy of magnesium, in the presence of aluminium. Obviously other mechanisms are probably affected, since at negative potentials there is a small but consistent increase in IK_1. The effect of aluminium is therefore not a direct block of IK_1 channels, such as obtained with barium (Giles & Shibata, 1985), since a direct blockade would linearize the entire current voltage relationship and reduce IK_1 amplitudes at all membrane potentials.

The aluminium-induced changes in the force of contraction were dependent on the season. Interestingly, Sanborn & Langer (1970) found seasonal variations in the response of rabbit ventricular septal preparations to lanthanum ions. Furthermore, the response of contractile force to lanthanum also showed a biphasic nature (Sanborn & Langer, 1970, Figure 1). We may therefore assume that the changes of responses with the seasons is not a peculiarity of aluminium, but rather a reflection of changes in underlying mechanisms, which control excitation contraction coupling.

The decrease in contractile force may be directly due to the decrease in calcium current. However, this would be partially offset by the decrease in the potassium current, since the latter would broaden the action potential. The decrease in the time dependent IK is much more significant than the small increases in IK_1, in the plateau range of potentials. The overall effect would most probably be broadening of the action potential, although we did not study this directly. A longer duration of depolarization could itself increase the force of contraction by prolonging the transient rise in intracellular calcium (Cannell *et al.*, 1987). A longer depolarization could also slow down the voltage-dependent extrusion of calcium via the Na/Ca exchanger (e.g. Fedida *et al.*, 1986), thereby contributing to the positive inotropic effect.

The secondary increase in contractile force was most probably not due to mechanisms depending on the sodium gradient, since aluminium had the same effects in the absence of any significant gradient. Thus, an inhibition of either the Na^+/Ca^{2+} exchanger or of the Na^+/K^+ pump cannot be the major mechanism for the increase in twitch amplitude. It is, however, worth noting that there is evidence for an inhibition of the Na^+/K^+-ATPase by aluminium (Lai *et al.*, 1980). However, if a block of the Na^+/K^+ pump were a major effect, we would still have obtained the same effect in the presence of caffeine. Furthermore, any substantial inhibition of the pump would have led to increases in the baseline, tonic tension. However, we cannot entirely rule out some contribution of pump or exchanger inhibition to the positive inotropic effect.

Interestingly, the results in low sodium rule out another possible inotropic mechanism. Recently, a stimulation of the Na/K/2Cl co-transporter has been suggested as underlying the positive inotropic effects of cardiac glycosides (Panet *et al.*, 1989). However, such a mechanism is also dependent on the sodium gradient.

The most likely effect of exposure to aluminium is an increase in the release of calcium from intracellular stores. Some displacement may occur from sarcolemmal binding sites (Langer, 1984), as proposed for the action of lanthanum (Mead & Clusin, 1985). Any massive 'dumping' of calcium, however, would have led to increases in baseline, tonic tension. This was not observed. Therefore, the increase in calcium release is probably effected by a larger amount of calcium released at each action potential. Aluminium may either increase the loading of internal stores, or enhance the amount of calcium released from these stores, or both.

The results with caffeine, which depletes SR stores (Niedergerke & Page, 1981; Hess & Wier, 1984), support the suggestion that aluminium acts by affecting internal calcium stores. A depletion of SR stores by caffeine was most probably the cause for the inversion of a positive to a negative inotropic effect of aluminium, since the depleted SR could no longer provide extra calcium for the secondary phase. This is based on the assumption that aluminium indeed acts intracellularly, as proposed for lanthanum ions (Peeters *et al.*, 1989). Interestingly, secondary positive inotropic effects of lanthanum were also abolished by caffeine (Mead & Clusin, 1985).

The measurement of twitch amplitude is obviously less sensitive than the direct measurement of intracellular calcium. There might be subtle changes which would slightly increase the level of free cytosolic calcium such that the twitch amplitude would be enhanced, without an increase in tonic tension. Even small elevations would greatly increase twitch amplitude, by indirectly causing large increases in SR calcium release during the action potential. Small increases in cytosolic calcium levels would occur if the cyclic AMP-dependent protein kinase in the SR membrane were slightly inhibited, thus reducing SR uptake following contraction. An inhibition of a kinase-dependent sarcolemmal calcium pump would also increase cytosolic calcium levels. This would be compatible with the other suggested effects of kinases associated with sarcolemmal currents. This would also be in line with other known actions of aluminium, such as the displacement of calcium from binding to calmodulin (Siegal & Haug, 1983).

Earlier work on the frog neuromuscular junction (Banin & Meiri, 1987) indicated an increase in transmitter release which was induced by aluminium. In the multicellular atrial trabeculae, functional nerve endings may be affected by aluminium, with an increased autonomic transmitter output. A differential effect on sympathetic and parasympathetic nerve endings could underlie the complex changes observed. Seasonal changes in the density of nerve endings or of postjunctional receptors could underlie the seasonal variability which we observed. These possibilities necessitate further investigation.

A final consideration relates to the finding that aluminium was found to induce membrane phase separation and rigidification in lipid vesicles of bovine origin (Deleers *et al.*, 1985; 1986). Non-specific alterations in membrane structure or organization could also explain the diversity of effects on the different ionic channels. A release of membrane bound calcium by alterations in the affinity to membrane phospholipids has also been raised as a possible mechanism for positive inotropic effects (Gervais *et al.*, 1977). The secondary increase in twitch amplitude may thus be related to an aluminium-induced displacement of membrane bound calcium.

In summary, several diverse effects of aluminium have been described. Although this diversity probably reflects a multiplicity in sites and mechanisms of action, it is tempting to speculate that a general derangement of phosphorylation by a variety of protein kinases is a cardinal intracellular complication which results from an acute exposure to aluminium.

We thank Professor Rami Rahamimoff for his comments on the manuscript. This work was supported in part by the Fund for Basic Research of the Israel Academy of Sciences and Humanities and by the Israel Institute for Psychobiology.

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(Received November 13, 1989
Revised August 21, 1990
Accepted October 9, 1990)

Br. J. Pharmacol. (1991), **102**, 492–496 © Macmillan Press Ltd, 1991

Strychnine-induced potassium current in isolated dorsal root ganglion cells of the rat

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1 Effects of strychnine (Str) on the dissociated dorsal root ganglion (DRG) cells of the rat have been investigated in whole-cells configuration by a conventional patch-clamp technique.

2 γ-Aminobutyric acid (GABA)-induced Cl^- current (I_{Cl}) increased sigmoidally with increasing concentration. The half-maximal response (K_a) was 3×10^{-5} M and the Hill coefficient was 1.5. Both Str and bicuculline inhibited the GABA-induced I_{Cl} in a concentration-dependent manner.

3 Str itself could elicit the current at concentrations over 10^{-5} M, at which concentrations the GABA response was completely suppressed. The concentration-response curve for the Str-induced current was bell-shaped, and a nearly maximum response occurred at 3×10^{-4} M. A transient 'hump' current appeared immediately after the wash-out of external solution containing high concentrations of Str over 3×10^{-4} M.

4 The Str-induced outward current and a transient 'hump' current were augmented by the removal of extracellular K^+ and were suppressed by the substitution of intracellular K^+ for Cs^+. But the current was not sensitive to extracellular Na^+, Ca^{2+} and Cl^-.

5 The reversal potential of Str-induced current (E_{Str}) was −75 mV, which was close to the K^+ equilibrium potential ($E_K = -76.3$ mV). The change of E_{Str} for a ten fold change in extracellular K^+ concentration was 58 mV, indicating that the membrane behaves like a K^+ electrode in the presence of Str. The reversal potential of the 'hump' current was also close to E_K.

6 The Str-induced outward current was antagonized by K^+ channel blockers such as Ba^{2+}, tetraethylammonium (TEA)-chloride, and 4-aminopyridine (4-AP) in a concentration-dependent manner.

7 The Str-induced K^+ current was not affected by internal perfusion of *bis*(γ-aminophenoxy)ethane-*N*,*N*,*N*′,*N*-tetraacetic acid (BAPTA), indicating that the Str response does not result in the activation of K^+ conductance by the intracellular Ca^{2+}.

Introduction

Glycine is an important inhibitory neurotransmitter in mammalian CNS neurones, especially at the motoneurones and interneurones in the spinal cord, and strychnine (Str) acts as a selective and competitive antagonist of the glycine receptor. The convulsant action of Str is due to interference with the postsynaptic inhibition mediated by glycine. Hence, Str has been used as a valuable pharmacological tool for studies of glycinergic inhibition (Curtis, 1969; Kuno & Weakly, 1972; Johnston, 1978; Zarbin *et al.*, 1981). However, in dissociated frog dorsal root ganglion neurones (Yakushiji *et al.*, 1987) and dissociated rat Purkinje cells (Kaneda *et al.*, 1989), Str was able to inhibit not only the glycine-induced Cl^- current (I_{Cl}) but also the GABA-induced I_{Cl} in a competitive manner at an almost equal concentration-range of the drug.

Recently, while we investigated the effect of Str on γ-aminobutyric acid (GABA)-induced I_{Cl} in dissociated rat dorsal root ganglion (DRG) cells, we observed that Str itself could induce a response at concentrations over 10^{-5} M. In the present study, therefore, we examined the electrical and pharmacological properties of the Str-induced current in dissociated DRG cells of the rat under voltage-clamp conditions.

Methods

Preparation

Dorsal root ganglia (DRG) were dissected from 2 weeks-old Wistar rats under ether anaesthesia. The isolated ganglia were kept in an incubation solution (see below) bubbled with 95% O_2 and 5% CO_2 at 31°C for 40–60 min and then the ganglia were successively treated enzymatically in the incubation solution with 0.33 mg ml^{-1} DISPASE (Godo Shusei) and 4 mg ml^{-1} collagenase (Sigma) at 31°C for 40 min. In a culture dish (35 mm Falcon #3801) filled with a standard external solution (see below), the DRG neurones were dissociated mechanically by gentle pipetting. The dissociated DRG cells adhered to the bottom of the dish within 30 min and were then prepared for the experiments at room temperature.

Solutions

The solutions used in the present experiments were composed as follows. Incubation solution (mM): NaCl 124, KCl 5, $CaCl_2$ 2.4, KH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 26 and glucose 10. Standard external solution: NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, and HEPES 10. Na^+-free external solution: NaCl in external solution was replaced with equimolar choline chloride. K^+-free external solution: KCl in the external solution was replaced with CsCl. Ca^{2+}-free external solution: $CaCl_2$ in the external solution was replaced with $CoCl_2$. Low Cl^- external solution: 80 mM NaCl in the external solution was replaced with equimolar Na-isethionate. The pH of all external solutions was adjusted to 7.4 with Tris-base. Internal solution (mM): KCl 50, K-gluconate 70, NaCl 30, $CaCl_2$ 0.25, $MgCl_2$ 1, Mg-ATP 2, HEPES 10, and EGTA 5. K^+-free internal solution: KCl and K-gluconate in the internal solution were replaced with CsCl. The pH of both internal solutions was adjusted to 7.2 with Tris-base.

Electrical measurements

Ionic current was measured in whole-cell voltage-clamp mode by the use of conventional patch-clamp technique (Hamill *et al.*, 1981). The resistance between the glass pipette filled with internal solution and the reference electrode in bathing medium was 4 to 8 MΩ. Both electrodes were led to a patch-clamp amplifier (List Medical, EPC-7), and the junction potential was compensated with an offset circuit. The current

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and voltage were monitored on a storage oscilloscope (Toshiba, 10M63) after being low-pass filtered at 1 kHz, and recorded simultaneously on a pen-recorder (Sanei, RECTI-HORIZ-8K) and a video cassette recorder (Mitsubishi, HV-F32) in a digital mode by a digital audio processor (Sony, PCM-501ES).

Drugs

Drugs used in the present experiments were γ-aminobutyric acid (GABA), 4-aminopyridine (4-AP) and tetraethylammonium (TEA)-chloride [Tokyo Kasei], strychnine hydrochloride (Str), bicuculline methioide (Bic) and *bis*(γ-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA, Sigma). All drugs were dissolved in the external or internal solution just before use. External test solution was exchanged by a rapid application technique termed 'Y-tube' method, which allows the exchange of solutions surrounding an isolated DRG cell within 10 to 20 ms (Akaike *et al.*, 1989; Murase *et al.*, 1989).

Results

GABA-induced response

The isolated DRG cells were perfused with standard external and internal solutions containing 161 and 82.5 mM Cl^-, respectively. The holding potential (V_H) was −50 mV. GABA was applied every 2 min as desensitization did not occur with this time interval.

The values of a transient peak and a successive steady-state current components of the GABA-induced Cl^- current (I_{Cl}) are plotted as a function of GABA concentration in Figure 1. In the figure all responses were normalized to the peak response induced by 10^{-5} M GABA. The threshold concentration of GABA was 3×10^{-6} M and the GABA-induced I_{Cl} increased in a sigmoidal fashion with increasing GABA concentration. In the GABA concentration-response curve, a half-maximal response (K_a) was 3×10^{-5} M and the Hill coefficient was 1.5.

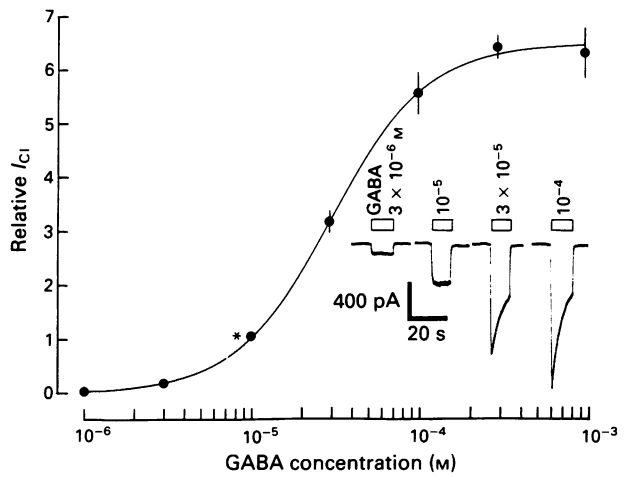


Figure 1 γ-Aminobutyric acid (GABA)-induced Cl^- current (I_{Cl}). Concentration-response relationship for GABA, in which all GABA responses at various concentrations were normalized to the peak response induced by 10^{-5} M GABA (symbol marked with asterisk). Holding potential (V_H) was −50 mV; $[Cl^-]_i$ = 82.5 mM; $[Cl^-]_o$ = 161 mM. Each point shows the average of 4–6 experiments, and bars indicate ±1 s.e. when larger than symbol. A continuous theoretical curve was drawn according to the following equation with $n = 1.5$, $K_a = 3 \times 10^{-5}$, and $I_{max} = 6.5$:

$$I = I_{max} \frac{C^n}{C^n + K_a^n}$$

where I is the observed GABA-activated current, I_{max} is the maximum current, C is the GABA concentration, K_a is the GABA concentration that evoked the half-maximal response, and n is the Hill coefficient. The inset shows the GABA-induced I_{Cl}s. Currents were obtained from the same cell. The cell was exposed to various concentrations of GABA for periods indicated by a horizontal bar above each response.

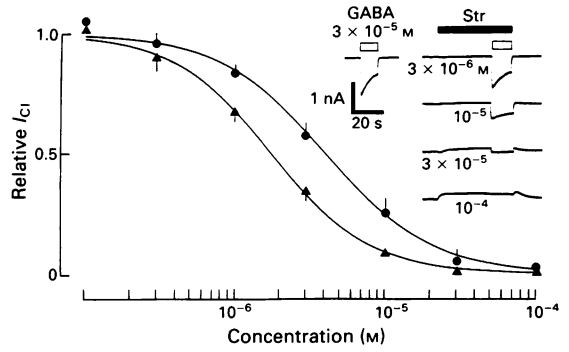


Figure 2 Inhibition of γ-aminobutyric acid (GABA)-induced I_{Cl} by strychnine (Str; ●) and bicuculline (▲). V_H was −50 mV. The preparation was pretreated with antagonists for 30 s. All responses induced by a mixture of 3×10^{-5} M GABA plus one of the antagonists at various concentrations were normalized to the peak current induced by 3×10^{-5} M GABA alone. Abscissa scale shows antagonist concentration. Each point shows the average of 4–6 experiments. The inset shows the inhibition of GABA-induced I_{Cl} by Str of various concentrations. All recordings shown in the inset were obtained from the same cell. Note that Str itself induces the outward current (inset).

Effects of convulsants on GABA-induced I_{Cl}

Figure 2 shows the concentration-dependency of inhibitory actions of strychnine (Str) and bicuculline (Bic) on the peak current component elicited by 3×10^{-5} M GABA. In this experiment, the dissociated DRG cells were pretreated with Str or Bic for 30 s, and successive applications of 3×10^{-5} M GABA plus one of the antagonists at various concentrations were made. All responses were normalized to the peak current induced by 3×10^{-5} M GABA alone. The concentrations for half-inhibition (IC_{50}) were 4.0×10^{-6} M for Str and 1.8×10^{-6} M for Bic. Unexpectedly, as shown in Figure 2 (inset), Str itself induced outward currents at concentrations higher than 10^{-5} M. On the other hand, Bic itself did not elicit any current even at the extremely high concentration of 10^{-4} M.

Strychnine-induced current (I_{Str})

Experiments were carried out at a V_H of −50 mV. Str was applied every 5 min, at which interval a constant response to Str could be repeatedly evoked for 1 h or more. The threshold concentration for the Str-induced current (I_{Str}) was around 10^{-5} M. The I_{Str} increased with increasing drug concentration in the range 10^{-5} to 3×10^{-4} M. Further increases in the Str concentration over 10^{-3} M decreased the peak current, and the peak current rapidly subsided to a steady plateau level during continuous application of the drug. Moreover, the 'wash-out' of external solution containing Str of concentrations higher than 3×10^{-4} M always induced a transient outward 'hump' current (Figure 3a). However, this 'hump' current was never induced by continuous applications for more than 1 min of Str at concentrations below 3×10^{-4} M. Figure 3b shows the Str-induced peak currents plotted as a function of Str concentration. A maximal response during a continuous application of the drug was regarded as the peak current. When all Str responses were normalized in relation to the peak current induced by 10^{-4} M Str, the concentration-response curve was bell-shaped. Glycine over the concentration range 10^{-8}–10^{-3} M did not induce any responses; glycine had no effect on I_{Str}.

The inset of Figure 4 shows the currents induced by 10^{-4} M Str at various V_H levels. In the current-voltage (*I-V*) relation-

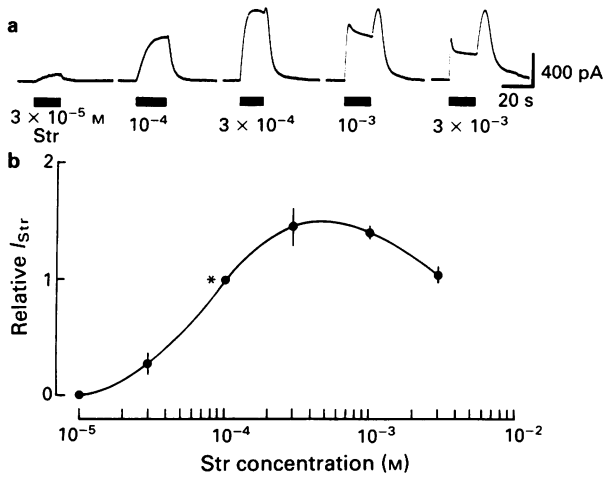


Figure 3 (a) Strychnine (Str)-induced outward currents in a dissociated DRG cell. V_H was −50 mV. The cell was exposed to various concentrations of Str for period indicated by a horizontal bar below each response. (b) Concentration-peak response curve for Str. The x axis denotes the Str concentration, and the y axis represents the relative peak Str response (I_{Str}). A maximal response during a continuous application of the drug was regarded as a peak response. Each point shows the average of 4–6 experiments. All Str responses were normalized to the peak current induced by 10^{-4} M Str (see symbol marked with asterisk). The continuous curve was drawn by eye.

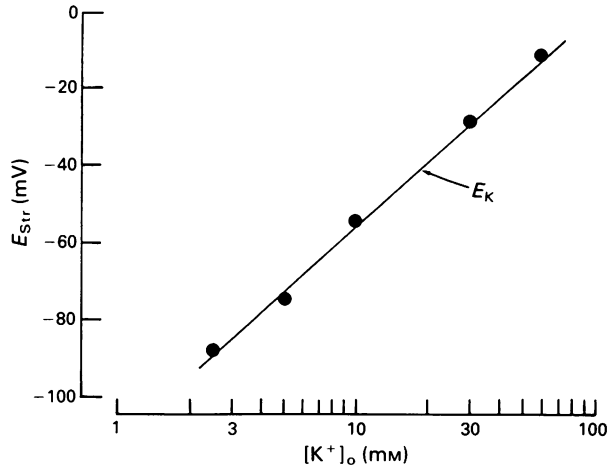


Figure 5 Effect of extracellular K^+ concentration ($[K^+]_o$) on the reversal potential of strychnine (Str)-induced current (E_{Str}). Dissociated DRG cells were perfused with external solutions containing K^+ of various concentrations. The intracellular K^+ concentration ($[K^+]_i$) was 120 mM throughout this experiment. Each point shows the average of 4–5 experiments. A straight line shows E_K.

ship of 10^{-4} M Str-induced response, the responses at various V_H were normalized to the peak response induced by Str at a V_H of −50 mV. The reversal potential of I_{Str} (E_{Str}) estimated from an intersect on the voltage axis in the *I-V* curve was −75.0 ± 2.0 mV (mean ± s.e.mean, $n = 5$). The experimental E_{Str} value was quite close to the K^+ equilibrium potential ($E_K = -76.3$ mV) calculated from the Nernst equation knowing the extra- and intracellular K^+ concentrations. Moreover, the 'hump' current induced by high concentrations of Str also reversed at almost E_K (not shown). The results indicate that the I_{Str} and the 'hump' current are carried by passing through K^+ channels.

When the dissociated DRG cells were perfused with an internal solution containing 120 mM K^+ and the external solutions containing various K^+ concentrations (i.e. $[K^+]_o = 2.5$, 5, 10, 30, and 60 mM), the measured E_{Str} values were −87.3 ± 2.1 mV ($n = 5$), −75.0 ± 2.0 mV ($n = 5$), −54.3 ± 0.9 mV ($n = 4$), −29.0 ± 1.5 mV ($n = 4$), and −12.0 ± 1.5 mV ($n = 4$) for 2.5, 5, 10, 30, and 60 mM $[K^+]_o$, respectively (Figure 5). In the E_{Str} and $[K^+]_o$ relationship, the change in E_{Str} for a ten fold change of $[K^+]_o$ was 58 mV, indicating that the cell membrane behaves like a K^+ electrode in the presence of Str.

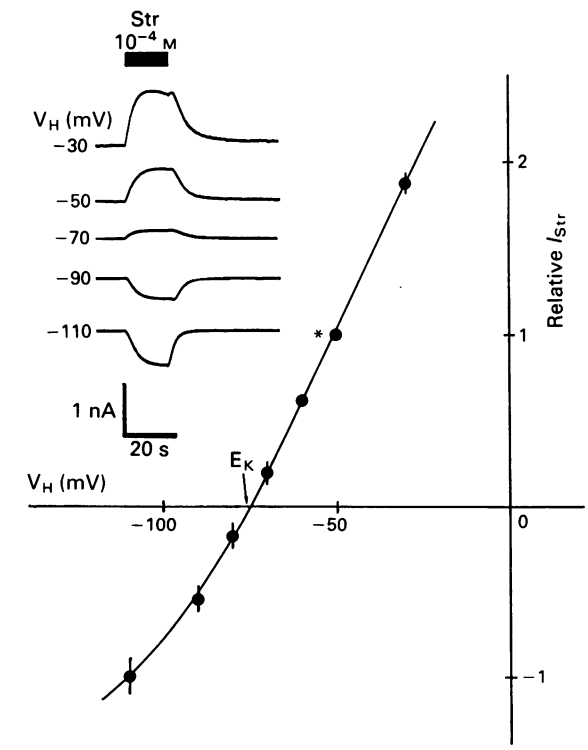


Figure 4 Current-voltage (*I-V*) relationship of 10^{-4} M strychnine (Str)-induced response. All responses were normalized to the peak response induced by Str at a V_H of −50 mV (see symbol marked with asterisk). Each point shows the average of 5 experiments. The inset shows 10^{-4} M Str-induced currents at various V_Hs in the same cell.

Effects of extracellular electrolytes on I_{Str}

In the presence of intra- and extracellular K^+ ($[K^+]_i$ and $[K^+]_o$, respectively), Str evoked the outward current at a V_H of −50 mV (Figure 6a). The Str-induced outward current was not affected by removal of extracellular Na^+, Ca^{2+}, and Cl^-, but was augmented by the absence of $[K^+]_o$, when KCl in the external solution was replaced with equimolar CsCl. In the presence of intracellular Cs^+, Str induced the inward current

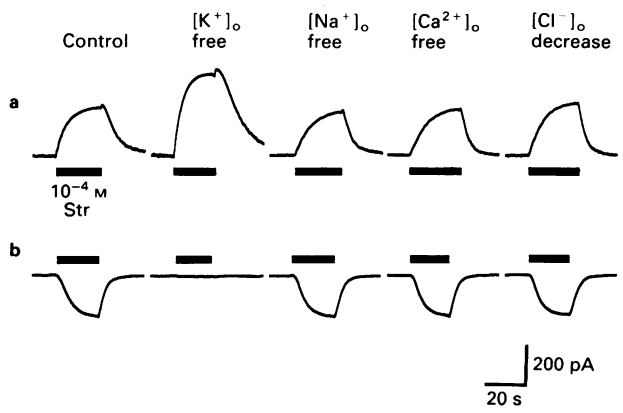


Figure 6 Effects of extracellular electrolytes on 10^{-4} M strychnine (Str)-induced currents. V_H was −50 mV. (a) The cell was perfused with the internal solution containing 120 mM K^+. (b) The cell was perfused with the internal solution containing 120 mM Cs^+ instead of K^+. The horizontal bars indicate the period of application of 10^{-4} M Str.

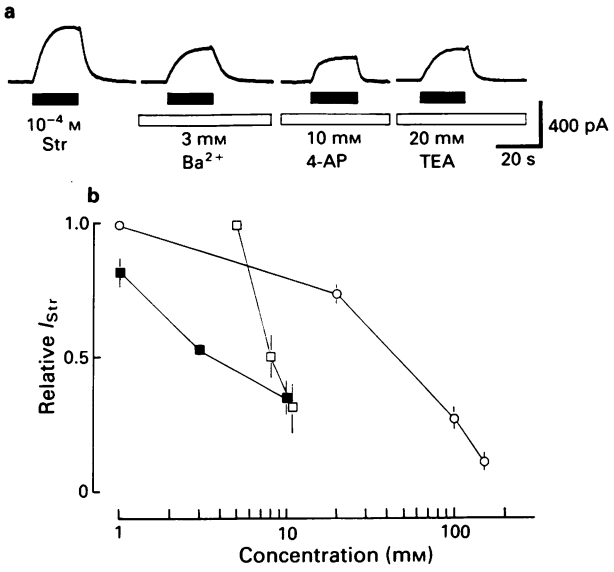


Figure 7 Effects of various K^+ channel blockers on the strychnine (Str)-induced outward current (I_{Str}). The cells were pretreated with standard external solution containing various K^+ channel blockers for 1 min, and successively 10^{-4} M Str plus one of the blockers were applied simultaneously. V_H was −50 mV. (a) All recordings were obtained from the same cell. Open and closed horizontal bars indicate the application of the blockers and Str, respectively. (b) All responses induced by a mixture of 10^{-4} M Str plus one of the blockers at various concentrations were normalized to peak current induced by 10^{-4} M Str alone. Abscissa scale shows the concentration of blockers. Each point shows the mean of 4 experiments, and vertical bars show ±1 s.e.mean when larger than symbol: (■) Ba^{2+}; (□) 4-aminopyridine (4-AP); (○) tetraethylammonium (TEA).

at the same V_H of −50 mV, and the inward current was completely abolished by the replacement of extracellular K^+ with Cs^+ (Figure 6b). These results also indicate that I_{Str} is carried by K^+.

Effects of various K^+ channel blockers on I_{Str}

In order to elucidate the pharmacological type of K^+ channel activated by Str, the effects of various K^+ channel blockers on the Str-induced outward current were tested at a V_H of −50 mV. The neurones were perfused with normal internal and external solutions. In these experiments, $BaCl_2$ and 4-AP were directly dissolved in the standard external solution. The external solution containing TEA was made by the replacement of NaCl in the external solution with equimolar TEA-Cl. Ba^{2+}, TEA, and 4-AP suppressed the Str-induced outward current in a concentration-dependent manner (Figure 7a). The half inhibition doses (IC_{50}) were 3.5 mM for Ba^{2+}, 8 mM for 4-AP, and 45 mM for TEA (Figure 7b).

Effects of internal perfusion of BAPTA on I_{Str}

When the Str-induced outward current was induced at a V_H of −50 mV, intracellular perfusion of BAPTA at concentrations of 5–10 mM had no effects on the amplitude and kinetics of the Str response in all cells tested ($n = 5$), indicating that the Str-induced K^+ response is not affected in the presence or the absence of intracellular Ca^{2+}.

Discussion

The present experiments clearly show that at high concentrations over 3×10^{-5} M Str induces K^+ currents in dissociated rat DRG cells. This K^+ current passes through K^+ channels as follows: (1) in the presence of intracellular K^+, the Str-induced outward current is augmented by removal of extracellular K^+; (2) in the absence of intracellular K^+, the Str-induced inward current is completely abolished by removal of extracellular K^+; (3) the reversal potential of Str-induced current (E_{Str}) is quite close to the E_K calculated from the Nernst equation.

When suppression of the peak and plateau currents appeared with concentrations of Str over 3×10^{-4} M, the wash-out of Str always induced a transient 'hump' current (Figure 3a). Such decreases of the peak and plateau currents may be due to blockade of the K^+ channel by the Str molecule itself, and the hump on the wash-out of high concentrations of Str is probably due to the activation of undesensitized receptors by Str, released from channels blocked by Str. Such reasoning may also apply to the hump following the wash-out of high concentrations of acetylcholine in the voltage-clamped frog endplate (Adams, 1975) and that of pentobarbitone in the frog DRG neurone (Akaike *et al.*, 1987).

A variety of voltage-dependent K^+ channels have been reported in various invertebrate and vertebrate neurones (Adams & Gage, 1978; Adams *et al.*, 1980; Segal & Barker, 1984; Cobbett *et al.*, 1989). At least five distinct K^+ currents have been identified as follows: a voltage-activated delayed K^+ current (I_K), a transient outward K^+ current (I_A) (Connor & Stevens, 1971; Neher, 1971; Neher & Lux, 1972), a non-inactivating outward K^+ current (I_M) (Brown & Adams, 1980; Adams *et al.*, 1982), a Ca^{2+}-activated outward K^+ current (I_{KCa}) (Meech & Standen, 1972; Brown & Griffith, 1983; Bourque *et al.*, 1985; Bourque, 1988), and an anomalous rectifier K^+ current (I_{AR}) which is a voltage- and time-dependent inward current triggered at hyperpolarized potentials (Purpura *et al.*, 1968; Hotson *et al.*, 1979; Halliwell & Adams, 1982). These have been differentiated on the basis of their voltage-sensitivity, Ca^{2+}-dependence and pharmacology. In molluscan neurones (Thompson, 1977), cultured rat hippocampal neurones (Segal *et al.*, 1984), and cultured neurones of the rat supraoptic nucleus area (Cobbett *et al.*, 1989), the extracellular application of TEA and 4-AP mainly attenuated the I_K and I_A, respectively. The extracellular application of Co^{2+}-, Mn^{2+}-, Cd^{2+}-, La^{3+}-, or organic Ca^{2+} blockers inhibited the I_{KCa} in *Helix aspersa* neurones (Meech & Standen, 1972), molluscan neurones (Thompson, 1977), hippocampal neurones of the guinea-pig (Brown & Griffith, 1983), and rat supraoptic nucleus neurosecretory neurones (Bourque *et al.*, 1985; Bourque, 1988). The I_M was depressed by adding Ba^{2+} to the bathing medium (Halliwell & Adams, 1982). The I_{AR} in hippocampal neurones was affected by the Na^+-free external solution or by the external solution containing Cs^+ (Halliwell & Adams, 1982; Segal & Barker, 1984). In the present preparation, the I_{Str} was not sensitive to extra- and intracellular Ca^{2+} but was suppressed by K^+ channel blockers in the order: Ba^{2+} > 4-AP > TEA. Moreover, there was a linear relationship between the Str-induced K^+ current and the holding potential, indicating that I_{Str} has no voltage-dependence. Interestingly, Str-binding sites exist in the membrane fraction associated with the glycine receptor (Young & Snyder, 1974). However, the present preparation has no glycine receptors, although Str increases K^+ conductance which is sensitive to K^+ channel blockers. Thus, the results suggest that the Str-induced K^+ current is probably due to activation of the Str receptor-K^+ channel complex which has no cooperative interaction with the glycine receptor.

It is of interest that Str acts not only as an antagonist to block the glycine- and GABA-induced I_{Cl} (Curtis *et al.*, 1968; 1971; Ryall *et al.*, 1972; Barron & Guth, 1987; Yakushiji *et al.*, 1987; Krishtal *et al.*, 1988; Akaike & Kaneda, 1989; Kaneda *et al.*, 1989) but also as an agonist to activate K^+ channels. However, at this moment, the functional role of the increase in K^+ conductance in DRG cells induced by Str itself is obscure. Further studies will be necessary to elucidate the mechanism or functional role of the Str-induced K^+ current.

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas (Nos. 02223105 and 02241101) and the Epilepsy Research Foundation to N.A.

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(Received June 18, 1990
Revised September 14, 1990
Accepted September 27, 1990)

Br. J. Pharmacol. (1991), **102**, 497–505 © Macmillan Press Ltd, 1991

Thromboxane (Tx) A_2 receptor blockade and TxA_2 synthase inhibition alone and in combination: comparison of anti-aggregatory efficacy in human platelets

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1 The present study has compared the relative anti-aggregatory effect of various compounds which interfere with thromboxane (Tx) A_2-dependent aggregation of human platelets in whole blood *in vitro*. These included the cyclo-oxygenase inhibitor aspirin, the TxA_2 synthase inhibitor dazoxiben, the TxA_2 (TP-) receptor blocking drug GR32191 and two compounds, R.68070 ((E)-5-[[[(3-pyridinyl) [3-(trifluoromethyl)phenyl]-methylen] amino]oxy] pentanoic acid) and CV-4151 ((E)-7-phenyl-7-(3-pyridyl)-6-heptenoic acid), which possess both TP-receptor blocking and TxA_2 synthase inhibitory activities in the same molecule.

2 GR32191, R.68070 and CV-4151 all antagonized aggregation to the TxA_2 mimetic U-46619, with pA_2 values of approximately 8.2, 5.4 and 4.8 respectively. This effect was specific, platelet aggregation induced by adenosine 5'-diphosphate (ADP) being unaffected by concentrations up to 10, 1000 and 300 μM respectively. In contrast, neither aspirin nor dazoxiben exhibited any measurable TP-receptor blocking activity.

3 The rank order of potency (pIC_{50}) for inhibition of TxA_2 formation in serum was R.68070 (7.4) > CV-4151 (6.9) > dazoxiben (5.7) > aspirin (5.3). In addition, all four drugs abolished collagen-induced platelet TxA_2 formation. In contrast, GR32191 produced no consistent inhibition of TxA_2 formation in either system up to concentrations of 10–30 μM.

4 The specificity of R.68070, CV-4151 and dazoxiben for TxA_2 synthase was indicated by their ability to increase serum levels of prostaglandin E_2 (PGE_2) and PGD_2 in parallel with decreases in TxA_2 formation. This profile was not observed with aspirin or GR32191. However, high concentrations of R.68070 (100 μM) and CV-4151 (1000 μM) necessary for maximum TP-receptor blocking activity, produced substantially smaller increases in PGE_2 and PGD_2, consistent with an aspirin-like effect of these compounds upon cyclo-oxygenase. With dazoxiben (1000 μM), PGE_2 and PGD_2 levels remained elevated.

5 Aspirin inhibited collagen-induced platelet aggregation, the effect correlating with inhibition of TxA_2 formation. Dazoxiben, whilst also achieving maximal inhibition of TxA_2 formation, produced significantly less inhibition of aggregation than aspirin. In contrast, GR32191 (0.1–10 μM), at concentrations specific for TP-receptor blockade, produced a significantly greater antagonism of collagen-induced platelet aggregation than aspirin. This additional effect of GR32191 was absent in platelets pretreated with aspirin, indicating the probable involvement of an endogenous anti-aggregatory cyclo-oxygenase product in response to collagen stimulation.

6 R.68070 and CV-4151 also inhibited collagen-induced aggregation, with very high concentrations of R.68070 (100 μM) producing an effect equivalent to that of GR32191.

7 In contrast, the combination of GR32191 with either dazoxiben, R.68070 or CV-4151, at concentrations specific for TxA_2 synthase, produced a synergistic inhibitory effect upon collagen-induced platelet aggregation which was greater than that achieved with either aspirin or any of the compounds used alone. Pretreatment of platelets with aspirin reversed this synergistic effect, consistent with it being dependent upon the formation and action of anti-aggregatory prostaglandins.

8 In conclusion, the present study has confirmed the superior platelet inhibitory profile of a combination of a TP-receptor blocking drug and a TxA_2 synthase inhibitor to that of either activity alone. However, the maximum inhibitory effect of the currently available compounds, R.68070 and CV-4151, which possess both activities in the same molecule, appears to be no greater *in vitro* than that obtained with the potent TP-receptor blocking drug, GR32191. This most probably reflects the inhibition by R.68070 and CV-4151 of platelet cyclo-oxygenase at the concentrations required for effective TP-receptor blockade which results in a reduction in the formation of anti-aggregatory prostanoids.

Introduction

Thromboxane A_2 (TxA_2) aggregates human platelets and constricts vascular smooth muscle (Hamberg *et al.*, 1975) through stimulation of specific thromboxane (TP-) receptors (Kennedy *et al.*, 1982). Evidence has accumulated which implicates it in the pathophysiology of thromboembolic diseases such as unstable angina, atherosclerosis and pulmonary embolism (Green & Vesterqvist, 1986; Fitzgerald *et al.*, 1987; Catella *et al.*, 1987). Thus, prevention of the actions of TxA_2 should provide an effective anti-thrombotic approach and various classes of pharmacological agent have been developed to achieve this goal.

Aspirin irreversibly acetylates cyclo-oxygenase (Roth *et al.*, 1975) and thus prevents formation of platelet-derived TxA_2. However, the beneficial inhibition of TxA_2 formation with aspirin may be compromised by the concomitant reduction in the formation of the anti-aggregatory prostaglandins (PG)D_2 and I_2, representing an important limitation of this class of anti-thrombotic drug. An alternative approach for reducing TxA_2 formation was through the development of specific inhibitors of the enzyme, thromboxane synthase (Vermylen *et*

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al., 1981). Dazoxiben (Tyler et al., 1981), an example of this class of drug, as a result of preventing TxA_2 formation, redirects PGH_2 metabolism towards increased formation of PGI_2 and PGD_2 (Defreyn et al., 1982; Orchard et al., 1985; Gresele et al., 1987). It is now recognised, however, that thromboxane synthase inhibition causes the accumulation of PGH_2, which is equipotent with TxA_2 at platelet TP-receptors (Le Breton et al., 1979; Hornberger & Patscheke, 1989) and can itself produce platelet aggregation and granule secretion (Bertele et al., 1981; Hornby & Skidmore, 1982). This effect of PGH_2 therefore counteracts the benefits arising from the redirection to anti-aggregatory prostanoids and limits the effectiveness of thromboxane synthase inhibitors (Vermylen et al., 1985). Yet a further strategy has focussed upon directly antagonizing the effects of TxA_2 by blocking its action at TP-receptors (Humphrey et al., 1990). In this respect, GR32191 (Figure 1) has been identified as a potent TP-receptor blocking drug upon human platelets and smooth muscle *in vitro* (Lumley et al., 1989) and is orally active with a long duration of action in man (Thomas & Lumley, 1990). In contrast to aspirin, which reduces PGD_2 and PGI_2 formation, GR32191 importantly preserves formation of these anti-aggregatory prostanoids (Hornby et al., 1989; Takahara et al., 1990).

Several lines of evidence now suggest that a superior anti-thrombotic profile can be achieved by combining a TP-receptor blocking drug and a thromboxane synthase inhibitor (Gresele et al., 1984). For example, in human platelet-rich plasma *in vitro*, the TP-receptor blocking drug, BM 13.177, markedly potentiates the anti-aggregatory activity of dazoxiben (Bertele & De Gaetano, 1982). Similarly, in an *in vivo* canine model of coronary thrombosis, the combination of a TP-receptor blocking drug with a thromboxane synthase inhibitor produced a substantially greater inhibition than that achieved with either compound alone, consistent with a pro-aggregatory effect of PGH_2 limiting the effectiveness of thromboxane synthase inhibitors *in vivo* (Fitzgerald et al., 1988). This latter effect has also been observed clinically, where the combined oral administration of BM 13.177 and dazoxiben to human volunteers produced inhibition of *ex vivo* collagen- and arachidonic acid-induced platelet aggregation and a prolongation of the bleeding time which were significantly greater than achieved following either treatment alone (Gresele et al., 1987). To date, the evidence for achieving a superior anti-thrombotic profile by combining a thromboxane synthase inhibitor with a TP-receptor blocking drug has been obtained by use of two separate chemical entities. Recently, two compounds, R.68070 (De Clerck et al., 1989) and CV-4151 (Imura et al., 1988) have been described which combine specific thromboxane synthase inhibitory and TP-receptor blocking activities in a single molecule (Figure 1). The aim of the present study, therefore, was to investigate whether R.68070 and CV-4151 were superior to GR32191 as inhibitors of platelet aggregation *in vitro*. The anti-aggregatory activity of dazoxiben and aspirin has also been examined for comparative purposes.

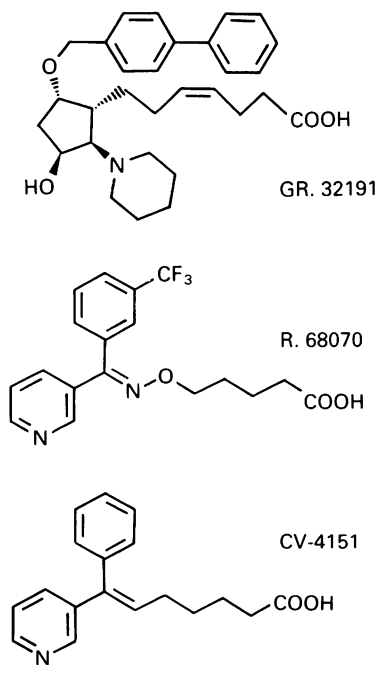


Figure 1 The chemical structures of GR32191, R.68070 and CV-4151.

Methods

Preparation of platelets

Human blood was collected from healthy male volunteers and anti-coagulated with trisodium citrate as described previously (Keery & Lumley, 1988). Citrated whole blood was centrifuged to obtain platelet-rich plasma (PRP; Lumley et al., 1989) for the preparation of human resuspended platelets in a Krebs-Henseleit buffer solution (Keery & Lumley, 1988).

Platelet aggregation

Platelet aggregation was quantified by monitoring the fall in single platelet count, produced by aggregatory agents in aliquots of whole blood or resuspended platelets, with an Ultra-Flo 100 (Becton & Dickinson) whole blood platelet counter (Lumley & Humphrey, 1981; Keery & Lumley, 1988). Unless otherwise stated, blood samples were treated with aspirin (2 mM) during the aliquoting procedure, to prevent any TxA_2 which might be formed contributing to the aggregatory response to exogenous agonists. Aliquots were placed in a shaking water bath at 37°C and incubated for 30 min before experimentation. In each sample, duplicate platelet counts were recorded before (control) and at intervals following addition of an aggregating agent until a nadir in the platelet count was achieved (Lumley & Humphrey, 1981; Keery & Lumley, 1988). The maximum fall in platelet count in each aliquot was expressed as a percentage of the control count to give percentage platelet aggregation. EC_{50} values (the concentration of agonist required to produce a 50% fall in platelet count) were determined by interpolation from semi-logarithmic plots of percentage aggregation versus concentration of agonist.

TP-receptor blocking activity on platelets

The potency of R.68070, CV-4151, dazoxiben and aspirin as TP-receptor blocking drugs was quantified in both whole blood, and in some experiments in resuspended platelets, by use of the TxA_2 (TP-receptor) agonist, U-46619 as described previously (Lumley et al., 1989). The effect of these agents was also investigated against collagen-induced aggregation. Platelet aggregation to collagen is mediated both by TxA_2 formation and adenosine diphosphate (ADP) release (Kinlough-Rathbone et al., 1977). The extent of the TxA_2 component was determined by constructing collagen concentration-effect curves (0.1–4.0 $\mu g\,ml^{-1}$) in the presence and absence of aspirin (2 mM). Further curves were then constructed in aliquots, in the presence of either GR32191, R.68070, CV-4151, dazoxiben or aspirin, or combinations of these compounds following a 10 min pre-incubation period. The inhibitory effect of each compound upon TxA_2 (measured as TxB_2) formation (see below) as well as aggregation was determined. The specificity of R.68070 (1000 μM), CV-4151 (300 μM), dazoxiben (100 μM) and aspirin (2 mM) was examined against ADP, by carrying out aggregation concentration-effect curves in the absence

(control) or presence of a single concentration of each compound.

In all experiments, inhibition of platelet aggregation, reflected as rightward displacements of agonist concentration-effect curves, was expressed in the form of concentration-ratios (CR values). These were derived by dividing the EC_{50} for an agonist in the presence of a treatment by that obtained in its absence. Interaction between U-46619 and TP-receptor blocking drugs was analysed by the method of Arunlakshana & Schild (1959) to obtain pA_2 values.

Thromboxane synthase inhibitory activity

The inhibition of human platelet TxA_2 synthase was assessed by measuring TxA_2 formation in whole blood, as its metabolically stable breakdown product TxB_2, using radioimmunoassay. The specificity of inhibitors for thromboxane synthase was assessed by determining, also by specific radioimmunoassay, the serum prostaglandin E_2 (PGE_2) and PGD_2 levels.

TxB_2, PGE_2 and PGD_2 formation in clotting blood Non-anti-coagulated human blood was rapidly divided into 0.5 ml aliquots in glass tubes each containing a single concentration of either R.68070, CV-4151, dazoxiben or aspirin (1 nM–1 mM), GR32191 (30 nM–300 μM) or, the corresponding vehicle equivalent to the highest concentration of each compound tested. Tubes were vortexed for approximately 2 s to ensure adequate mixing of the compound with the blood and then incubated in a water bath at 37°C for 60 min to allow complete clot formation. Thirty minutes into the incubation period, the clot was released from the wall of the tube with a metal rod to enable clot retraction to occur. Upon completion of the incubation period, blood samples were centrifuged at 900 *g* for 15 min at 25°C (MSE Mistral 3000) to obtain serum.

Collagen-induced TxB_2 formation The effect of GR32191, R.68070, CV-4151, dazoxiben and aspirin was also determined upon TxB_2 formation induced by addition of collagen (4 $\mu g\,ml^{-1}$) to human citrated whole blood. The samples used were taken from aggregation experiments. Following measurement of peak aggregation, blood samples were treated with indomethacin (5 μM) to prevent any further TxB_2 formation and placed on ice (2–4°C). Samples were then transferred to Eppendorf tubes and centrifuged at 5600 *g* for 3 min at 25°C (Eppendorf Model No. 5414) to obtain plasma.

Preparation of serum and plasma samples for radioimmunoassay Aliquots (100 μl) of serum or plasma were transferred to Eppendorf tubes and 500 μl of absolute ethanol added to precipitate plasma proteins. The tubes were then capped, vortexed for 3 s and centrifuged at 5600 *g* for 3 min at 25°C (Eppendorf Model No. 5414) to remove the protein precipitate. Approximately 400 μl of the alcoholic supernatant was then carefully removed and transferred to a plastic container (Sarstedt) and stored at −20°C. Recovery of [^{3}H]-TxB_2, [^{3}H]-PGE_2 and [^{3}H]-PGD_2 added to serum or plasma and taken through this process was >97%.

Subsequently, TxB_2, PGE_2 and PGD_2 levels were determined by specific radioimmunoassay and expressed in $ng\,ml^{-1}$ of serum or plasma. For calculation of inhibitory potency, TxB_2 formation at each concentration of a compound was expressed as a percentage of the maximum produced in corresponding vehicle-treated control blood samples. The molar concentration of inhibitor required to produce 50% inhibition of TxB_2 formation was derived graphically by interpolation and expressed logarithmically (pIC_{50}).

Expression of results

Data are expressed as either the arithmetic or geometric mean values with 95% confidence intervals, or as the arithmetic mean value ± s.e.mean from (n) experiments. Individual data were compared by means of a paired Student's t test where $P < 0.05$ was taken to indicate a significant difference.

Drugs used

R.68070 (10 mM; (E)-5-[[[(3-pyridinyl) [3-(trifluoromethyl) phenyl]-methylen]amino]oxy]pentanoic acid; Janssen) and CV-4151 (10 mM; (E)-7-phenyl-7-(3-pyridyl)-6-heptenoic acid; Glaxo Group Research Limited) were dissolved in 10% w/v sodium bicarbonate solution and 0.9% w/v sodium chloride solution (saline) added to give a final bicarbonate concentration of 1%. Subsequent dilutions were made with saline. Dazoxiben hydrochloride (10 mM; Pfizer Central Research) was dissolved in saline and further dilutions prepared with the same vehicle. Equine collagen tendon (1 $mg\,ml^{-1}$, Hormon-Chemie) was used as a suspension and dilutions prepared in the buffer (pH 2.8) provided. Absolute ethanol (99.7% v/v; James Burrough, Essex) was used for the precipitation of proteins in human plasma and serum. Details of the preparation of U-46619 (11,9 epoxymethano-PGH_2), GR32191, ADP, indomethacin, aspirin and trisodium citrate are given elsewhere (Keery & Lumley, 1988; Lumley *et al.*, 1989). The value in parentheses after a drug name refers to the concentration of stock solution prepared. In the text, concentrations of drugs are expressed as those in whole blood or resuspended platelets. All drugs were kept on ice during an experiment.

Radioimmunoassay materials included [^{3}H]-TxB_2 (100 $Ci\,mmol^{-1}$; New England Nuclear, Southampton, Hants) and [^{3}H]-PGE_2 (184 $Ci\,mmol^{-1}$; Amersham International Plc, Amersham). Non-radioactive standard TxB_2 (1 $mg\,ml^{-1}$) and PGE_2 (1 $mg\,ml^{-1}$) were obtained from Upjohn Diagnostics, Michigan, USA. Lyophilised antisera (rabbit) to TxB_2 (Metachem Diagnostics Ltd, Northampton) and PGE_2 (ICN Biomedicals Ltd, High Wycombe, Bucks) were used. PGD_2 measurements were made with a commercially available assay kit (Amersham International Plc, Amersham).

Results

Antagonism of U-46619-induced human platelet aggregation in whole blood and resuspended platelets

In human citrated whole blood, U-46619 (0.03–1.0 μM) produced concentration-dependent platelet aggregation, as measured by the disappearance of single platelets, with a mean (95% confidence interval) EC_{50} value of 0.18 (0.12–0.25 μM; $n = 14$). GR32191 (0.01–10 μM), R.68070 (10–100 μM) and CV-4151 (10–300 μM) all antagonized U-46619-induced aggregation producing concentration-related parallel rightward displacements of the agonist concentration-effect curve. As reported previously (Lumley *et al.*, 1989), aggregation to U-46619 in the presence of GR32191 was slowed compared with controls and the maximum effect was suppressed. However, neither of these effects was observed with R.68070 or CV-4151.

Analysis of the data yielded apparent pA_2 values which indicated the potency of GR32191 to be some 500 and 3000 times greater than R.68070 and CV-4151 respectively (Table 1). Slopes of the Schild regression not significantly different from unity indicated a surmountable, competitive interaction between U-46619 and both R.68070 and CV-4151, but not with GR32191 (Table 1). In contrast to the effects of these compounds, dazoxiben (100–300 μM) and aspirin (2 mM) were without effect upon U-46619-induced aggregation, with mean CR values of 2.0 (1.1–3.7; $n = 3$) and 0.9 (0.6–1.4; $n = 4$) respectively.

pA_2 values were also determined in resuspended platelets. Analysis of the data yielded mean pA_2 values of 8.8 (8.6–8.9) for GR32191 and 5.7 (4.9–6.6) and 5.2 (4.5–5.8) for R.68070 and CV-4151 respectively. Thus, whilst in the absence of plasma proteins the potency of each compound was increased

Table 1 A comparison of the TP-receptor blocking activity and the thromboxane A_2 synthase inhibitory activity of GR32191, R.68070, CV-4151, dazoxiben and aspirin upon human platelets in whole blood *in vitro*

	Antagonism of U-46619-induced aggregation			*Inhibition TxB_2 formation in clotting blood*	
Compound	pA_2[a]	*Slope*[a]	(n)	pIC_{50}[b]	(n)
GR32191	8.2 (7.9–8.6)	1.3 (1.1–1.5)	(6)	<3.5	(5)
R.68070	5.4 (5.2–5.6)	1.1 (0.8–1.3)	(4)	7.4 (7.2–7.6)	(9)
CV-4151	4.8 (4.3–5.3)	1.1 (0.7–1.6)	(4)	6.9 (6.7–7.1)	(9)
Dazoxiben	<3.5	—	(3)	5.7 (5.5–5.9)	(4)
Aspirin	<2.7	—	(3)	5.3 (5.1–5.4)	(5)

Values are [a] arithmetic and [b] geometric means (95% confidence interval) from (*n*) experiments.

by some 2–3 fold over that in whole blood (Table 1), the relative potencies were unaffected.

In both whole blood and in resuspended platelets, GR32191 (10 μM), R.68070 (100–1000 μM), CV-4151 (100–300 μM), dazoxiben (100 μM) and aspirin (2 mM) alone were without effect upon ADP (0.3–3.0 μM)-induced aggregation (mean CR values of <2 for each drug; $n = 4$), nor did they produce any direct effect on the baseline platelet count.

Inhibition of TxB_2 formation in human clotting whole blood

In human clotted whole blood, the mean serum TxB_2 formation was 250 ± 22 ng ml^{-1} ($n = 10$). When blood was clotted in the presence of R.68070, CV-4151, dazoxiben or aspirin (1 nM–1 mM), each compound produced a concentration-related inhibition of serum TxB_2 formation. All compounds were able to abolish TxA_2 formation with the minimum concentration required for a maximal effect (mean inhibition >95%) being obtained at 1 μM R.68070, 10 μM CV-4151, 10 μM dazoxiben and 100 μM aspirin. pIC_{50} values determined from the inhibition curves indicated that R.68070 was some three time more potent than CV-4151, which in turn was at least ten times more potent than dazoxiben in inhibiting serum TxB_2 formation (Table 1). Aspirin was approximately three times weaker than dazoxiben (Table 1). In marked contrast GR32191, up to concentrations of 30 μM, was without effect on TxB_2 formation (12 ± 6% inhibition; $n = 5$). Even at a concentration of 300 μM, a mean ($n = 5$) inhibition of TxB_2 formation of only 39 ± 7% was observed. Thus, the rank order of potency for inhibiting TxB_2 formation in human clotting blood was R.68070 > CV-4151 > dazoxiben > aspirin ≫ GR32191, with R.68070 and CV-4151 being some three to four orders of magnitude more potent than GR32191.

Since inhibition of serum TxB_2 production could be produced by inhibition of either TxA_2 synthase or cyclooxygenase, the enzymic specificity of each compound was determined by measuring both serum PGE_2 and PGD_2 in parallel with TxA_2 (TxB_2). In the absence of an inhibitor (vehicle-treated blood), mean ($\pm$s.e.mean; $n = 9$) serum levels of PGE_2 and PGD_2 were 14.2 ± 5.5 and 5.5 ± 1.4 ng ml^{-1} respectively. Over the concentration range of each compound producing inhibition of TxB_2 (Figure 2), R.68070, CV-4151 and dazoxiben produced a progressive increase in the serum levels of PGE_2 and PGD_2 which mirrored the corresponding reduction in TxB_2 formation (Figure 2). The maximum increases in PGE_2 and PGD_2 were fairly comparable between compounds, and were approximately ten and five fold greater respectively than those measured in the absence of the compounds (Figure 2). At concentrations of R.68070 and CV-4151 supramaximal for inhibition of TxB_2 formation (100 and 1000 μM), no further increase in either PGE_2 or PGD_2 formation occurred, rather the levels of PGE_2 and PGD_2 were substantially reduced (Figure 2). For example, at 100 and 1000 μM CV-4151, PGE_2 formation was reduced by 50 and 95% respectively of the mean level measured in the presence of 1 μM of the compound. A similar profile was obtained with R.68070 (Figure 2). However, in the presence of dazoxiben, even at concentrations as high as 1000 μM, PGE_2 and PGD_2 remained elevated. In contrast to the thromboxane synthase inhibitors, aspirin and GR32191 did not increase the serum concentrations of either PGE_2 or PGD_2 (Figure 2).

Effect of GR32191, R.68070, CV-4151, dazoxiben and aspirin upon collagen-induced platelet aggregation and TxB_2 formation

Collagen (0.1–40 μg ml^{-1}) added to human citrated whole blood produced concentration-related platelet aggregation (mean EC_{50} in the range 0.5–0.7 μg ml^{-1}) and increases in TxB_2 formation (Figure 3a). However, whilst near maximal aggregation to collagen was observed at 4 μg ml^{-1}, TxB_2 production continued to increase reaching 312 ± 22 ng ml^{-1} ($n = 11$) at 40 μg ml^{-1} collagen (Figure 3a). Over the concentration range 2 μM–2 mM, aspirin produced a progressive inhibition of collagen-induced TxB_2 formation with a maximum effect at 20 μM and a mean pIC_{50} of 5.5 (5.2–5.8; $n = 5$). This effect was associated with a progressive rightward displacement of the collagen aggregation concentration-effect curve, with mean CR values of 1.3 (0.7–2.4; $n = 3$) and 4.0 (2.5–6.3; $n = 3$) obtained at 2 and 20 μM aspirin. The latter effect was not significantly increased by the use of higher concentrations of aspirin (mean ($n = 3$) CR values at 200 μM and 2 mM were 4.2 (3.2–5.6) and 4.1 (2.4–7.1) respectively). Aspirin therefore fully reveals the extent of the PGH_2/TxA_2-mediated component in collagen-induced aggregation.

In contrast to aspirin, GR32191 up to a concentration of 10 μM, produced no consistent inhibition of collagen (0.4–40 μg ml^{-1})-induced TxB_2 formation (Figure 3b). Despite this, GR32191 (0.01–10 μM) produced concentration-related rightward displacements of the collagen aggregation-concentration effect curve. At concentrations of 0.1–10 μM, the inhibitory effect of GR32191 was significantly greater ($P < 0.05$) than that of aspirin (Figure 3b; Table 2). This additional effect of GR32191 was absent if platelets were pre-exposed to aspirin (2 mM), the mean CR value of 5.3 (3.3–8.7; $n = 4$) for GR32191 (10 μM) in the presence of aspirin then being not significantly different from the effect obtained with aspirin alone (4.1(3.6–4.7); $n = 4$) in this sub-group of experiments.

Unlike GR32191, R.68070 (0.1 μM), CV-4151 (1 μM) and dazoxiben (1 μM) each produced substantial (>90%) inhibition of collagen-induced TxB_2 formation. Associated with this inhibition was a rightward displacement of the collagen-induced aggregation curve (Table 2). However, even at concentrations abolishing TxA_2 formation (mean inhibition >98% obtained at 1 μM R.68070, 10 μM CV-4151 and 10 μM dazoxiben) all three compounds produced maximum CR values of <3.0 which was significantly less than that produced

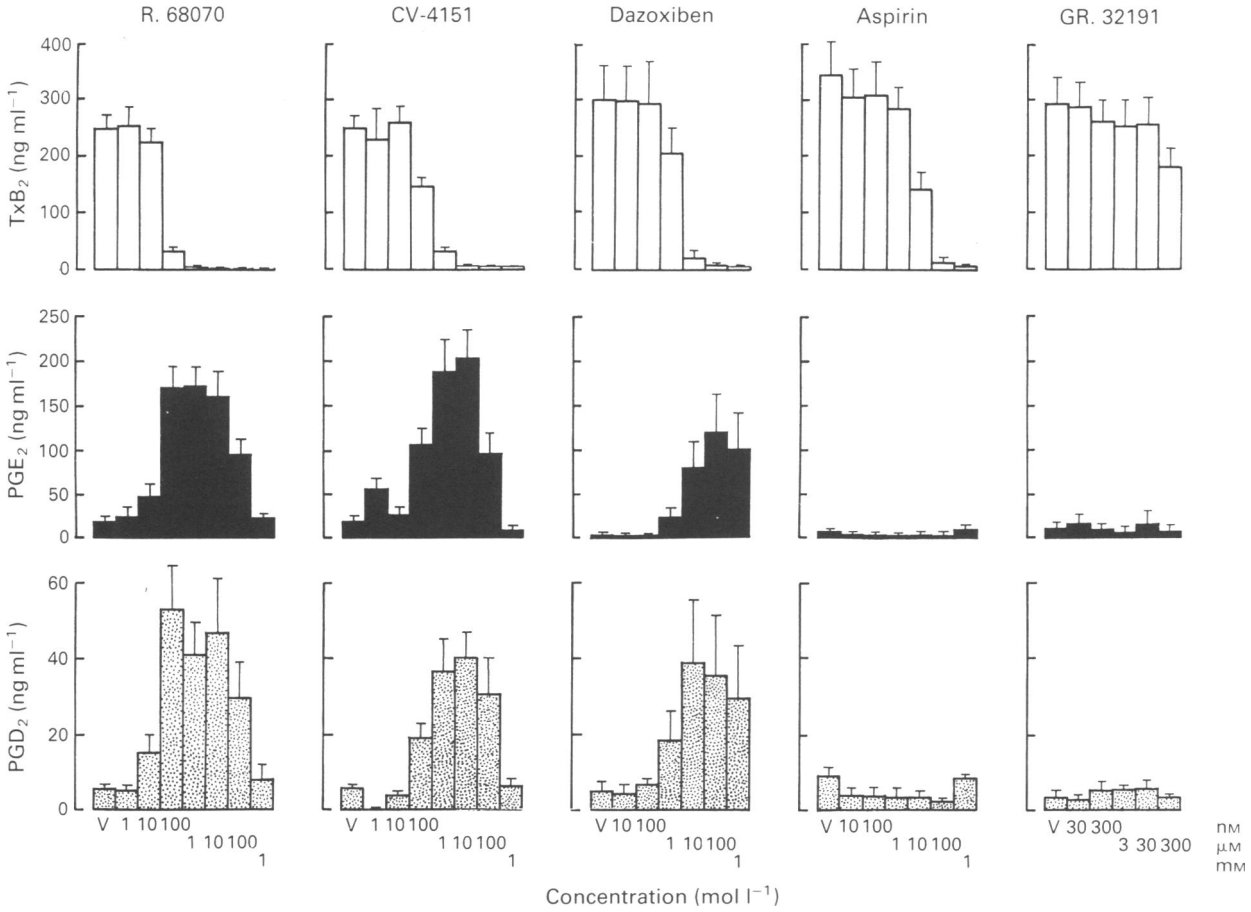


Figure 2 The effects of R.68070, CV-4151, dazoxiben, aspirin and GR32191 upon the concentration (ng ml^{-1} serum) of thromboxane B_2 (TxB_2), prostaglandin E_2 (PGE_2) and PGD_2 in human clotting whole blood. Columns show the mean from at least 3 experiments for each compound; vertical bars show s.e.mean. Responses are shown in the presence of increasing concentrations of each drug or vehicle (V) equivalent to the highest drug concentration.

by a maximally effective concentration of aspirin (Table 2). When higher concentrations (up to 100 μM) of R.68070 and CV-4151 were used, a second 'phase' of inhibition of collagen-induced aggregation was seen, the effect eventually exceeding that produced by aspirin, and in the case of R.68070, equalling that produced by GR32191 (Table 2). However, upon further increases in the concentration of R.68070 (1000 μM), the inhibitory effect upon collagen-induced aggregation was reversed towards that obtained with aspirin (Table 2).

Effect of combining GR32191 with either dazoxiben, R.68070 or CV-4151 upon collagen-induced platelet aggregation

The combination of TP-receptor blocking and thromboxane synthase inhibitory activities in both R.68070 and CV-4151 failed to improve upon the inhibitory action of a TP-receptor blocking drug alone (Table 2). It was therefore decided to test the 'combination' hypothesis by using separate drug entities to

Table 2 A comparison of the effect of GR32191, R.68070, CV-4151 and dazoxiben with that of aspirin upon collagen-induced platelet aggregation in human whole blood

	Collagen concentration-ratio[a] (CR) *Drug concentration* (μM)						
Drug	*Aspirin*[b] (2 mM)	0.01	0.1	1.0	10	100	1000
GR32191	4.1 (3.7–4.6)	1.8[c] (1.3–2.5)	4.9* (4.3–5.6)	7.2* (5.7–9.1)	6.0* (5.1–7.0)	NT	NT
R.68070	4.0 (3.2–5.0)	NT	2.4[c] (2.0–2.9)	2.5 (2.0–3.0)	4.8 (3.6–6.6)	7.3* (5.1–10.4)	4.7[c] (1.9–11.6)
CV-4151	4.3 (3.2–5.7)	1.4 (0.9–2.0)	1.9 (1.5–2.3)	2.1 (1.6–2.8)	2.7 (2.3–3.1)	5.4 (3.9–7.4)	NT
Dazoxiben	3.8 (3.2–4.6)	1.1 (0.8–1.6)	1.0[c] (0.9–1.04)	1.8 (1.5–2.3)	2.1 (1.8–2.4)	2.9[c] (1.8–4.8)	NT

[a] Collagen CR values are expressed as the geometric mean (95% confidence intervals) where values represent data from at least 10 experiments unless indicated otherwise ([c] = 3–6 experiments). [b] Data for aspirin are shown for each separate batch of experiments. NT = not tested.

* Significantly greater than aspirin ($P < 0.05$).

For each drug all values are compared with the corresponding aspirin value by means of Student's paired t test.

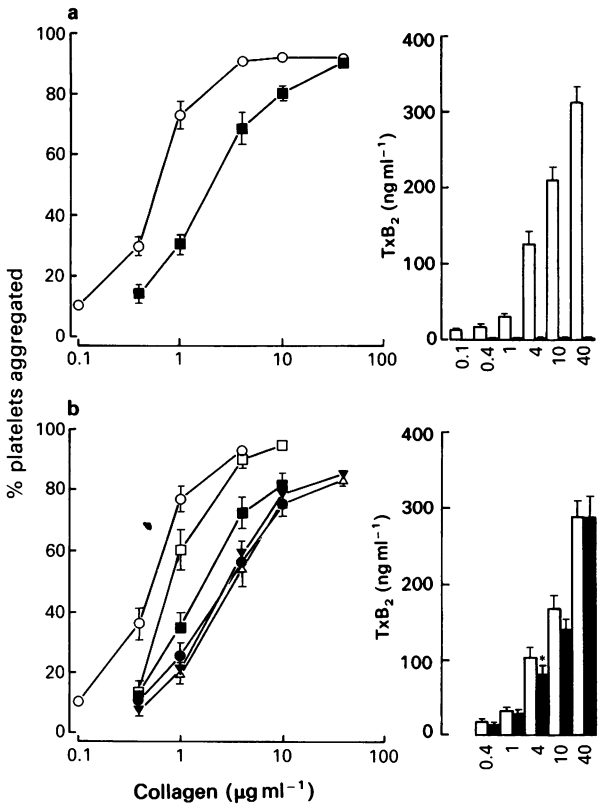


Figure 3 The effect of (a) aspirin and (b) GR32191 upon collagen-induced platelet aggregation and thromboxane B_2 (TxB_2) formation in human whole blood. In (a), the control concentration-effect curve (○) and that in the presence of aspirin (2 mM; ■) are the mean from 11 experiments with s.e.mean shown by vertical bars; the effect of aspirin (filled columns) upon control collagen-induced TxB_2 production (open columns) is taken from the same experiments. In (b), the effect of GR32191 (0.01, □; 0.1, ●; 1.0, ▼ and 10 μM, △) upon collagen-induced platelet aggregation are the mean from at least 6 experiments. The control and aspirin symbols are as shown in panel (a). The effect of GR32191 (10 μM; filled columns) upon collagen-induced TxB_2 production (open columns) are taken from a separate ($n = 11$) series of experiments where vertical bars represent s.e.mean. * Significantly reduced compared to control ($P < 0.05$).

optimise the required level of each activity. Thus, a maximally effective, but specific, TP-receptor blocking concentration of GR32191 (10 μM) was combined with a maximally effective, but specific (see Figure 2), TxA_2 synthase inhibitory concentration of either dazoxiben (10 μM), R.68070 (1 μM) or CV-4151 (10 μM). The combination of GR32191 and dazoxiben resulted in a superior effect to aspirin or to either compound alone (Figure 4), a mean collagen CR value of 13.4 being obtained (Table 3). Similar effects were seen with both R.68070 and CV-4151 in combination with GR32191 (Table 3). The same

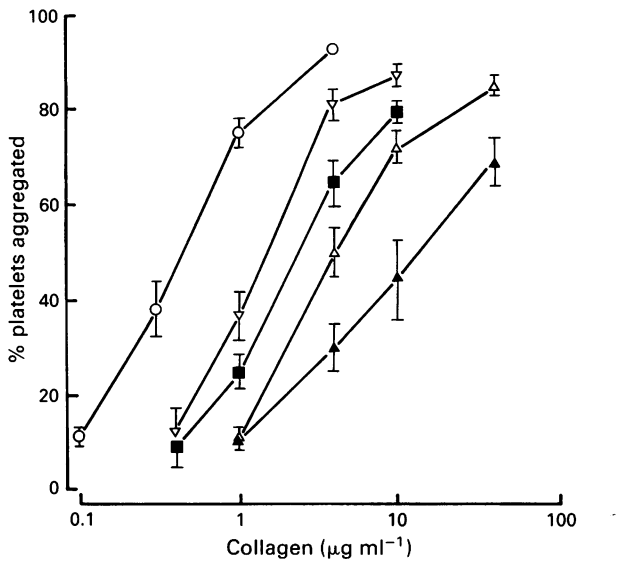


Figure 4 The effect of a combination of dazoxiben (10 μM) and GR32191 (10 μM) on collagen-induced platelet aggregation in human whole blood. Responses are shown in the absence (○) and presence of aspirin (■, 2 mM), dazoxiben (▽), GR32191 (△) and a combination of dazoxiben and GR32191 (▲). Each point is the mean of 4–6 determinations where vertical bars represent s.e.mean.

magnitude of inhibitory effect was also obtained even when a 100 fold lower concentration of GR32191 (0.1 μM) was combined with dazoxiben (10 μM) (mean collagen CR value 13.6 (7.9–23.2; $n = 8$)). The 'combination' CR values were not significantly different from each other, but significantly different ($P < 0.05$) from the CR value produced either by each compound alone, or that produced by aspirin (Table 3). In platelets pre-incubated with aspirin (2 mM), the superior effect of a combination of GR32191 with either R.68070 or dazoxiben was absent, the collagen concentration-ratios for the combination then being similar to those obtained with aspirin alone.

Discussion

The present study has compared the relative effectiveness of various drugs upon TxA_2-induced human platelet aggregation *in vitro*. The drugs studied, which interfere by different mechanisms with the action of TxA_2, were the TP-receptor blocking drug GR32191, the TxA_2 synthase inhibitor dazoxiben, the cyclo-oxygenase inhibitor aspirin and two compounds with both TP-receptor blocking and TxA_2 synthase inhibitory activity in the same molecule, namely R.68070 and CV-4151. Examination of the ability of each drug to block platelet TP-receptors and inhibit platelet TxA_2 synthase revealed a relatively high degree of specificity. Thus, GR32191 antagonized platelet aggregation induced by U-46619, but not that induced

Table 3 A comparison of the effect of GR32191 alone and in combination with either dazoxiben, R.68070 or CV-4151 upon collagen-induced platelet aggregation in human whole blood

TxSI (μM) used in combination with GR32191	*Collagen concentration-ratio (CR)* *Aspirin (2 mM)*	*GR32191 (10 μM)*	*TxSI alone*	*TxSI + GR32191*
Dazoxiben (10)	4.2 (3.8–4.7)	6.0 (5.1–7.0)	2.1 (1.8–2.4)	13.4* (11.1–16.0)
R.68070 (1.0)	4.5 (3.5–5.7)	6.2 (3.6–10.4)	2.4 (1.8–3.1)	16.1* (9.9–26.0)
CV-4151 (10)	5.3 (3.4–8.4)	6.7 (4.1–10.8)	2.5 (2.1–2.9)	14.3* (7.2–28.3)

Collagen CR values shown are geometric mean values (95% confidence interval) from at least 8 experiments. TxSI = TxA_2 synthase inhibitor.
* Significantly greater than either aspirin or each drug alone ($P < 0.05$).

by ADP. GR32191 was the most potent TP-receptor blocking drug tested ($pA_2 = 8.2$), being some 500 and 3000 times more potent than R.68070 and CV-4151 respectively in human whole blood. Despite their low potency, R.68070 and CV-4151 were specific platelet TP-receptor antagonists. In contrast, dazoxiben displayed no antagonist effect against U-46619. In terms of TxA_2 synthase inhibitory activity, R.68070 was the most potent compound tested ($IC_{50} = 40$ nM), being 3 times more active than CV-4151 and 30 times more active than dazoxiben. Both the TxA_2 synthase inhibitory and TP-receptor blocking potencies of these drugs agree well with the published data (Terashita *et al.*, 1986; Imura *et al.*, 1988; De Clerck *et al.*, 1989). All three compounds were specific for thromboxane synthase, since concentrations of PGE_2 and PGD_2 increased as TxB_2 levels decreased. However, at high concentrations of both R.68070 and CV-4151 (100 μM and above), PGE_2 and PGD_2 formation were also inhibited indicating a possible action of these two compounds at the cyclo-oxygenase enzyme. In contrast, GR32191 displayed negligible TxA_2 synthase inhibitory activity whilst aspirin produced an effect upon TxA_2 production consistent with cyclo-oxygenase inhibitory activity. Thus, the initial part of the study confirmed the pharmacological mode of action of each drug and we therefore went on to examine their ability to inhibit collagen-induced platelet aggregation.

Collagen is a major component of the sub-endothelium of blood vessels and is considered to play a physiologically important role in platelet activation (Baumgartner, 1977; Nievelstein & de Groot, 1988). When collagen is added to blood, platelets adhere to collagen fibrils and this contact phase is associated with activation of phospholipases C and A_2 and the release from membrane phospholipids of arachidonic acid (Blackwell *et al.*, 1977; Sano *et al.*, 1983). This is then converted to TxA_2 by the sequential action of the cyclo-oxygenase and TxA_2 synthase enzymes. However, aggregation to collagen is also mediated by platelet-derived adenosine diphosphate (ADP) (Kinlough-Rathbone *et al.*, 1977). The extent of the TxA_2-mediated component in the response to collagen can be revealed by using a maximally effective concentration of aspirin. Thus, aspirin produces an approximately 4 fold rightward displacement of the collagen aggregation concentration-effect curve. The remaining aggregation can be shown to be mediated predominantly by ADP (Kinlough-Rathbone *et al.*, 1977). In the present study GR32191, over the concentration-range 0.01–10 μM, resulted in a progressive rightward displacement of the collagen concentration-effect curve. At 10, 1 and even 0.1 μM GR32191 antagonism of collagen-induced aggregation was significantly greater than that of aspirin (2 mM). For this to occur, GR32191 must be affecting the ADP-induced component of collagen-induced aggregation, either directly or indirectly. However, GR32191 (10 μM) has been shown to be specific having no effect upon aggregation induced by ADP (Lumley *et al.*, 1989). It is most likely, therefore, that this additional inhibition seen with GR32191 is indirect, being induced by an endogenous anti-aggregatory agent or agents. Since the superior effect of GR32191 could be prevented by pretreating platelets with aspirin, this suggests the involvement of an anti-aggregatory cyclo-oxygenase product such as PGI_2 or PGD_2. Indeed, both prostaglandins can be detected following collagen-induced aggregation of human platelets (Orchard *et al.*, 1985). Whereas aspirin will inhibit the production of these anti-aggregatory prostanoids, GR32191 has no such effect and therefore will allow their anti-aggregatory effects to be expressed. If PGI_2 and PGD_2 have anti-aggregatory activity *in vivo*, then by preserving their action, GR32191 would be predicted to have an important clinical advantage over aspirin (Lumley *et al.*, 1990).

The present study has also demonstrated a superior antagonist effect of a TP-receptor blocking drug upon collagen-induced aggregation when compared with that of a maximally effective concentration of a specific TxA_2 synthase inhibitor. The inability of this latter class of drugs to antagonize fully the TxA_2-induced component of collagen- or arachidonic acid-induced platelet aggregation has been reported by others (Bertele *et al.*, 1981; 1982; Fitzgerald *et al.*, 1985; Gresele *et al.*, 1987). The reason for such a weak inhibitory profile has been suggested to be due to accumulating PGH_2, which occurs in the presence of TxA_2 synthase inhibition. PGH_2 is itself a potent stimulator of the platelet TP-receptor, and can substitute for TxA_2 at TP-receptors to cause aggregation (Bertele *et al.*, 1981; Hornby & Skidmore, 1982). It has, however, been argued that the accumulating PGH_2 would be redirected to anti-aggregatory PGI_2 and PGD_2 which would augment the effect of reduced TxA_2 formation with TxA_2 synthase inhibitors (Vermylen *et al.*, 1981; Defreyn *et al.*, 1982). Evidence for a rediversion of prostaglandin endoperoxides to PGI_2 has been demonstrated following administration of TxA_2 synthase inhibitors to man (Fitzgerald *et al.*, 1983). Despite this effect, the clinical studies performed to date with this class of drugs have failed to demonstrate any marked anti-thrombotic effect (Fiddler & Lumley, 1990). The efficacy of the TxA_2 synthase inhibitors would therefore appear to depend upon the balance between the pro-aggregatory effect of PGH_2 and the anti-aggregatory effect of PGI_2 and PGD_2.

Evidence consistent with such a pro-aggregatory effect of PGH_2 reducing the full anti-aggregatory potential of a TxA_2 synthase inhibitor has been obtained in the present study. Thus, when GR32191 was combined with a maximally effective concentration of dazoxiben, a significant potentiation of the inhibitory effect of the latter compound was observed. The collagen curve was displaced markedly to the right of that in the presence of either aspirin or GR32191. As discussed above in terms of the enhanced platelet inhibitory effect of GR32191, this effect of the combination of drugs involves inhibition of the non-TxA_2-mediated component of collagen-induced aggregation. Since the concentrations of dazoxiben and GR32191 used were without effect themselves upon ADP-induced aggregation, and since the enhanced effect could be largely prevented by aspirin treatment of platelets, these findings are again consistent with the action of PGI_2 and/or PGD_2. It has in fact been reported (Orchard *et al.*, 1985) that concentrations of both PGI_2 and PGD_2 are enhanced when platelet aggregation is induced with collagen in the presence of dazoxiben *in vitro*.

Both R.68070 and CV-4151, up to concentrations of 1 and 10 μM respectively, produced rightward displacements of the collagen concentration-effect curve which were less than that achieved with aspirin. At these concentrations, the compounds produced a maximum inhibition of TxB_2 production. Interestingly, the magnitude of the inhibitory effect against collagen-induced aggregation of the compounds was very similar to that produced by maximally effective concentrations of dazoxiben (10–100 μM). Thus, it is suggested that up to concentrations of 1 and 10 μM R.68070 and CV-4151 respectively, these drugs produce their effects against collagen-induced aggregation solely through TxA_2 synthase inhibition. When 10 and 100 μM R.68070 were employed, an effect against collagen-induced aggregation equivalent to aspirin and GR32191 was obtained with the two concentrations respectively. Since inhibition of TxA_2 synthesis was already maximal, the effect of R.68070 was most probably due to TP-receptor blocking activity, which would be present at these concentrations. In the case of CV-4151, a weaker effect than that achieved with R.68070 was always observed, probably reflecting the weaker TP-receptor blocking potency of the compound.

R.68070 and CV-4151 possess both TxA_2 synthase inhibitory and TP-receptor blocking activity. Whilst this latter action is relatively weak for both compounds, it would have been anticipated that if a sufficiently high concentration of each drug were used, a superior effect to GR32191 alone and one approaching that of GR32191 and dazoxiben would have been achieved. This was not the case. In fact, a high concentration of R.68070 produced smaller inhibition of collagen-induced aggregation. The most likely explanation for this

effect is the lack of specificity of high concentrations of R.68070 for TxA_2 synthase. This was indicated by the reduced formation of both PGE_2 and PGD_2 in human clotting blood with 100 and 1000 μM of R.68070 and CV-4151. Thus, as the concentrations of R.68070 and CV-4151 are increased, so cyclo-oxygenase becomes inhibited and the peak concentrations of PGI_2 and/or PGD_2 may actually fall, resulting in a reduced anti-aggregatory effect. In keeping with this hypothesis, when a lower, but still maximal and specific, TxA_2 synthase inhibitory concentration of each drug was combined with a maximal TP-receptor blocking concentration of GR32191, an inhibition of collagen-induced aggregation equivalent to that of the combination of GR32191 and dazoxiben was seen. The effect, where examined, was also greatly reduced by aspirin pretreatment. Thus, in the present *in vitro* study, the non-specific effect of both R.68070 and CV-4151 meant that their true anti-aggregatory potential could not be realised. In the presence of TxA_2 synthase inhibition, the redirection of PGH_2 to anti-aggregatory prostanoids in whole blood *in vitro* is limited by the absence of a major source of prostacyclin synthase. However, *in vivo* it is conceivable that more of the accumulating PGH_2 would be redirected to PGI_2, thus enhancing the anti-aggregatory potential of the combined TP-receptor blocker/TxA_2 synthase inhibitors.

In summary, the effectiveness of various classes of compounds which interfere with the formation and action of TxA_2 have been examined upon collagen-induced human platelet aggregation. At maximal, but specific concentrations, the order of effectiveness was GR32191 combined with either dazoxiben, R.68070 or CV-4151, > GR32191 = R.68070 > CV-4151 > aspirin > dazoxiben. Thus, the concept of a combination of TxA_2 synthase inhibitory and TP-receptor blocking activity being superior to either agent alone has been substantiated. However, R.68070 and CV-4151, compounds which possess both activities, were not able to achieve the maximum possible effect, most probably due to a non-specific action on cyclo-oxygenase when used at the high concentrations necessary to achieve TP-receptor blockade. It remains to be seen in clinical studies whether either drug can produce a superior anti-thrombotic effect to a specific TP-receptor blocking drug such as GR32191.

We acknowledge the skilled technical assistance of Davina Humphreys, Kate LaPorte, Marcelle Morrell and Jason Mundin. We would also like to thank members of the Chemistry Research Department, Glaxo for synthesis of some of the compounds used in this study and the staff of the Human Pharmacology Unit, Glaxo for supplying human blood samples. We acknowledge the generous gifts of R.68070 from Dr F. De Clerck (Janssen) and dazoxiben from Dr M.J. Randall (Pfizer). Finally we thank Susan Cook and Jayne Wells for typing the manuscript and Mr K. Mantle for preparation of the artwork.

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(Received June 25, 1990
Revised October 1, 1990
Accepted October 19, 1990)

Br. J. Pharmacol. (1991), **102**, 506–510 © Macmillan Press Ltd, 1991

Calcitonin gene-related peptide and human epicardial coronary arteries: presence, release and vasodilator effects

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1 In the present study, the levels of calcitonin gene-related peptide (CGRP)-like immunoreactivity (-LI) in human cardiopulmonary tissue were determined in combination with studies on CGRP-LI release from the left anterior descending coronary artery (LAD) and functional effects of CGRP on coronary arterial tone.

2 The highest levels of CGRP-LI were found in the LAD followed in declining order by the bronchus, right atrium, pulmonary artery, lung and left ventricle.

3 Exposure to capsaicin evoked a clear-cut increase in CGRP-LI outflow, suggesting release from isolated large specimen of the LAD. This release was Ca^{2+}-dependent and was markedly attenuated by incubation with the mitochondrial Ca^{2+}-inhibitor, ruthenium red. Exposure to potassium also released CGRP-LI in a Ca^{2+}-dependent fashion from the LAD.

4 In functional experiments on human epicardial coronary arteries with an inner diameter of 0.4 to 0.8 mm, human CGRP alpha and beta relaxed the potassium-precontracted arteries equipotently. Substance P (SP) also relaxed these precontracted arteries but the relaxation could be prevented by incubation with methylene blue, an inhibitor of endothelium derived relaxing factor (EDRF)-mechanisms, which did not influence the effect of CGRP.

5 Capsaicin evoked a ruthenium red-sensitive relaxation of the potassium-precontracted arteries. However, ruthenium red did not affect the relaxations induced by CGRP or SP. Furthermore, the capsaicin effect was not influenced by methylene blue.

6 It is concluded that CGRP-LI is present in human cardiopulmonary tissue and can be released upon exposure to high concentrations of capsaicin as well as potassium. CGRP causes relaxation of arteries independently of EDRF activation and closely resembles the vasodilator effects of capsaicin. This supports the view that the coronary vasodilatation observed upon sensory nerve activation is mediated by CGRP. Ruthenium red inhibits capsaicin-induced CGRP-LI release and functional effects and may thus serve as an experimental tool in evaluating the function of capsaicin-evoked stimulation of peripheral nerve terminals.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino-acid-residue peptide generated by alternate splicing of calcitonin gene transcripts (Amara *et al.*, 1982, Rosenfeld *et al.*, 1983; see Fischer & Born, 1987). CGRP-like immunoreactivity (-LI) is present together with substance P (SP) in the cardiovascular system of a variety of species, including man (see Franco-Cereceda, 1988), and exerts potent dilator effects on human coronary arteries both *in vitro* (Franco-Cereceda & Lundberg, 1987) and *in vivo* (McEwan *et al.*, 1986). At least two molecular forms of CGRP are expressed in man, CGRP alpha and CGRP beta (Amara *et al.*, 1985, Petermann *et al.*, 1987), which differ in three amino acids (see Fischer & Born, 1987).

Capsaicin, the irritant agent in hot peppers, causes activation of cardiac C-fibre endings and has been widely used experimentally to study the local effects of mediators released from capsaicin-sensitive sensory nerves (see Holzer, 1988; Maggi & Meli, 1988). In the pig *in vivo*, local bolus injections of capsaicin into the left anterior descending coronary artery (LAD) caused coronary vasodilatation, presumably through the release of sensory peptides (Franco-Cereceda, 1988). The actual contribution of the co-stored SP and CGRP in the vasodilator response to capsaicin has been evaluated both in porcine and human coronary arteries (Franco-Cereceda & Rudehill, 1989; Franco-Cereceda, 1988). However, although CGRP-LI is released from the guinea-pig heart and probably mediates the capsaicin-evoked stimulation of atrial contractility (Franco-Cereceda, 1988), release of CGRP-LI from human cardiac tissue has not been demonstrated. Furthermore, a comparison of the dilator responses to CGRP alpha and CGRP beta has not been made. Nor have the effects of ruthenium red, an inhibitor of mitochondrial Ca^{2+}-fluxes (Moore, 1971; Rahaminoff & Alnaes, 1973) which blocks capsaicin-induced excitation of sensory nerves (Maggi *et al.*, 1988a,b), on capsaicin-evoked vasodilatation been studied.

Methods

This study was approved by the Ethics Committee of the Karolinska Hospital. A total of 46 epicardial coronary arterial segments with an inner diameter of 0.4 to 0.8 mm were obtained from 12 organ donor patients (4 males, 8 females, age 40 ± 3 years) with total cerebral infarction due to intracranial bleeding and/or traumata. The arteries were dissected out of the left ventricle of the heart with the aid of a microscope and mounted in 2 ml organ baths on two L-shaped holders (0.3 mm in diameter). A resting tension of 5 mN was then applied by adjusting one of the metal holders (see Högestätt *et al.*, 1983). The other holder was connected to a Grass FTO3C force-displacement transducer and a Grass Polygraph, model 7 B, for recordings of isometric tension. The vessels were kept in Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42 and glucose 5.6. The Tyrode solution was aerated with 95% O_2 and 5% CO_2 at 37°C. After 60 min of equilibration with adjustment to compensate for the spontaneous decline in arterial tension, circular contractions were induced by Tyrode solution in which the NaCl had been replaced with equimolar amounts of KCl to give a final concentration of 60 mM potassium. Only vessels giving two reproducible contractions were used and each experimental procedure was examined in arteries from at least four patients. The vessels were dissected out approximately 15–30 min after the artificial ventilation had been turned off and careful micro-

scopic examination was made to exclude vessels with atherosclerosis.

The tissue content of CGRP- and neuropeptide Y (NPY)-LI was analysed in human cardiopulmonary tissue obtained from 8 of the patients (3 males, 5 females, age 41 ± 6 years) by radioimmunoassay (RIA). The tissues were extracted in 2 ml (tissue specimen < 0.1 g) or 20 volumes (tissue specimen > 0.1 g) of 1 M acetic acid. After heating for 10 min to 90°C, the tissues were homogenized in a Polytron. The homogenates were subsequently centrifuged and the supernatants collected and lyophilized before RIA. CGRP-LI was analysed with an antiserum raised against human CGRP alpha (RAS 6009; Peninsula, CA, U.S.A.; for characterization, see Franco-Cereceda, 1988) while the content of NPY-LI was analysed with antiserum N1 (Theodorsson-Norheim *et al.*, 1985). Detection limits were 4 and 8 fmol in a 0.5 ml sample for CGRP- and NPY-LI, respectively.

In addition, large pieces of the LAD (89 ± 29 mg; $n = 6$) were used to study the release of CGRP-LI. The arteries were perfused at a rate of 1 ml min^{-1} for 20 min with Tyrode solution and then exposed to capsaicin (10^{-5} M) or potassium (60 mM) for 20 min (the exposure time and concentrations used were chosen based on previous animal experiments; see Franco-Cereceda, 1988). This was also carried out in Ca^{2+}-free Tyrode solution containing 0.1 mM EGTA (capsaicin and potassium) and in Tyrode solution containing 10^{-4} M ruthenium red (capsaicin). Perfusate fractions were collected in a beaker placed on ice to which acetic acid was added to give a final concentration of 0.2 M. The perfusate samples were desalted with the aid of SEP-PAK C_{18} cartridges (Wu *et al.*, 1983), lyophilized and redissolved in appropriate buffer for the determination of CGRP-LI (see above). Due to the low peptide content observed in preliminary tissue determinations of CGRP-LI, these larger-sized arteries were chosen for release experiments although no functional recordings could be obtained with the currently available experimental set-up.

Values are given as means ± s.e.mean. The Mann-Whitney U-test was used for statistical analysis and $P < 0.05$ was considered significant.

Human CGRP alpha and beta and SP were obtained from Peninsula (Belmont, CA, U.S.A.), methylene blue from Sigma (St Louis, MO, U.S.A.) and ruthenium red from Serva (Heidelberg, F.R.G.). All these compounds were dissolved in saline. Capsaicin was obtained from Fluka (Basel, Switzerland) and dissolved in a stock solution of 10 mg ml^{-1} with 70% ethanol and further diluted with saline.

Results

The levels of CGRP-LI were found to be 3–10 times higher in the LAD (2.1 ± 0.2 pmol g^{-1}) than in the bronchus, right atrium, pulmonary artery, lung and left ventricle (Table 1). NPY-LI was also present in the cardiopulmonary tissue, with somewhat higher levels in the LAD (10.7 ± 2.2 pmol g^{-1}) than in the right atrium, left ventricle and, especially, the lung (Table 1).

Table 1 Content of calcitonin gene-related peptide (CGRP) and neuropeptide Y-like immunoreactivity (NPY-LI) in some human cardiopulmonary tissues

	CGRP-LI	*NPY-LI*
	(pmol g^{-1})	
LAD	2.12 ± 0.2	10.7 ± 2.2
Bronchus	0.76 ± 0.1	ND
Right atrium	0.39 ± 0.09	10.3 ± 1.9
Pulmonary artery	0.25 ± 0.02	ND
Left ventricle	0.20 ± 0.07	8.3 ± 0.9
Lung	0.26 ± 0.04	1.8 ± 0.7

The tissues were obtained from 8 patients (3 males, 5 females, age 41 ± 6 years). LAD = left anterior descending coronary artery. Data are given as means ± s.e.mean and represent 3–8 specimens in each group. ND = not determined.

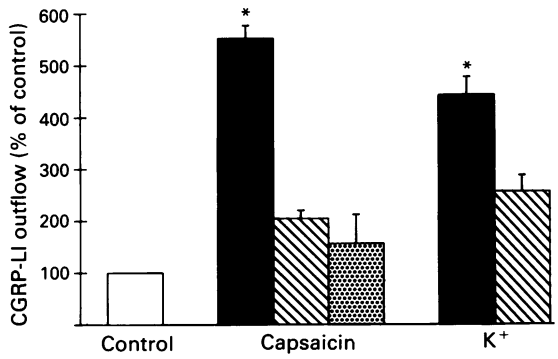


Figure 1 Effects of exposure to capsaicin (10^{-5} M for 20 min) or potassium (60 mM for 20 min; K^+) on isolated left anterior descending coronary arteries (solid columns) compared to basal outflow in ordinary Tyrode solution (open column). Also shown is the inhibitory effect on calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) release by capsaicin and potassium in Ca^{2+}-free Tyrode solution (hatched columns) as well as the inhibitory effect of ruthenium red (10^{-4} M added 15 min before; stippled column) on the capsaicin-induced CGRP release. Values are given as means of 3–5 experiments in each group; vertical bars show s.e.mean. * $P < 0.05$, Mann Whitney U-test, compared to controls.

Basal CGRP-LI outflow from the LAD was 11 ± 3 fmol per fraction ($n = 5$, one basal value was below the detection limit). Exposure to capsaicin (10^{-5} M for 20 min) evoked a clear-cut increased outflow of CGRP-LI, suggesting release from the

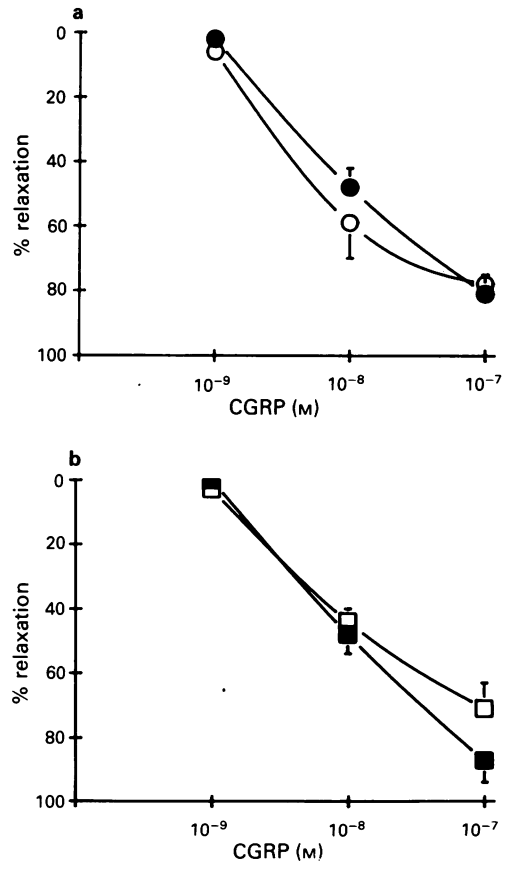


Figure 2 Relaxation of potassium (60 mM)-precontracted human coronary arteries induced by increasing concentrations of human calcitonin gene-related peptide (CGRP) alpha (○) and beta (●; a). Also shown is the lack of effects of incubation with methylene blue (10^{-5} M; ■) and ruthenium red (10^{-4} M; □) on the relaxant effect of human CGRP alpha (b).

LAD (Figure 1). This release was significantly reduced ($P < 0.05$) by both Ca^{2+}-free Tyrode solution and incubation with 10^{-4} M ruthenium red. Exposure to potassium (60 mM for 20 min) also caused a release of CGRP-LI, which was attenuated by Ca^{2+}-free Tyrode solution (Figure 1). Based on the tissue concentrations of CGRP-LI, capsaicin evoked a release of approximately 15–20% of the total tissue content of CGRP-LI.

In the functional experiments, potassium (60 mM) induced a contraction by 3.6 ± 0.2 mN in control arteries. Incubation with ruthenium red (10^{-4} M) or methylene blue (10^{-5} M) for 15 min did not statistically influence the contractile response to potassium (2.9 ± 0.7 and 3.2 ± 0.6 mN after ruthenium red and methylene blue incubation, respectively).

Human CGRP alpha and beta (10^{-11} to 10^{-7} M) evoked a concentration-dependent, long-lasting relaxation of the precontracted arteries (Figures 2, 3). On a molar basis, there was no difference in the response to the two forms of CGRP. Incubation with ruthenium red or methylene blue did not influence

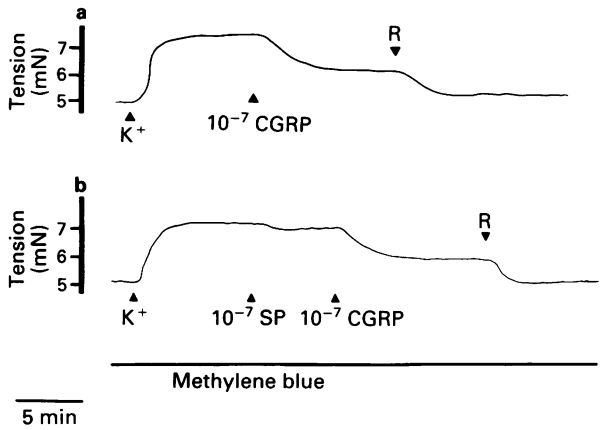


Figure 3 Original tracings from two arterial segments with an inner diameter of about 0.5 mm showing the relaxant effects of calcitonin gene-related peptide (CGRP) alpha (a) and the inhibitory effect of methylene blue (10^{-5} M) on the substance P (SP)- but not CGRP-induced relaxation (b). The vessels were precontracted by 60 mM potassium (K^+). R indicates rinsing. The bar represents 5 min.

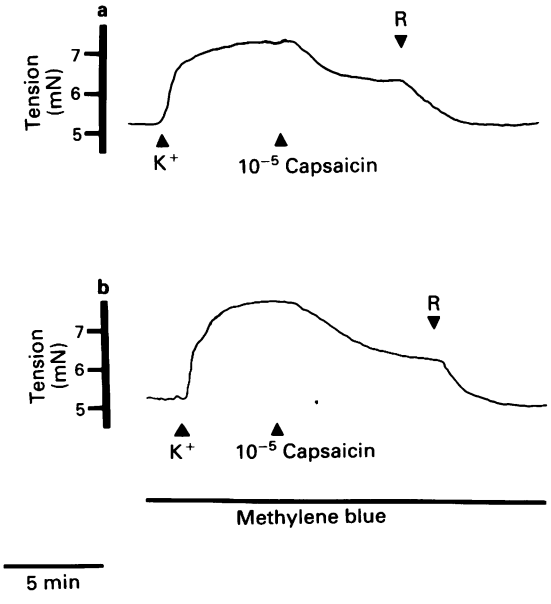


Figure 4 Original recordings showing the relaxant effect of capsaicin (10^{-5} M) on potassium (60 mN; K^+)-precontracted coronary arteries in ordinary Tyrode solution (a) and in Tyrode solution containing methylene blue (10^{-5} M; b). R indicates rinsing, the bar represents 5 min.

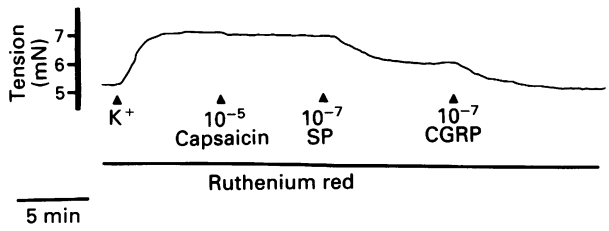


Figure 5 Original recording showing the lack of effect of ruthenium red (10^{-4} M) on the relaxation evoked by substance P (SP) and calcitonin gene-related peptide (CGRP), while the effect of capsaicin is completely abolished. The vessels were precontracted by 60 mM potassium (K^+). The bar represents 5 min.

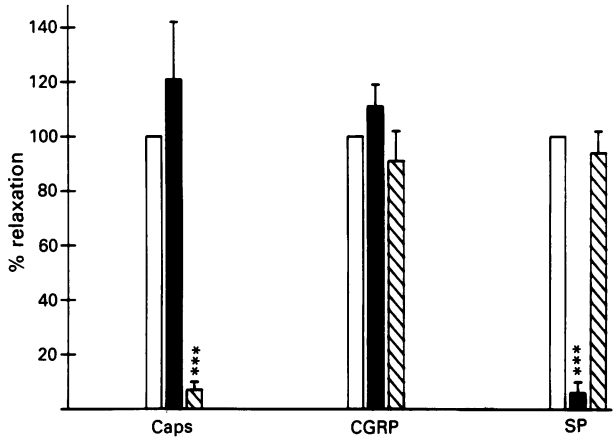


Figure 6 Summary of the effects of capsaicin (10^{-5} M), calcitonin gene-related peptide (CGRP) alpha (10^{-7} M) and substance P (SP, 10^{-7} M), expressed as percentage relaxation of potassium (60 mM)-evoked contractions. Open columns represent the control group, solid columns represent the relaxation after methylene blue incubation (10^{-5} M) and hatched columns represent the relaxations in the presence of ruthenium red (10^{-4} M). Data are given as means of 4–8 observations in each group; vertical bars show s.e.mean. *** $P < 0.001$, Mann Whitney U-test.

the relaxant effects of CGRP alpha (Figures 2, 5, 6) on the precontracted arteries. SP (10^{-7} M) caused relaxation by 51 ± 17% of the precontracted arteries ($n = 10$). This effect remained unchanged in the presence of ruthenium red (48 ± 4%; $n = 6$) but was almost completely abolished after incubation with methylene blue (3 ± 2%; $n = 7$; Figure 6). Capsaicin (10^{-5} M) evoked relaxation of the potassium-precontracted arteries amounting to 34 ± 6% ($n = 8$). While this effect remained unchanged in the presence of methylene blue (28 ± 9%; $n = 8$; Figures 4, 6), ruthenium red markedly reduced the vasodilatation evoked by capsaicin (2 ± 1%; $n = 4$; Figures 5, 6).

Discussion

The present data confirm and expand earlier findings concerning the vasodilator effects of CGRP on human coronary arteries (Franco-Cereceda & Lundberg, 1987; McEwan *et al.*, 1986). Furthermore, this is the first demonstration of CGRP-LI release from human cardiac tissue that accords with previous reports on CGRP-LI release from rat and guinea-pig hearts (see Franco-Cereceda, 1988).

CGRP-LI was detected in human cardiopulmonary tissue, as determined by RIA, with the highest levels in the LAD. This is in conformity with the abundance of CGRP-immunoreactive nerve fibres around human coronary vessels (Franco-Cereceda, 1988) and suggests a primary action of CGRP on cardiac vascular tone rather than direct effects on

cardiac myocyte function, although CGRP has been shown to exert a positive inotropic effect on the human isolated atrium (Franco-Cereceda *et al.*, 1987a). Moreover, the intracoronary administration of CGRP caused no changes in heart rate at concentrations used to evoke an up to 40% increase in diameter in the LAD *in vivo* (McEwan *et al.*, 1986). The predominantly perivascular localization of CGRP-LI contrasts with the sympathetically stored NPY (Lundberg *et al.*, 1982), which seemed to be more homogeneously distributed in the heart since the levels in the atrium, ventricle and LAD were similar. Furthermore, it is also clear that NPY-LI was present in all cardiac tissues examined in considerably higher concentrations than CGRP-LI. The CGRP-LI in the heart is probably sensory and may arise from cell-bodies in the thoracic spinal ganglia and/or vagal (nodose/jugular) ganglia. It has been shown that the SP content in the heart is of dual vagal and spinal origin (Dalsgaard *et al.*, 1986) and the same may thus apply to the CGRP-LI in the heart. This is also supported by the lack of CGRP-LI in local parasympathetic ganglia in the heart and stellate ganglia (Franco-Cereceda, 1988).

Capsaicin causes activation of C-fibre afferents and is associated with the release of sensory neuropeptides from a variety of organs in several species (see Holzer, 1988; Maggi & Meli, 1988). In the guinea-pig Langendorff-perfused heart, administration of capsaicin and potassium evoked a Ca^{2+}-dependent CGRP-release (Franco-Cereceda, 1988) which did not occur after systemic pretreatment with capsaicin when C-fibre afferents had degenerated (Papka *et al.*, 1984). Also in the human LAD exposure to capsaicin and potassium were associated with an increased outflow of CGRP-LI in a Ca^{2+}-dependent manner, suggesting activation of cardiac sensory nerves. In agreement with this, capsaicin has been shown by electrophysiological techniques to activate sensory C-fibre endings in the heart ventricle (Coleridge *et al.*, 1964; Bishop *et al.*, 1983). The relative amount of CGRP-LI released by capsaicin in the present study (15–20% of the total tissue content) is high compared to, for example, the guinea-pig heart but is likely to be due to the high concentration (10^{-5} M) of capsaicin used here in combination with the long exposure time (20 min).

It has recently been reported that ruthenium red, an inhibitor of mitochondrial calcium fluxes (Moore, 1971), reduced the excitatory effects of capsaicin on sensory nerves (Maggi *et al.*, 1988a,b). The effect of ruthenium red on neuronal Ca^{2+} channels is not known but, judging from the present experiments, it is likely that capsaicin activates Ca^{2+} mechanisms which are sensitive to ruthenium red (see also below). Although only tested on capsaicin in this study (due to a limited amount of tissue), ruthenium red seems to have a rather selective effect on capsaicin-induced functional responses and peptide release (Amann & Lembeck, 1989; Franco-Cereceda *et al.*, 1989).

Human CGRP alpha and beta were equipotent in inducing relaxation of the precontracted arteries. This finding is in line with the close structural similarity between the two forms of CGRP, which differ in only 3 of the 37 amino acids (Steenbergh *et al.*, 1986). It remains to be established, however, which of the two forms is actually present in peripheral sensory nerve terminals in the heart. In conformity with the present findings on coronary vasodilator actions of CGRP alpha and beta, the two peptides were equally potent in causing positive inotropic effects on the isolated electrically driven human right atrium as well as in relaxing human isolated skeletal muscle arteries (Franco-Cereceda *et al.*, 1987b).

It has been suggested that CGRP is a more potent vasodilator of human coronary arteries than SP, based on *in vitro* studies on potassium-precontracted vessels (Franco-Cereceda & Rudehill, 1989). In view of the present findings concerning activation of sensory nerve terminals and release of CGRP-LI from the arteries by potassium and the rapid development of tachyphylaxis to SP effects (Franco-Cereceda & Rudehill, 1989), the relaxation evoked by SP may have been underestimated. In fact, on intracoronary administration, SP seems to be a slightly more potent vasodilator than CGRP (Crossman *et al.*, 1989). However, the CGRP/SP-containing capsaicin-sensitive nerves in the heart are generally located outside the media of the blood vessels (Franco-Cereceda, 1988). To activate the endothelium-dependent mechanisms, SP may therefore have to pass diffusion barriers when released *in vivo*, compared to CGRP, which, at least in human coronary arteries, acts directly on the vascular smooth muscle (see below; Franco-Cereceda & Rudehill, 1989). A recent report (Minami *et al.*, 1989) demonstrated the ability of extraluminally applied SP to cause vasodilatation through release of endothelium derived relaxing factor (EDRF) at considerably higher concentrations than when applied intraluminally, although such mechanisms as metabolic degradation, apart from diffusion barriers, could not be excluded.

The possible influence of the vascular endothelium in the relaxant response to CGRP has attracted a great deal of attention. Thus, there are several reports on both endothelium-dependent and endothelium-independent CGRP relaxation of blood vessels (see Franco-Cereceda, 1988; Owman, 1988). It is doubtful whether the CGRP-evoked relaxation of human coronary arteries *in vitro* is mediated through EDRF since the response was not changed by methylene blue (present study) and gossypol (Franco-Cereceda & Rudehill, 1989), both inhibitors of EDRF mechanisms (see Hamasaki & Tai, 1985; Luscher & Vanhoutte, 1988). The lack of an SP effect after incubation with methylene blue indicated successful inhibition of the release of EDRF (see Föstermann *et al.*, 1986).

The extent to which CGRP or co-stored tachykinins mediate the vasodilatation induced by capsaicin has been evaluated earlier in human coronary arteries by the use of gossypol and SP tachyphylaxis (Franco-Cereceda & Rudehill, 1989), and CGRP was proposed as a more likely candidate than SP in this response. After incubation with methylene blue the dilator effects of capsaicin remained unchanged and thus resembled the effect of CGRP but not of SP. Duckles (1986) and Illes & Falkenhausen (1986) reported that the capsaicin-induced relaxation of cerebral blood vessels *in vitro* was not influenced by removal of the endothelium (which abolished the effects of exogenously applied SP) or by a SP antagonist. Also in porcine coronary arteries, removal of the endothelium abolished the relaxant effects of SP but not of capsaicin or CGRP (Franco-Cereceda, 1988), which favours a predominant role of CGRP in this reaction. The lack of capsaicin effects after ruthenium red incubation is probably related to inhibition of CGRP release (see above) and not to postjunctional effects or metabolic degradation of CGRP since the relaxation in response to both SP and CGRP remained unchanged. Ruthenium red could then be used to evaluate mechanisms and functional effects observed upon selective activation of cardiac capsaicin-sensitive sensory nerves under different experimental conditions.

In conclusion, the capsaicin-evoked vasodilatation of human epicardial coronary arteries is mediated through the release of CGRP-LI present in cardiac tissue. Of the co-stored peptides, CGRP and SP, the relaxation caused by sensory nerve activation in the heart is probably mediated through CGRP. The extent to which other agents, as well as ischaemia, which is known to stimulate cardiac sensory nerves (Bishop *et al.*, 1983), cause a release of CGRP with subsequent alterations in cardiac blood flow, remains to be established. It is tempting, however, to speculate about such a local efferent function of cardiac C-fibre afferents.

The present study was supported by the Heart-Lung Foundation, the Laerdal Foundation, the Swedish Medical Research Council (14X-6554), the Lars Hierta Foundation, the Tore Nilsson Foundation, the Swedish Society of Medicine, the American Council for Tobacco Research, the Magnus Bergvall Foundation and funds from the Karolinska Institute.

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(Received March 30, 1990
Revised August 15, 1990
Accepted September 29, 1990)

Br. J. Pharmacol. (1991), **102**, 511–517 © Macmillan Press Ltd, 1991

Novel selective agonists and antagonists confirm neurokinin NK_1 receptors in guinea-pig vas deferens

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1 This study investigated the recognition characteristics of neurokinin receptors mediating potentiation of the contractile response to field stimulation in the guinea-pig vas deferens.

2 A predominant NK_1 receptor population is strongly suggested by the relative activities of the common naturally-occurring tachykinin agonists, which fall within less than one order of magnitude. This conclusion is supported by the relative activities of the synthetic NK_1 selective agonists substance P methyl ester, [Glp^6,L-Pro^9]-SP$_{(6-11)}$ and δ-aminovaleryl-[L-Pro^9,N-MeLeu^{10}]-SP$_{(7-11)}$ (GR73632) which were 0.78, 9.3 and 120 as active as substance P, respectively. Furthermore, the NK_2 selective agonist [Lys^3, Gly^8,-R-γ-lactam-Leu^9]-NKA$_{(3-10)}$ (GR64349) was active only at the highest concentrations tested (> 10 μM), and the NK_3 selective agonist, succ-[Asp^6,N-MePhe^8]-SP$_{(6-11)}$ (senktide) was essentially inactive (10 nM–32 μM).

3 [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP$_{(1-11)}$ antagonized responses to neurokinin A, neurokinin B, physalaemin, eledoisin, [Glp^6,D-Pro^9]-SP$_{(6-11)}$, GR73632 and GR64349 (apparent pK_Bs 5.6–6.2), but was less potent in antagonizing responses to substance P, substance P methyl ester and [Glp^6,L-Pro^9]-SP$_{(6-11)}$ (apparent pK_Bs ≤ 5.0–5.0).

4 In contrast, the recently developed NK_1-selective receptor antagonist [D-Pro^9[Spiro-γ- lactam]Leu^{10}, Trp^{11}]-SP$_{(1-11)}$ (GR71251) did not produce agonist-dependent pK_B estimates. Schild plot analysis indicated a competitive interaction with a single receptor population where the antagonist had an estimated overall pK_B of 7.58 ± 0.13 for the four agonists of differing subtype selectivity tested (GR73632, GR64349, substance P methyl ester and neurokinin B). This estimate is similar to that we obtained for NK_1-mediated (substance P methyl ester) contraction in the guinea-pig ileum preparation (pK_B = 7.86 ± 0.05).

5 Tachykinin action appears not to depend on release of a number of intermediary mediators including acetylcholine, histamine or cyclo-oxygenase products, nor to involve interaction with neuronal mechanisms including α_2-adrenoceptor feedback, noradrenergic Uptake-1 or opioid-release, since antagonism or inhibition of these mechanisms did not modify responses to tachykinins.

6 We conclude that tachykinin action in the field-stimulated guinea-pig vas deferens preparation is mediated through interaction with a predominant neurokinin NK_1 receptor population and this preparation can therefore be used to study NK_1 modulation of sympathetic neurotransmission.

Introduction

There is now considerable support for the proposed existence of three functional neurokinin receptor subtypes, NK_1, NK_2 and NK_3 (see, Henry, 1987). The evidence for this proposal has come, until comparatively recently, almost exclusively from the determination of activities of naturally-occurring tachykinin agonists including the mammalian neurokinins, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) in functional studies in the periphery and radio-ligand binding studies both in the periphery and in the CNS (for reviews see, Watson, 1984; Lee *et al.*, 1986; Regoli *et al.*, 1987; Quirion & Dam, 1988).

The existence of three neurokinin receptor subtypes has been further substantiated through use of synthetic subtype selective neurokinin receptor agonists. Such agonists that have proven valuable for classificational purposes include; the NK_1 selective agonists substance P methyl ester (SPOMe, Cascieri *et al.*, 1981; Watson *et al.*, 1983), [Glp^6,L-Pro^9]-SP$_{(6-11)}$ (septide, Piercey *et al.*, 1985; Laufer *et al.*, 1986a), and [Sar^9, Met(O$_2$)^{11}]-SP (Drapeau *et al.*, 1987), the NK_2 selective agonist [β-Ala^8]-NKA$_{(4-10)}$ (Rovero *et al.*, 1989), and the NK_3 selective agonists succ-[Asp^6,N-MePhe^8]-SP$_{(6-11)}$ (senktide, Wormser *et al.*, 1986) and [N-MePhe^7]-NKB (Drapeau *et al.*, 1987).

Selective and potent competitive neurokinin receptor antagonists are particularly necessary in neurokinin receptor studies in order to provide confirmatory data to support the current putative classificational schemes largely based on agonist activities. The earlier developed neurokinin receptor antagonists, however, have been of little value for such purposes. For example, many antagonists were found to be low in potency, showed no clear neurokinin subtype selectivity, and several produced agonist-dependent potency estimates in hitherto presumed mono-receptor systems for reasons that are unclear (for discussion see, Bailey *et al.*, 1986). Furthermore, a number of early antagonists additionally have actions not related to receptor antagonism (Karlsson *et al.*, 1984; Lembeck *et al.*, 1986), and several show antagonism at other receptors such as those for bombesin (Folkers *et al.*, 1984; Jensen *et al.*, 1984; Yachnis *et al.*, 1984; Mizrahi *et al.*, 1985).

Very recently several novel agonists and antagonists have been proposed as having high potency and neurokinin receptor subtype selectivity. Such agonists include the NK_1 selective agonist δ-aminovaleryl-[L-Pro^9, N-Me-Leu^{10}]-SP$_{(7-11)}$ (GR73632) and the NK-2 selective agonist, [Lys^3,Gly^8-R-γ-lactam-Leu^9]-NKA$_{(3-10)}$ (GR64349) (Hagan *et al.*, 1989). Novel neurokinin receptor antagonists include the NK_1 selective antagonist [D-Pro^9[Spiro-γ-lactam]Leu^{10},Trp^{11}]-SP$_{(1-11)}$; GR71251 (Hagan *et al.*, 1990; Ward *et al.*, 1990), and the NK_2 selective antagonist cyclo(Gln,Trp,Phe,Gly,Leu,Met) (L659,877; McKnight *et al.*, 1988; Williams *et al.*, 1988).

The contractile response to sympathetic field-stimulation of the guinea-pig vas deferens is potentiated by substance P (SP) and related tachykinins (von Euler & Hedqvist, 1974a,b). However, since its original classification as an SP-P (= NK_1) (Watson *et al.*, 1983), this preparation has been little studied in terms of neurokinin receptor subtypes or involvement of other

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mediators in responses to tachykinins. Pharmacological investigation of the site of action of tachykinins (Ellis & Burnstock, 1989) and the autoradiographic localisation of neurokinin receptors (Mussop *et al.*, 1989) have, however, recently been reported. The rat vas deferens in contrast has been used extensively to study sympathetic neuromodulation, though here tachykinin action is mediated through interaction with NK_2 receptors.

We describe, in this paper, an extensive re-examination of neurokinin receptors in the field-stimulated guinea-pig vas deferens *in vitro* and we report, for the first time, activities of some novel agonist and antagonist analogues in this preparation.

Preliminary accounts of this work have been communicated to The British Pharmacological Society (Hall *et al.*, 1987) and to X Satellite IUPHAR (Sydney) (Hall *et al.*, 1988).

Methods

Tissue preparation and recording arrangements

Male Dunkin-Hartley guinea-pigs (450–800 g) were killed by stunning and exsanguination. The abdomen was opened by mid-line incision, and the two vasa deferentia were removed, cleared of superficial blood vessels and connective tissue and divided into separated prostatic and epididymal sections by length. Each section was mounted on a stainless-steel tissue holder, with a platinum ring electrode around the base of the preparation and with the upper section of the preparation tied with 0.1 mm fully-softened stainless-steel wire. The preparations were attached to Grass FT03B force-displacement transducers under an initial resting tension of 0.5–1.0 g, the wire being used both to attach the tissue to the strain gauge and to act as the upper electrode. Four preparations were set up in parallel in 1 ml silanised glass organ baths, with contractions recorded on Grass model 79C polygraphs after d.c. amplification. Preparations were stimulated via Grass SD9 stimulators individual to each preparation, collectively gated by a single Grass S44 stimulator at 0.05 Hz generating a 0.25 ms gating pulse. Stimulation parameters were a modification of those used by Watson *et al.* (1983) and were trains of 5 pulses at 20 Hz at a 1 ms pulse-width every 20 s. At submaximal voltage (40–60 V) the tissue-electrode-bath assembly drew 20–30 mA. The neurally-mediated 'twitch' response so obtained may in fact be regarded as a short tetanus since some summation occurs.

Experiments were carried out in Krebs solution (composition mM: Na^+ 140, K^+ 5.9, Cl^- 104.8, $H_2PO_4^-$ 1.2, HCO_3^- 24.9, Ca^{2+} 2.6, Mg^{2+} 1.15, SO_4^{2-} 1.15, glucose 10), maintained at 37°C and oxygenated with 95% O_2:5% CO_2.

Relative activities of tachykinin agonists

Tachykinins were assayed for their ability to potentiate responses to nerve stimulation at normally four dose-levels (10 nM–10 μM) in a fully randomised (concentration and agonist) serial block design, with each preparation receiving each concentration of each agonist. A 40–60 s contact time and 7 min dose cycle with SP (1 μM) applied at the start of each block of four doses was used to allow normalisation of responses which are expressed in terms of % increase in twitch height over basal. Similar results were obtained on expression of the data by a number of different methods. Random orders for dosing were determined from computer-generated numbers. In all experiments, the Krebs solution contained atropine (1 μM), mepyramine (1 μM) and hexamethonium (10 μM).

Six of the naturally-occurring tachykinins SP, NKA, NKB, physalaemin (Phy), eledoisin (Ele) and kassinin (Kas) were assayed along with the synthetic NK_1 selective analogue SPOMe.

The D- and L-isomers of [Glp^6,*Pro^9]-SP$_{(6-11)}$, and the NK_1 and NK_2 selective analogues GR73632 and GR64349 respectively, as well as [pGlu^5,MePhe^8,Sar^9]-SP$_{(5-11)}$ (DiMeC7), were assayed in two separate series of experiments, along with SP. The NK_3 selective analogue, senktide, was found to be virtually inactive (10 nM–32 μM) and thus was not included in any formal assay procedure.

Estimation of pK_B values for neurokinin receptor antagonists

[D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP$_{(1-11)}$ (10 μM) was tested against responses to several tachykinins. After establishing control submaximal responses to 2 or 3 dose-levels of selective tachykinins (SP, NKA, NKB, Phy, Ele, SPOMe, [Glp^6,L-Pro^9]-SP$_{(6-11)}$, [Glp^6,D-Pro^9]-SP$_{(6-11)}$, GR73632 and GR64349), the antagonist was left in contact with the preparation for 7 min and suitable concentrations of agonist applied to enable estimation of dose-ratios after antagonist application. The rapid reversibility of antagonist effects on washout allowed the assay of two agonists in each preparation. Schild plot analysis was not possible with this antagonist because of its low potency coupled with the shallow agonist log concentration-response curve which thus did not permit accurate estimation of small dose-ratios.

For studies with the NK_1-selective antagonist, GR71251, four preparations were set up in parallel and control cumulative concentrations-response curves to the NK_1 selective agonists GR73632 and SPOMe, the NK_2 selective agonist GR64349, and the relatively non-selective agonist NKB were obtained in individual preparations. A cumulative dosing protocol was used in these experiments in order to determine antagonist effects on the whole concentration-response curve. Cumulative concentration-response curves were then repeated after a 7 min equilibration with GR71251 (0.32, 1.0 or 3.2 μM). A 20–60 min dose-cycle was used, depending on agonist washout time (see, Results). Washout of antagonist effect was established in each preparation, and the procedure was repeated with higher antagonist concentrations (allowing up to three antagonist concentrations to be tested in each preparation).

In all antagonist experiments, the Krebs solution contained atropine (1 μM), hexamethonium (10 μM), mepyramine and cimetidine (both 1 μM). Mepyramine and cimetidine were included in view of the known histamine-releasing properties of [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP$_{(1-11)}$ and GR71251 (Folkers *et al.*, 1984; Hagan *et al.*, 1990).

Controls for antagonist experiments

Control cumulative concentration-response curves were determined in the absence of antagonist throughout the experimental periods in order to investigate possible time effects and desensitization on tachykinin concentration-response curves. Confirmation of competitive antagonism with GR71251 against NK_1-mediated contraction of the whole guinea-pig ileum was obtained by Schild plot analysis for the antagonist against SPOMe in the presence of hexamethonium (10 μM), atropine, mepyramine, ibuprofen, naloxone and guanethidine (all 1 μM). Three-point concentration-response curves were determined in the absence, and then after a 7 min incubation with GR71251 at increasing antagonist concentrations (0.1–10 μM). Reversibility of antagonism was established in each individual preparation.

Test for involvement of other mediators or interaction with other mechanisms

Responses to single sub-maximal concentrations of SP, Ele, Phy, NKA and NKB were obtained first in the absence, and then after a 30 min equilibration with atropine (1 μM) or mepyramine (1 μM), with each preparation receiving every tachykinin. Two-point concentration-response curves were

obtained to SP, Ele and Phy and were repeated after a 30 min incubation with cimetidine (1 μM). Also, ipromidine (10 μM) was tested for activity in all preparations. The involvement of cyclo-oxygenase products in responses to SP, Ele and Phy was investigated by comparing concentration-response curves (10 nM–10 μM) obtained before and after a 30 min equilibration with ibuprofen (1 μM). Responses to sub-maximal concentrations of SP, Ele and Phy were obtained in the absence, and then after a 30 min equilibration with naloxone (1 μM) or yohimbine (0.32 μM). Full concentration-response curves were obtained for SP, Phy and [Glp^6,D-Pro^9]-SP$_{(6-11)}$ and then repeated after a 30 min equilibration with cocaine (2 μM), a blocker of the noradrenergic Uptake-1 system, in the presence of yohimbine (0.1 μM) to circumvent α_2-adrenoceptor prejunctional autofeedback caused by accumulation of noradrenaline.

Source of materials

Agents were obtained as follows: substance P, eledoisin, physalaemin, kassinin, neurokinin A, neurokinin B, substance P methyl ester, [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP$_{(1-11)}$, [pGlu^5,MePhe^8,Sar^9]-SP$_{(5-11)}$ and yohimbine hydrochloride (Peninsula Laboratories Europe). Ibuprofen, atropine sulphate, naloxone and hexamethonium bromide, cocaine hydrochloride (Sigma, U.K.), mepyramide maleate (May & Baker, U.K.), cimetidine and ipromidine (Smith, Kline & French, U.K.). All salts used were of analytical grade and were obtained from B.D.H., U.K. δ-Aminovaleryl-[L-Pro^9,N-Me-Leu^{10}]-SP$_{(7-11)}$ (GR73632), [Lys^3,Gly^8-R-γ-lactam-Leu^9]-NKA$_{(3-10)}$ (GR64349) and [D-Pro^9[Spiro-γ-lactam]Leu^{10},Trp^{11}]-SP$_{(1-11)}$ (GR71251) were kind gifts from Glaxo Group Research, Ware, Hertfordshire, U.K. Succ-[Asp^6,N-MePhe^8]-SP$_{(6-11)}$ (senktide), [Glp^6,L-Pro^9]-SP$_{(6-11)}$ and [Glp^6,D-Pro^9]-SP$_{(6-11)}$ were kind gifts from Dr B. Williams, Merck, Sharp and Dohme, U.K.

Neurokinin B was dissolved in dimethylsulphoxide (DMSO). All other peptides were dissolved in 0.01 M acetic acid. Ibuprofen was dissolved in 5% Na_2CO_3. All other agents were dissolved in distilled water. Peptides were stored deep frozen under N_2 with dilutions discarded at the end of each experiment.

Analysis of results

Responses of the prostatic and epididymal sections of the vas deferens did not differ, so data from both sections were pooled. Tests for significance were made using Student's t test for independent or paired samples or by Analysis of Variance as appropriate. pK_B estimates for antagonist experiments were determined from individual dose-ratios using the Gaddum-Schild equation for competitive inhibition at equilibrium, $pK_B = \log_{10}(x - 1) - \log_{10}[A]$, where x is the dose-ratio and [A] the applied antagonist concentration. Data described as apparent pK_Bs are where Schild plot analysis was not carried out; otherwise pK_B values are quoted where analysis showed slopes of the Schild plots not to differ significantly from unity. Log_{10} activity estimates for agonists were calculated for individual preparations at the arbitrary level of 100% potentiation of the twitch response.

Results

General

Tachykinins were without consistent effect on baseline tension of the guinea-pig vas deferens; this contrasts with tachykinin actions in the rat vas deferens where marked contraction of the smooth muscle is seen in response to tachykinins (data not shown). In experiments using both serial and cumulative dosing procedures, potentiation of contractile responses to field stimulation by all tachykinins was rapid in onset with

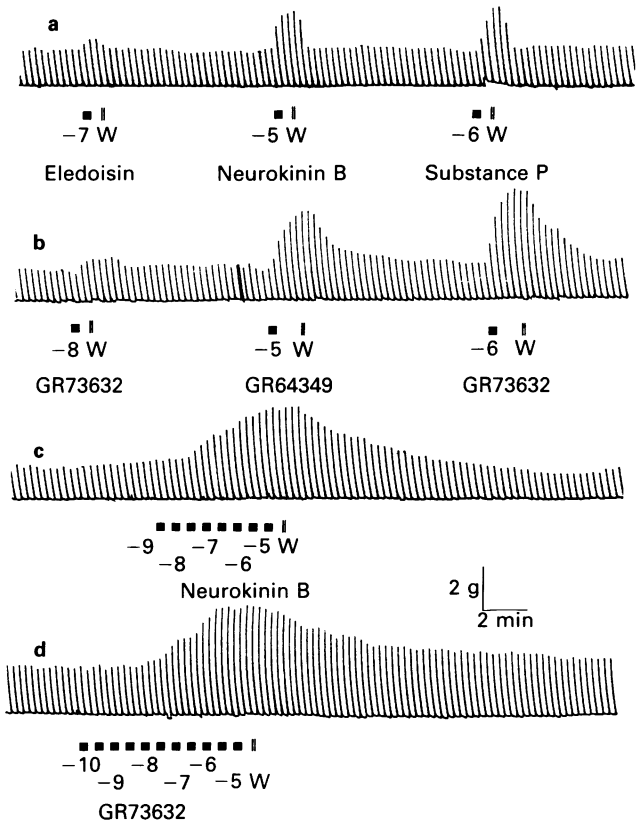


Figure 1 Typical traces showing potentiation of the contractile response to field stimulation in the guinea-pig vas deferens. Responses to naturally-occurring (a and c) and synthetic (b and d) tachykinin analogues were obtained with a serial (a and b) or cumulative (c and d) dosing protocol. Responses shown are to 5-pulse trains at 20 Hz of 1 ms duration every 20 s. Note the delayed onset and offset of response to the synthetic agonists GR73632 and GR64349 compared with the naturally-occurring agonists substance P, eledoisin and neurokinin B. W indicates washout. Agonist concentrations are given as $\log_{10}$.

peak responses to each agonist concentration occurring within ca. 40–60 s, though responses to GR73632 and GR64349 were often somewhat slower in onset than the other agonists (see Figure 1). Responses to the novel NK_1 and NK_2 selective agonists GR73632 and GR64349 respectively, however, were consistently slower in offset than responses to the other tachykinins both after serial and cumulative dosing protocols. For example, washout of agonist responses after cumulative concentration-response curves showed the following times to return to pre-agonist twitch level; GR73632 and GR64349, 37 ± 7 min and 22 ± 6 min respectively compared to 12 ± 4 min and 12 ± 3 min for SPOMe and NKB respectively (see also Figure 1). For comparison, from an earlier series of experiments (see, Hall *et al.*, 1990) washout times after cumulative concentration-response curves to SP, NKA, NKB and Phy were 11 ± 2 min, 14 ± 3 min, 9 ± 3 min and 15 ± 4 min respectively.

Inhibitors or antagonists of several mechanisms implicated in tachykinin actions in other peripheral preparations were without significant effect on responses to various tachykinins. Thus, atropine (1 μM) or mepyramine (1 μM) were without effect on responses to SP, Ele, Phy, NKA and NKB; cimetidine (1 μM), ibuprofen (1 μM), naloxone (1 μM) or yohimbine (0.32 μM) were without effect on responses to SP, Ele or Phy and cocaine (2 μM) was without effect on responses to SP, Phy or [Glp^6,D-Pro^9]-SP$_{(6-11)}$ (data not shown).

Relative activities of tachykinin agonists

The log concentration-response relationships shown in Figure 2 are for three separate series of experiments where, in each series, several tachykinins were assayed along with SP. The

Table 1 Relative activities of tachykinins in the field-stimulated guinea-pig vas deferens

Agonist	*Activity* ($-\log_{10}$)	s.e.mean	*Relative activity*	n
Substance P	6.2	0.07	1.00	52
Neurokinin A	5.9	0.16	0.56	24
Neurokinin B	5.7	0.12	0.36	24
Physalaemin	7.0	0.06	7.20	24
Kassinin	6.1	0.10	0.80	24
Eledoisin	6.3	0.10	1.50	24
Substance P methyl ester	6.0	0.07	0.78	24
[Glp^6,L-Pro^9]-SP$_{(6-11)}$	6.9	0.15	9.30	20
[Glp^6,D-Pro^9]-SP$_{(6-11)}$	5.3	0.11	0.23	20
Senktide	<4.5	—	<0.03	4
GR73632	8.7	0.08	120.00	8
GR64349*	5.6	0.05	0.05	8
[pGlu^5,Me-Phe^7,Sar^8]-SP$_{(5-11)}$	5.2	0.37	0.37	8

Activities of naturally-occurring and synthetic tachykinin agonists in the field-stimulated guinea-pig vas deferens preparation. The activity estimates shown, were calculated for individual preparations at the arbitrary level of 100% potentiation of the twitch response, and are given as the $-\log_{10}$ of the concentration required (M). Relative activities are expressed as compared to substance P (activity = 1.00) in that series of experiments.

* It should be noted that this analogue was inactive at $\leq 1\,\mu$M, but had marked activity at $> 10\,\mu$M (see, Results).

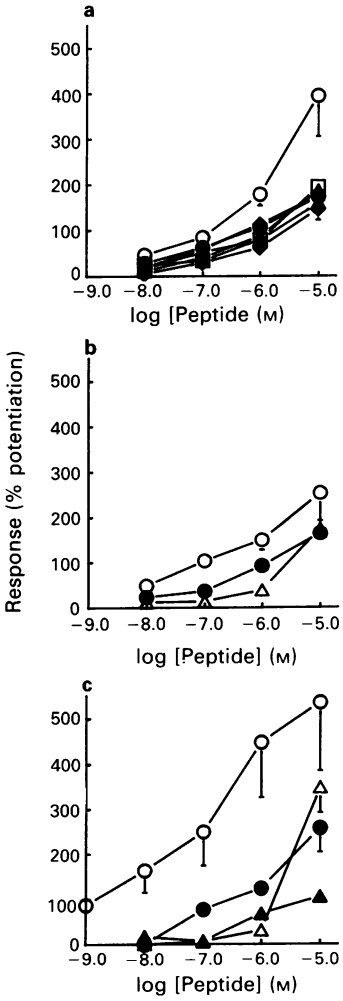


Figure 2 Log concentration-response curves to tachykinins from three series of experiments in the field-stimulated guinea-pig vas deferens preparation. (a) Substance P (●), neurokinin A (□), neurokinin B (◆), kassinin (△), eledoisin (◇), physalaemin (○) and substance P methyl ester (▲); (b) substance P (●), [Glp^6,L-Pro^9]-SP$_{(6-11)}$ (○) and [Glp^6,D-Pro^9]-SP$_{(6-11)}$ (△), and (c) substance P (●),[pGlu^5,MePhe^7, Sar^9]-SP$_{(5-11)}$ (▲), GR73632 (○) and GR64349 (△). Senktide was inactive (0.1 nM–32 μM). Experiments were carried out in Krebs solution at 37°C containing atropine and mepyramine (both 1 μM) and hexamethonium (10 μM). Each point is the mean for 8–24 preparations; s.e.mean shown by vertical bars. Responses are expressed as % increase in twitch height over basal normalised to responses to substance P (1 μM).

log concentration-response curves are rather shallow in all cases, with thresholds ca. 10 nM and maxima greater than 10 μM for active analogues, though true maxima could not be estimated for reasons of cost, solubility and possible solvent effects. It is evident that though tachykinins of roughly equal activity have log concentration-response curves with similar slopes, those of both the more active and least active agents in each series diverge from parallelism. Activity estimates shown in Table 1 are expressed relative to SP in that individual series. The absolute potency of SP did not differ significantly ($P > 0.05$) between the different series.

Estimated pK_B values for neurokinin receptor antagonists

Table 2 shows estimates of apparent pK_B values for the antagonist [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP$_{(1-11)}$ when tested against responses to several tachykinin agonists. This antagonist gave lower apparent pK_B estimates when tested against SP, SPOMe and [Glp^6,L-Pro^9]-SP$_{(6-11)}$ than against the other agonists. It should be pointed out that rigorous Schild plot analysis of antagonism by [D-Arg^1,D-Pro^2,D-Trp^{7,9}, Leu^{11}]-SP$_{(1-11)}$ over a reasonable concentration-range was not possible because of the low potency of the antagonist in this system, with the consequence inaccuracies in estimation of small dose-ratios. However, this antagonist at 10 μM did not produce a parallel shift to the right of the concentration-response line to all those agonists against which it was active

Table 2 Estimated apparent pK_B values for [D-Arg^1,D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP$_{(1-11)}$ against tachykinins in the guinea-pig vas deferens

Agonist	pK_B	s.e.mean	n
Substance P methyl ester	<5.0	—	6
Substance P	5.0	0.2	6
[Glp^6,L-Pro^9]-SP$_{(6-11)}$	5.0	0.2	6
Neurokinin A	5.6	0.1	6
Eledoisin	5.6	0.2	6
GR64349	5.9	0.1	5
Physalaemin	5.8	0.1	5
GR73632	6.0	0.1	5
[Glp^6,D-Pro^9]-SP$_{(6-11)}$	6.1	0.3	4
Neurokinin B	6.2	0.2	6

pK_B[D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]-SP$_{(1-11)}$ values were estimated from calculation of dose-ratios in individual preparations using the Gaddum-Schild equation $pK_B = \log(x - 1) - \log[Ant]$ where x is the dose-ratio and [Ant] the applied antagonist concentration.

<5.0 denotes inactive at 10 μM.

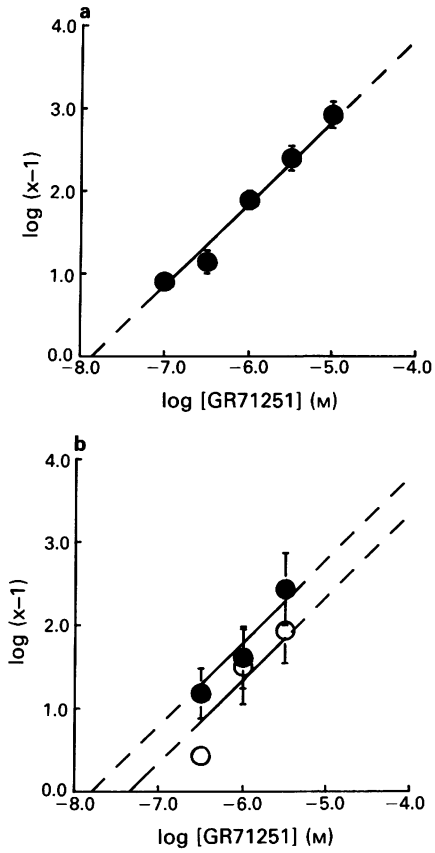


Figure 3 Schild plots for the NK_1 selective antagonist GR71251 against (a) the NK_1 selective agonist substance P methyl ester (●) in the guinea-pig ileum in the presence of atropine, (b) the NK-1 selective agonist GR73632 (●), and the NK_2 selective agonist GR64349 (○) in the guinea-pig vas deferens. The slope estimates (b = 1.06, 1.25 and 1.25 for substance P methyl ester, GR73632 and GR64349 respectively) did not differ significantly from 1.00 ($P > 0.05$), so unity slopes were imposed in the Schild plot shown. Estimated pK_B values are given in Table 3.

which was rapidly reversible on washout. The antagonist had no action *per se* on the twitch response.

The novel NK_1 selective antagonist, GR71251, was much more potent as an antagonist but, in addition showed concentration-related inhibition of the twitch response at high concentrations (above 3.2 μM). Consequently a relatively lower concentration range (0.32–3.2 μM) was employed in obtaining the Schild plots shown in Figure 3b against the NK_1 selective agonist, GR73632, and the NK_2 selective agonist, GR64349. Table 3 shows pK_B estimates for GR71251 against a range of tachykinin agonists, and Analysis of Variance showed no significant evidence of agonist-dependent pK_B estimates (mean overall $pK_B = 7.58 \pm 0.13$, $n = 48$; $P > 0.05$). The maximum potentiation of responses to field stimulation by the four agonists was unchanged in the presence of this antagonist ($P > 0.05$). For the purpose of comparison, GR71251 was also tested, over a more extensive concentration range (0.1–10 μM), against NK_1 mediated (SPOMe) contraction of the guinea-pig ileum in the presence of atropine, and Schild plot analysis (Figure 3a) indicated a competitive interaction with a pK_B estimate of 7.86 (± 0.05, $n = 20$). In the ileum preparation, this antagonist was without effect on basal tension in its own right. In both preparations, the antagonism was fully reversible on washout. The overall pK_B for GR71251 in the vas deferens did not differ significantly from that obtained in the ileum ($P > 0.05$).

Table 3 Estimated pK_B values for GR71251 against tachykinins of differing subtype selectivities in the guinea-pig vas deferens

Agonist	pK_B	s.e.mean	n
Substance P methyl ester	7.61	0.25	10
GR73632	7.65	0.25	16
GR64349	7.43	0.22	15
Neurokinin B	7.57	0.28	7
Overall estimate	7.58	0.13	48

pK_B values for GR71251 were estimated from calculation of dose-ratios in individual preparations using the Gaddum-Schild equation $pK_B = \log(x - 1) - \log[Ant]$ where x is the dose-ratio and [Ant] the applied antagonist concentration. Analysis of Variance showed no significant difference ($P > 0.05$) between agonists, so an overall pK_B estimate is shown.

Discussion

This study investigated neurokinin receptors mediating potentiation of the twitch response to field-stimulation of the guinea-pig vas deferens *in vitro*.

Inhibitors and antagonists of several intermediary mediators implicated in tachykinin actions in other systems were without effect on responses to tachykinins. This suggests a direct interaction of tachykinins with neurokinin receptors at a pre- and/or post-junctional site. The site of action of tachykinins in the guinea-pig vas deferens is unclear. A post-junctional site on the smooth muscle has been suggested by Ellis & Burnstock (1989) who reported potentiation of the contractile response to exogenously applied α,β-methylene ATP (though not to noradrenaline) by SP, but failed to measure an increase in release of ATP or [^{3}H]-noradrenaline overflow. However, although we confirm that there is no increase in [^{3}H]-noradrenaline overflow in this preparation in response to several tachykinins of differing subtype selectivities, we fail to confirm potentiation of the contractile response to exogenously applied α,β-methylene ATP by SP or other tachykinins (data not shown). The presence of neurokinin receptors (NK_1) both on sensory nerve endings in the outer longitudinal muscle of the guinea-pig vas deferens, and post-junctional neurokinin (NK_1) receptors on the innner longitudinal smooth muscle has been proposed from autoradiographic studies (Mussop *et al.*, 1989). It therefore seems unresolved whether both pre- (on sensory or sympathetic) and post-junctional receptors are functionally involved in the potentiation of twitch responses by tachykinins. Atropine and hexamethonium were without effect on responses to tachykinins, thus discounting an interaction with parasympathetic nerves (data not shown). It should be noted in relation to later discussion, that autoradiographic studies detected only NK_1 binding sites in this preparation (Mussop *et al.*, 1989).

Neurokinin receptor subtypes; agonist studies

The relative activities of the naturally-occurring tachykinin agonists fall within less than one order of magnitude which suggests the presence of a predominant NK_1 receptor population according to the criteria originally defined by Lee *et al.* as SP-P (Lee *et al.*, 1982). These present data in the guinea-pig vas deferens confirm an early report by Watson *et al.* (1983), who, using the limited number of tachykinin analogues available at the time, also proposed the presence of an SP-P (NK_1) receptor population in this preparation.

This conclusion is further substantiated by results obtained with several subtype selective synthetic agonist analogues. The well established NK_1 receptor agonist SPOMe had an activity of 0.78 relative to SP, a value similar to that reported previously in this preparation (0.52; Watson *et al.*, 1983). [Glp^6,L-Pro^9]-$SP_{(6-11)}$ had an activity of 9.3 and the novel NK_1 receptor agonist, GR73632 an activity of 120. Furthermore, the ratio of activity of the L- and D-isomers of [Glp^6,*Pro^9]-$SP_{(6-11)}$ was 41, which also indicates interaction with an NK_1

receptor population (Lee *et al.*, 1986; Bristow *et al.*, 1987; Fletcher *et al.*, 1987). The established NK_3 selective agonist, senktide, was inactive (10 nM–32 μM). This agonist is inactive in several other NK_1 preparations (Laufer *et al.*, 1986a,b; Wormser *et al.*, 1986). The novel NK_2 agonist GR64349 had very little activity over the concentration-range 10 nM–1 μM, but at concentrations greater than 10 μM, potentiation of the twitch response was seen to give a very steep log concentration response curve in this region and approached the magnitude of effect of several other agonists. These latter results with GR64349 could be interpreted to indicate the presence of either a relatively subsidiary NK_2 sub-population in the preparation, or alternatively an interaction of GR64349 with NK_1 receptors as might be expected at such a high concentration of agonist, even if the agent is relatively selective. GR64349 has ca. 0.01 the activity of SP in some other NK_1 preparations (Hagan *et al.*, 1989). The former conclusion is supported by the antagonist data with GR71251 discussed below.

The agonist data discussed seem clearly to indicate the presence of a predominant NK_1 receptor population mediating the action of tachykinins. It should also be noted that we have previously investigated, in the guinea-pig vas deferens, factors related to in the interpretation of agonist studies for receptor classification, particularly those involving possible failure to reach an equilibrium or known biophase concentration due to diffusional barriers or degradation (for discussion, see Kenakin, 1987). We have studied the effect of several peptidase inhibitors on tachykinin activity estimates and kinetics of clearance in the guinea-pig vas deferens, and conclude that agonist breakdown is not a primary determinant of onset of agonist activity or peak response, though slower processes such as offset may well be affected (Hall *et al.*, 1990; see also, Watson, 1983a). This is unlike the situation in other slower responding preparations where peak responses to agonists may indeed be modified by agonist breakdown (eg Stephens-Smith *et al.*, 1988).

In the present series of experiments, it was shown that, in the absence of peptidase inhibitors, the offset times of responses after washout for the synthetic agonists GR73632 and GR64349 were more prolonged as compared to the other agonists. These observations offer support for the claims that these agonists are metabolically stable (Hagan *et al.*, 1989; see, Hall *et al.*, 1990).

Neurokinin receptor subtypes; antagonist studies

The neurokinin receptor antagonist [D-Arg^1,D-Pro^2,D-$Trp^{7,9}$, Leu^{11}]-$SP_{(1-11)}$ was tested against several tachykinins of differing subtype selectivities (see also, Watson, 1983b). The antagonist (10 μM), though active against most agonists tested, produced lower apparent pK_B when tested against SP, the NK_1 selective agonist, [Glp^6,L-Pro^9]-$SP_{(6-11)}$ and was inactive when tested against the NK_1 selective agonist, SPOMe. It should be noted that the antagonist was active when tested against the NK_1 selective agonist, GR73632, with an apparent pK_B of 6.0. That the agonist-dependent apparent pK_B estimates obtained with [D-Arg^1,D-Pro^2,D- $Trp^{7,9}$,Leu^{11}]-$SP_{(1-11)}$ could indicate the presence of a mixed NK_1 and NK_2 neurokinin receptor population is unlikely in view of this latter observation along with the data obtained with GR71251 discussed below.

The putative NK_1 selective antagonist, GR71251 (Hagan *et al.*, 1990; Ward *et al.*, 1990), was tested against agonists of differing subtype selectivity (SPOMe, GR73632, GR64349 and NKB) in the guinea-pig vas deferens. Schild plot analysis indicated a competitive interaction with a single population of receptors having recognition properties (pK_B = 7.58) virtually identical to those described previously in the guinea-pig ileum in the presence of atropine (pK_B = 7.72; Hagan *et al.*, 1990; Ward *et al.*, 1990), and confirmed in this study (pK_B = 7.86). GR71251 has a reported pK_B of 4.8 at NK_2 receptors (Ward *et al.*, 1990). In view of the similar pK_B estimates being obtained for the four different agonists in the guinea-pig vas deferens, and that the overall estimate is similar to that obtained against NK_1-mediated contraction of the guinea-pig ileum, these data indicate that GR71251 interacts with a homogeneous NK_1 receptor population in this preparation. Further, the pK_B estimate obtained against GR73632 was not significantly different from that obtained against GR64349, showing an interaction of the latter agonist with NK_1 receptors in this preparation.

In conclusion, evidence obtained in this present series of experiments suggests a direct interaction of tachykinins with a homogeneous NK_1 receptor population mediating potentiation of the contractile response to field stimulation in the guinea-pig vas deferens. This preparation has value as a model system where NK_1 modulation of sympathetic neurotransmission is to be studied.

We thank Glaxo Group Research for the kind gifts of GR71251, GR64349 and GR73632 and Merck, Sharp & Dohme for the kind gifts of succ-[Asp^6,N-$MePhe^8$]-$SP_{(6-11)}$, [Glp^6,L-Pro^9]-$SP_{(6-11)}$ and [Glp^6,D-Pro^9]-$SP_{(6-11)}$. Part of this work was carried out during the tenure of a M.R.C. Scholarship to J.M.H. We thank Simon Ireland for helpful comments on the manuscript.

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(Received August 9, 1990
Revised September 27, 1990
Accepted October 9, 1990)

Br. J. Pharmacol. (1991), **102**, 518–522 © Macmillan Press Ltd, 1991

Pharmacological profile of various κ-agonists at κ-, μ- and δ-opioid receptors mediating presynaptic inhibition of neurotransmitter release in the rat brain

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1 The potency, relative efficacy and selectivity of a series of κ-opioid receptor agonists at the μ-, δ- and κ-opioid receptors mediating inhibition of electrically-induced (radiolabelled) neurotransmitter release from superfused rat brain slices was determined.

2 With regard to their potencies at κ-receptors mediating inhibition of striatal [^{3}H]-dopamine release, the highest pD_2 value (8.7) was found for bremazocine and the lowest (7.1) for U50488; the pD_2 values for ethylketocyclazocine (EKC), tifluadom, U69593 and PD117302 were between 8.0 and 8.3. There were no marked differences between the relative efficacies of the κ-agonists (maximum inhibition being 60–70%). In contrast to the other κ-agonists, at a concentration of 1 μM, PD117302 caused a significant (25–40%) increase of the spontaneous efflux of tritium.

3 None of the κ-agonists significantly affected striatal [^{14}C]-acetylcholine (ACh) release, with the exception of a slight inhibitory effect of EKC. The δ-receptor-mediated inhibitory effect of [D-Ala^2, D-Leu^5] enkephalin (DADLE) on [^{14}C]-ACh release was antagonized in a concentration-dependent manner by bremazocine (0.1 and 1.0 μM) and also partially by EKC (1 μM), but not by the other κ-agonists. The pA_2 value for bremazocine as an antagonist at the δ-receptors involved was 8.0, compared to 7.6 for naloxone.

4 None of the κ-agonists significantly affected cortical [^{3}H]-noradrenaline (NA) release, with the notable exception of tifluadom, which strongly inhibited release by activating μ-receptors. The μ-receptor-mediated inhibitory effect of Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) on [^{3}H]-NA release was antagonized in a concentration-dependent manner by bremazocine and EKC, but not by the other κ-agonists. The pA_2 value for bremazocine as an antagonist at the μ-receptors involved was 8.2, compared to 8.6 for naloxone.

5 Thus, whereas U69593 and PD117302 display high potency and selectivity towards κ-opioid receptors, the potent benzomorphan κ-agonists bremazocine and EKC also appear to be strong μ-opioid receptor antagonists.

Introduction

Both in peripheral tissues and in the central nervous system opioid receptors can be separated into at least three pharmacologically distinct types, μ-, δ- and κ-receptors (Wood, 1982; Paterson *et al.*, 1983; Martin, 1984; Goldstein & James, 1984; Zukin & Zukin, 1984). Previous studies on presynaptic modulation of neurotransmitter release from rat brain slices have shown that activation of different opioid receptor subtypes may have different functional consequences. Thus, dynorphin and some other agonists with κ-opioid receptor selectivity, such as bremazocine and U50488, were found to inhibit the depolarization-induced release of [^{3}H]-dopamine from rat striatal slices, with little or no effect on the release of [^{14}C]-acetylcholine (ACh) (Mulder *et al.*, 1984; 1989; Schoffelmeer *et al.*, 1988; Werling *et al.*, 1988). On the other hand, Leu-enkephalin and many of its analogues appear to inhibit striatal ACh release through activation of δ-opioid receptors, without affecting dopamine release (Mulder *et al.*, 1984; 1989; Schoffelmeer *et al.*, 1988). Finally, μ- but not δ- or κ-receptor activation results in an inhibition of [^{3}H]-noradrenaline (NA) release from rat cortical or hippocampal slices and synaptosomes (Mulder *et al.*, 1984; 1987; Jackisch *et al.*, 1986; Werling *et al.*, 1987; Schoffelmeer *et al.*, 1988).

In the present study we have determined the potency, relative efficacy and selectivity of a series of κ-agonists at different opioid receptors mediating inhibition of neurotransmitter release in various regions of the brain. The drugs examined were the benzomorphans bremazocine (Römer *et al.*, 1980) and ethylketocyclazocine (EKC), the benzodiazepine analogue tifluadom (Römer *et al.*, 1982) and the cyclohexyl benzeneacetamide derivatives U50488 (Von Voigtlander *et al.*, 1983), U69593 (Lahti *et al.*, 1985; Clark *et al.*, 1988a) and PD117302 (Leighton *et al.*, 1987; Clark *et al.*, 1988b). Previous studies, investigating opioid agonist-induced inhibition of neurally-evoked contractions in smooth muscle preparations have shown that, in addition to acting as κ-agonists, bremazocine and other benzomorphans may behave as δ- and/or μ-opioid receptor antagonists (Gillan *et al.*, 1981; McKnight *et al.*, 1985; Corbett & Kosterlitz, 1986; Miller *et al.*, 1986; Sheehan *et al.*, 1986; Takemori *et al.*, 1986). Ligand-receptor binding studies have revealed that bremazocine and other benzomorphan κ-opioid agonists display quite high affinities for δ- and/or μ-binding sites in the brain (Gillan & Kosterlitz, 1982; Paterson *et al.*, 1983). Furthermore, a neurophysiological study (Dunwiddie *et al.*, 1987) has demonstrated that bremazocine, but not U50488, is a potent antagonist at μ-opioid receptors in the rat hippocampus. Therefore, in the present study the different κ-agonists were examined for possible antagonist actions at μ- and δ-opioid receptors mediating presynaptic inhibition of neurotransmitter release.

Methods

Preparation and incubation of brain slices

Male Wistar rats (160–200 g body weight) were decapitated and the brains rapidly removed. Neocortical and/or neostriatal slices (approx. 0.3 × 0.3 × 2 mm) were prepared, labelled and superfused essentially as described previously (Stoof *et al.*, 1982; Mulder *et al.*, 1984; 1989; Schoffelmeer *et al.*, 1988). In

brief, neocortical slices (about 100 mg fresh tissue weight) were incubated for 15 min in 2.5 ml Krebs-Ringer-bicarbonate medium containing 0.1 μM [^{3}H]-NA to label noradrenergic nerve terminals selectively. Similarly, striatal slices were incubated in medium containing 0.1 μM [^{3}H]-dopamine and 1 μM [^{14}C]-choline, resulting in a selective labelling of dopaminergic and cholinergic nerve terminals, respectively.

Superfusion of brain slices and addition of drugs

After labelling the slices were transferred to each of 24 chambers (volume 0.2 ml; 3–4 mg of tissue per chamber) of a superfusion apparatus and subsequently superfused (0.25 ml min^{-1}) with medium (gassed with 95% O_2 : 5% CO_2) at 37°C. After 40 min of superfusion (i.e. t = 40 min) the superfusate was collected in 10 min samples.

At t = 50 min the slices were exposed to electrical stimulation (biphasic square wave pulses, 1 Hz, 2 ms duration) for 10 min to induce calcium-dependent release of the radiolabelled neurotransmitters. For [^{3}H]-NA release from neocortical slices a current of 15 mA was used and for [^{3}H]-dopamine and [^{14}C]-ACh release from striatal slices 30 mA.

When tested as agonists the drugs were added to the superfusion medium 10 min before electrical stimulation of the slices and remained present until the end of the experiment. When tested as antagonists at μ- and δ-receptors the κ-agonists were added to the medium 20 min before stimulation and the respective μ- and δ-agonists, i.e. Tyr-D-Ala-Gly-(NMe) Phe-Gly-ol (DAMGO; 1 μM) and [D-Ala^2, D-Leu^5]enkephalin (DADLE; 1 μM), were added 10 min later. In each experiment quadruplicate observations were made. At the end of the experiment the radioactivity remaining in the tissue was extracted with 0.1 M HCl. In one series of experiments (see Figure 2) the μ- and δ-antagonist actions of bremazocine were analyzed further by a cumulative dose-response technique, developed previously for studying the pharmacological characteristics of presynaptic receptors in superfused brain slices and described in detail elsewhere (Frankhuyzen *et al.*, 1982). In brief, cortical slices labelled with [^{3}H]-NA or striatal slices labelled with [^{3}H]-dopamine and [^{14}C]-choline were subjected to continuous electrical field stimulation (biphasic square wave pulses, 1 Hz, 2 ms duration) from 40 min after beginning the superfusion (t = 40 min). In experiments with cortical slices a current of 15 mA was used and the superfusion medium routinely contained the NA uptake inhibitor desipramine (3 μM). In experiments with striatal slices the stimulation current was 30 mA and the superfusion medium contained the dopamine uptake inhibitor nomifensine (1 μM). Increasing concentrations of the μ-agonist DAMGO and the δ/μ-agonist DADLE were added cumulatively to the medium at 10 min intervals, from t = 50 to t = 80 min, either in the absence or presence of bremazocine or naloxone added to the medium from t = 20 min. In each experiment the inhibitory effect of DAMGO or DADLE was determined (in duplicate), by comparing the stimulation-evoked release of radiolabelled transmitter in its presence with the release found in control superfusion chambers not exposed to the agonist.

Calculation of release data

The radioactivity in the superfusion samples and tissue extracts was determined by liquid scintillation counting. The efflux of radioactivity during each 10 min collection period was expressed as fractional release, i.e. as a fraction of the amount of radioactivity present in the tissue at the beginning of the respective collection period. To calculate the electrically-evoked release of the radiolabelled neurotransmitter, the spontaneous efflux of radioactivity was subtracted from the total overflow of radioactivity during stimulation and the subsequent 10 min. The spontaneous efflux of radioactivity from striatal slices labelled with [^{3}H]-dopamine and [^{14}C]-ACh was 0.25–0.30% min^{-1} (^{3}H) and 0.20–0.25% min^{-1} (^{14}C), respectively, of total tissue radioactivity; the spontaneous efflux of tritium from cortical slices labelled with [^{3}H]-NA was 0.15–0.20% min^{-1}.

The electrically-evoked release of radioactivity (in excess of spontaneous efflux) from striatal slices amounted to 3–4% ([^{3}H]-dopamine) and 6–8% ([^{14}C]-ACh), respectively, of total tissue content and from cortical slices labelled with [^{3}H]-NA, 3.5–4.5%.

A detailed description of the calculation of the data obtained in the experiments using the cumulative dose-response technique has been published elsewhere (Frankhuyzen *et al.*, 1982). In these experiments the spontaneous efflux of radioactivity was determined separately in chambers in which the slices were not exposed to electrical stimulation.

The concentration-effect curves, Hill slopes and pD_2-values ($pD_2 = -\log EC_{50}$) were derived from the release data by the non-linear curve-fitting programme ALLFIT. Statistical analysis of the data was carried out with a two-way analysis of variance, followed by Duncan's multiple range test, using SPSS/PC + V2.0 (SPSS, Inc.).

Radiochemicals and drugs

1-[7-^{3}H]-noradrenaline (37 Ci mmol^{-1}), [7,8-^{3}H]-dopamine (47 Ci mmol^{-1}) and [methyl-^{14}C]-choline (50 mCi mmol^{-1}) were purchased from the Radiochemical Centre (Amersham) and DAMGO and DADLE from Bachem. The following drugs were kindly donated: bremazocine and tifluadom by Sandoz, ethylketocyclazocine by Stirling-Winthrop, U50488 (*trans* - 3,4 -dichloro - N - methyl - N - [2 - (1 - pyrrolidinyl) - cyclohexyl]benzene-acetamide) and U69593 by (5α,7α,8β-(−)-N-methyl - N - [7 - (1 - pyrrolidinyl) - 1 - oxaspiro(4,5)dec - 8 - yl] phenyl-benzeneacetamide) Upjohn and PD117302 ((±)-trans - N - methyl - N[2 - (1 - pyrrolidinyl) - cyclohexyl]benzo[b] thiophene-4-acetamide) by Parke-Davis.

Results

Striatal [^{3}H]dopamine release: κ-receptor agonism

All of the κ-agonists inhibited the electrically-evoked release of [^{3}H]-dopamine from striatal slices (Figure 1). At a concentration of 1 μM the κ-agonists slightly decreased (by 10–15%) the spontaneous efflux of radioactivity, with the exception of PD117302, which increased spontaneous efflux by 25–40%.

The concentration-response curves shown in Figure 1 were constructed by use of a non-linear curve-fitting programme and the computed maximal inhibitory effects and pD_2 values are given in Table 1. The highest pD_2 value (8.7) was found for bremazocine and the lowest (7.1) for U50488; the pD_2 values of the other κ-agonists were between 8.0 and 8.3 (Table 1). The maximal inhibitory effect of the κ-agonists was between approximately 60 and 70%, with the exception of PD117302 (78.5%) (Table 1).

Table 1 Inhibitory effects of various κ-opioid receptor agonists on the electrically-evoked release of [^{3}H]-dopamine from rat striatal slices and their apparent affinities (pD_2 values) for the κ-receptors involved

κ-Agonist	*Maximal inhibition*	*pD_2 value*
Bremazocine	57.3 ± 0.3%	8.7
Ethylketocyclazocine	62.9 ± 2.6%	8.0
Tifluadom	62.9 ± 0.3%	8.3
U50488	70.1 ± 2.5%	7.1
U69593	69.3 ± 0.5%	8.0
PD117302	78.5 ± 5.4%	8.3

Data were derived from the concentration-response curves (Figure 1), computed by use of the non-linear curve-fitting programme ALLFIT and represent means ± s.e.mean of 12–16 observations. The variability in the pD_2 values was small (s.e.mean values 0.1–0.2)

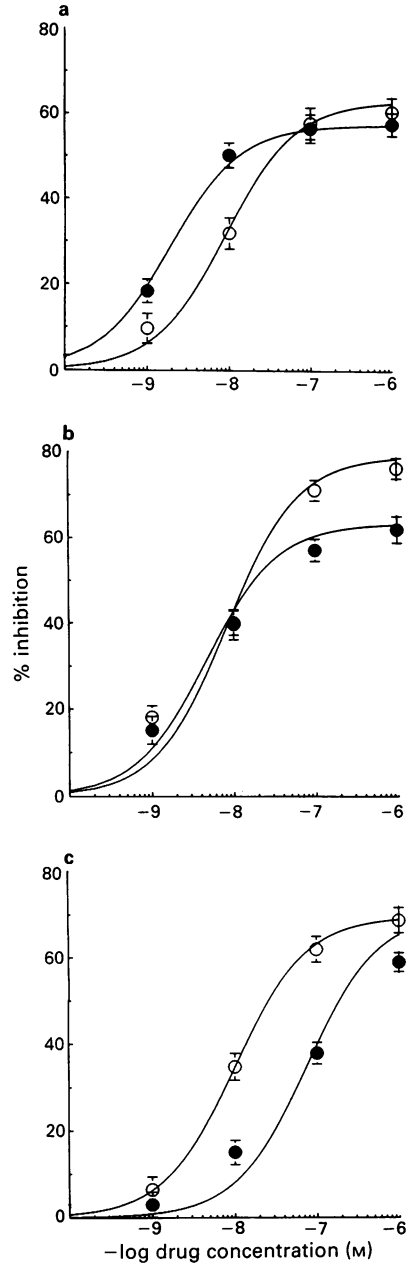


Figure 1 Inhibitory effects of various κ-opioid agonists on the electrically-evoked release of [^{3}H]-dopamine from rat striatal slices. The concentration-response curves were constructed by use of the non-linear curve fitting programme ALLFIT. The data points represent means of 12–16 observations (3–4 separate experiments); vertical bars show s.e.mean. (a) (●) bremazocine; (○) ethylketocyclazocine; (b) (●) tifluadom; (○) PD117302 and (c) (●) U50488; (○) U69593.

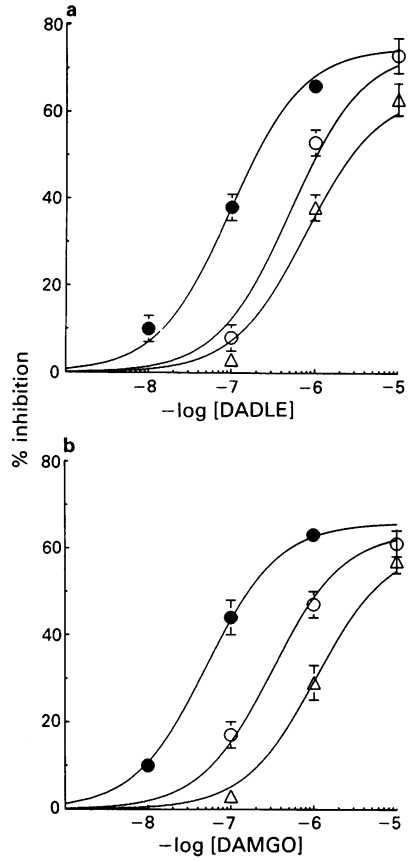


Figure 2 Competitive antagonism by bremazocine of (a) the δ-opioid receptor-mediated inhibitory effect of [D-Ala^2, D-Leu^5]enkephalin (DADLE) on (electrically-evoked) [^{14}C]-acetylcholine from striatal slices and (b) the μ-opioid receptor-mediated inhibitory effect of Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) on [^{3}H]-noradrenaline release from cortical slices. These experiments were carried out by use of a cumulative dose-response technique (see Methods). Data are means of 6 determinations (3 separate experiments); vertical bars show s.e.mean. (a) (●) DADLE alone; (○) DADLE + 10^{-7} M naloxone; (△) DADLE + 10^{-7} M bremazocine and (b) (●) DAMGO alone; (○) DAMGO + 10^{-8} M naloxone; (△) DAMGO + 10^{-7} M bremazocine.

Striatal [^{14}C]-acetylcholine release: δ-receptor agonism and antagonism

None of the κ-agonists significantly affected the electrically-evoked release of [^{14}C]-ACh from striatal slices, with the exception of EKC, which caused a slight inhibition (of about 25% at a concentration of 1 μM) (Table 2). This inhibitory effect of EKC was fully antagonized by 1 μM naloxone (data not shown). None of the drugs appreciably affected the spontaneous efflux of radioactivity.

Table 2 Antagonism by some κ-agonists of the δ-opioid receptor-mediated inhibitory effect of [D-Ala^2, D-Leu^5]enkephalin (DADLE) on the electrically-evoked release of [^{14}C]-acetylcholine ([^{14}C]-ACh) from rat striatal slices

	[^{14}C]-*ACh release (as % of control) in presence of:*			
κ-Agonist	κ-Agonist (1 μM)	DADLE (1 μM)	DADLE + 0.1 μM κ-agonist	DADLE + 1 μM κ-agonist
Bremazocine	89.4 ± 2.7	38.8 ± 2.4	62.3 ± 2.9**	79.3 ± 3.4**
EKC	73.7 ± 3.5†	30.3 ± 3.0	33.8 ± 2.5	48.3 ± 2.6*
Tifluadom	87.6 ± 2.9	38.0 ± 1.9	37.5 ± 2.0	44.3 ± 3.1
U50488	92.0 ± 2.5	41.3 ± 2.6	39.7 ± 3.3	40.6 ± 2.8
U69593	102.0 ± 2.7	31.8 ± 2.4	31.9 ± 3.0	30.7 ± 2.7
PD117302	95.3 ± 2.2	36.7 ± 2.5	40.9 ± 2.7	34.0 ± 2.2

Data represents means ± s.e.mean of 12–16 observations from 3–4 separate experiments.
† $P < 0.001$ (vs. control).
* $P < 0.01$ (vs. DADLE alone).
** $P < 0.001$ (vs. DADLE alone).

Table 3 Antagonism by some κ-agonists of the μ-opioid receptor-mediated inhibitory effect of Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) on the electrically-evoked release of [^{3}H]-noradrenaline ([^{3}H]-NA) from rat cortical slices

	[^{3}H]-NA release (as % of control) in presence of:			
κ-Agonist	κ-Agonist (1 μM)	DAMGO (1 μM)	DAMGO + 0.1 μM κ-agonist	DAMGO + 1 μM κ-agonist
Bremazocine	90.6 ± 2.6	31.2 ± 2.3	79.7 ± 2.9**	93.5 ± 3.2**
EKC	91.3 ± 2.8	27.9 ± 2.5	44.9 ± 3.1*	96.6 ± 3.5**
Tifluadom	39.6 ± 3.1†	22.9 ± 2.5	25.8 ± 2.7	27.7 ± 2.1
U50488	95.0 ± 2.6	24.0 ± 1.8	23.4 ± 2.8	25.6 ± 2.4
U69593	93.1 ± 3.0	32.6 ± 2.7	33.2 ± 3.0	40.3 ± 3.3
PD117302	94.5 ± 2.5	22.3 ± 2.6	23.1 ± 2.1	25.0 ± 2.9

Data represent means ± s.e.mean of 12–16 observations from 3–4 separate experiments. EKC = ethylketocyclazocine.
† $P < 0.001$ (vs. control).
* $P < 0.01$ (vs. DAMGO alone).
** $P < 0.001$ (vs. DAMGO alone).

DADLE (1 μM) inhibited [^{14}C]-ACh release by 60–70% and this inhibitory effect was antagonized in a concentration-dependent manner by bremazocine (0.1 and 1.0 μM). At a concentration of 1 μM EKC also partially antagonized the effect of DADLE, but the other κ-agonists did not display any antagonist action at this concentration (Table 2).

Cortical [^{3}H]-noradrenaline release; μ-receptor agonism and antagonism

None of the κ-agonists significantly affected the electrically-evoked release of [^{3}H]-NA from cortical slices, with the notable and unexpected exception of tifluadom, which strongly inhibited release ([^{3}H]-NA release 40.0 ± 2.8% of control at a concentration of 1 μM; $n = 12$). The inhibitory effect of 1 μM tifluadom was fully antagonized by naloxone ([^{3}H]-NA release 72.6 ± 4.5 and 102.2 ± 3.8% of control in the presence of, respectively, 0.01 and 0.1 μM naloxone). The spontaneous efflux of tritium was not significantly changed by tifluadom, nor by the other κ-agonists.

DAMGO (1 μM) strongly inhibited [^{3}H]-NA release (by 70–80%) and this inhibitory effect was antagonized in a concentration-dependent manner by both bremazocine and EKC (Table 3). At the highest concentration tested (1 μM) none of the other κ-agonists affected the inhibitory effect of DAMGO.

Further analysis of the δ- and μ-receptor antagonist actions of bremazocine: comparison with naloxone

In a final series of experiments the antagonist activity of bremazocine (0.1 μM) was tested against increasing concentrations (1 nM to 1 μM) of either DADLE ([^{14}C]-ACh release; δ-receptors) or DAMGO ([^{3}H]-NA release; μ-receptors), by a cumulative dose-response technique. For comparison, the antagonist activity of naloxone was also determined at concentrations of either 0.1 μM ([^{14}C]-ACh release) or 0.01 μM ([^{3}H]-NA release). The virtually parallel shifts to the right of the concentration-response curves (Figure 2) indicate that bremazocine is a competitive antagonist at both δ- and μ-opioid receptors. The pA_2 values for antagonism at the δ-receptors mediating inhibition of striatal [^{14}C]-ACh release, derived from the data of Figure 2, were 8.0 and 7.6 for bremazocine and naloxone, respectively. The pA_2 values for antagonism at the μ-receptors mediating inhibition of cortical [^{3}H]-NA release (Figure 2) were 8.2 (bremazocine) and 8.6 (naloxone).

Discussion

It has been well established that the inhibitory effects of opioids on the depolarization-induced release of [^{3}H]-dopamine and [^{14}C]-ACh from rat striatal slices are mediated exclusively by κ- and δ-receptors, respectively, whereas [^{3}H]-NA release from rat cortical slices is inhibited by opioids only if they display μ-agonist activity (Mulder *et al.*, 1984; 1989; Werling *et al.*, 1987; 1988; Schoffelmeer *et al.*, 1988). In the present study we have used these selective functional paradigms of the three major opioid receptor types in the brain to examine the potencies, relative efficacies and selectivities of a number of κ-opioid agonists, belonging to different chemical classes, with respect to these receptors.

As expected, all of the κ-agonists inhibited [^{3}H]-dopamine release, without pronounced differences in their relative efficacies, with the apparent exception of PD117302. It should be noted, however, that at the concentration which caused maximal inhibition of electrically-evoked [^{3}H]-dopamine release, PD117302 also significantly increased the spontaneous efflux of radioactivity. This almost certainly results in an overestimation of the inhibitory effect on evoked release, suggesting that in fact the maximal inhibitory effects of all κ-agonists tested fall within the range of 60–70%, i.e. under the experimental conditions used in the present study. With the exception of U50488, which had the lowest potency (pD_2 7.1), all κ-agonists showed pD_2-values of 8.0 or higher, up to 8.7 for bremazocine. A relatively low potency of U50488 compared to the benzomorphans was also demonstrated by others using peripheral tissue preparations (Hayes & Kelly, 1985; Miller *et al.*, 1986; Verlinde & DeCanter, 1988). The more recently developed κ-agonists U69593 and PD117302, as well as U50488, were the most selective ones, since they did not affect striatal [^{14}C]-ACh release or cortical [^{3}H]-NA release, nor did they possess δ- or μ-antagonist activity at concentrations up to 1 μM.

The benzomorphans, bremazocine and EKC, and the benzodiazepine analogue, tifluadom, appear to be less selective than the cyclohexyl benzeneacetamide derivatives but in different ways. Although bremazocine did not act as an agonist at the δ- and μ-opioid receptors mediating inhibition of neurotransmitter release, it appeared to be a strong antagonist at these receptors. This finding is in agreement with various studies (see Introduction), carried out with peripheral tissues or with ligand-receptor binding or neurophysiological studies in the brain. In the present study the pA_2 values of bremazocine as an antagonist at μ- and δ-receptors were estimated against DAMGO and DADLE as respective agonists. Although DADLE, in addition to being a δ-opioid receptor agonist, displays μ-agonist activity it was used in the present study as a selective agonist to activate the δ-receptors mediating inhibition of striatal [^{14}C]-ACh release, because of its relatively high potency at these receptors and the fact that μ-opioid receptor activation does not appreciably affect striatal [^{14}C]-ACh release (Schoffelmeer *et al.*, 1988; Mulder *et al.*, 1989). Compared to naloxone, the apparent affinity of bremazocine for μ-receptors was found to be about two times lower and that for δ-receptors about two times higher. The pA_2 values found for naloxone with respect to δ- (pA_2 7.6) and μ-receptors (pA_2 8.6) are in accordance with the well-known

preference of this antagonist for μ-opioid receptors (Paterson *et al.*, 1983).

EKC appeared to inhibit striatal [^{14}C]-ACh release slightly, an effect that was antagonized by naloxone and might reflect an agonistic effect on δ-receptors. Like bremazocine, EKC was found to be an antagonist at both δ- and μ-receptors. The pA_2 values of EKC were not determined, but the data suggest that EKC is equipotent with bremazocine as an antagonist at μ-receptors, but is far less effective at δ-receptors. Thus, in addition to being a potent κ-agonist, EKC appears to possess partial δ-agonist as well as potent μ-antagonist properties.

Tifluadom did not significantly affect [^{14}C]-ACh release, but it slightly counteracted the inhibitory effect of DADLE, which might indicate a slight δ-antagonist action. Surprisingly, tifluadom was found to be a rather potent agonist at the μ-opioid receptors mediating presynaptic inhibition of cortical [^{3}H]-NA release. Among the κ-agonists examined, tifluadom appears to be unique in combining agonist action at μ- and κ-opioid receptors without an appreciable effect on δ-receptors and in that sense it resembles the pharmacological profile of the opioid peptide dynorphin$_{1-13}$ (Mulder *et al.*, 1989).

In summary, in contrast to bremazocine, EKC and tifluadom, which appear to display notable differences in their pharmacological profile with respect to opioid receptors, U50488, U69593 and PD117302 are highly selective for κ-receptors. In terms of potency, efficacy and selectivity U69593 and PD117302 in particular appear to be valuable pharmacological tools for studying the physiological functions of κ-opioid receptors, although at concentrations in the micromolar range the latter drug appears to interfere with striatal dopaminergic neurotransmission in a manner not related to its κ-agonist action.

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(Received March 19, 1990
Revised September 28, 1990
Accepted October 3, 1990)

Br. J. Pharmacol. (1991), **102**, 523–529 © Macmillan Press Ltd, 1991

Characterization of the palytoxin-induced sodium conductance in frog skeletal muscle

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1 The effects of palytoxin (PTX) on transmembrane potentials and currents of frog skeletal muscle were analyzed by intracellular microelectrode techniques and the double sucrose-gap voltage clamp method.

2 PTX irreversibly depolarized the membrane. The depolarization was Na-sensitive.

3 Under voltage clamp, PTX induced an inward resting current which did not inactivate, was inhibited by external Na^+ removal and was a function of external Na concentration.

4 This resting current could be carried either by Na^+, Li^+, K^+ or by guanidinium according to the permeability sequence $K^+ < Li^+ < Na^+ < Gua^+$.

5 The PTX-induced current was only weakly sensitive to tetrodotoxin. It was reversibly and dose-dependently inhibited by amiloride with a one to one stoichiometry and a K_D of 0.3 mM.

6 Acidic pH partially inhibited the current induced by PTX which was also highly sensitive to external Cd^{2+} and La^{3+}. The inhibitory sequence for divalent cations was: $Mg^{2+} < Ca^{2+} = Ba^{2+} = Mn^{2+} < Cd^{2+}$; with $La^{3+} > Cd^{2+}$.

7 The amplitude of the PTX-induced I_{rest} was markedly reduced in the absence of external Ca^{2+}.

8 PTX induced a Na^+ resting conductance in frog skeletal muscle. The size of the channel induced by PTX is larger than the guanidinium ion. External membrane Ca^{2+} might be a cofactor involved in the mode of action of PTX.

Introduction

Palytoxin (PTX), a toxin isolated from a marine coelenterate by Moore & Scheuer, (1971), causes an irreversible sodium-dependent depolarization in many excitable cells (Dubois & Cohen, 1977; Sauviat & Pichon, 1977; Weidmann, 1977; Pichon, 1982; Tesseraux *et al.*, 1983; Sauviat *et al.*, 1987). It has been proposed that PTX acts as a Na ionophore in both *Torpedo* membrane vesicles (Stengelin *et al.*, 1983) and cardiac muscle (Sauviat *et al.*, 1987; Muramatsu *et al.*, 1988; Ikeda *et al.*, 1988). In the cockroach giant axon the PTX-induced depolarization was decreased in the presence of tetrodotoxin (Sauviat & Pichon, 1977; Pichon, 1982) while in frog nerve, it was sensitive to saxitoxin (Castle & Strichartz, 1988). Although it appears that sodium plays a central role in the action of PTX, the mechanism by which the toxin depolarizes excitable cells remains unidentified. The goal of the present work was to characterize the toxin-induced conductance in frog skeletal muscle membrane and to identify its mode of action.

Methods

Voltage clamp experiments were performed at 10–12°C on fine, cut end skeletal muscle fibres (100–200 μm in diameter, 5–6 mm in length) isolated from sartorius muscle of *Rana esculenta*. The double sucrose-gap technique with vaseline seals was used (Pater & Sauviat, 1987). The membrane voltage was held at −80 mV and the resting current (I_{rest}) recorded. In experiments performed on intact cleaned sartorius muscle, conventional intracellular microelectrode techniques were used (electrode resistance 20 to 30 MΩ; tip potential $\leqslant \pm 3$ mV) to record transmembrane potentials with a differential voltage follower and a fast flow experimental chamber similar to the recording system described by Sauviat (1990). The permeability ratio between sodium (Na^+) and the substituted ion (X^+) was calculated according to the Goldman-Hodgkin-Katz equation:

$$\Delta E_{rev} = RT/zF \ln(Px^+[X^+]_o)/(P_{Na^+}[Na^+]_o)$$

in which ΔE_{rev} was the reversal potential difference between the two ions; Px^+/P_{Na^+} the permeability ratio; $[X^+]_o$ and $[Na^+]_o$ the external concentration of both ions; T the absolute temperature; F the Faraday constant; z the valency of the ion and R the perfect gas constant.

It should be noted that each figure is representative of five to six experiments unless otherwise stated. Results are expressed as mean values ± s.e. of the mean; (*n*) corresponds to the number of preparations tested.

Transmembrane potentials and currents were displayed on an oscilloscope (Nicolet 310) and recorded on the mass storage unit of a desk-top computer (Hewlett Packard 9826) and plotted on an XY plotter (Ifelec or Hewlett Packard 7470) for further analysis.

The composition of the Ringer solution was (mM): NaCl 110.5, KCl 2.5 and $CaCl_2$ 2. The pH of the solution was maintained at 7.2 with HEPES buffer (5 mM). In experiments where differences in pH were studied, Tris acetylglycine KOH buffer (5 mM) was used to provide acidic pH and more basic pH values of 8 and 9.6 were obtained by use of TES buffer (5 mM) and $NaHCO_3$/NaOH buffer (5 mM) respectively. In Na-free solution, NaCl was isosmotically replaced by mannitol or, in some experiments by guanidinium chloride, LiCl or KCl. Tetrodotoxin (TTX) was used in the range 10^{-7} to 10^{-5} M. Cadmium, barium, magnesium, maganese and lanthanum were used as $CdCl_2$, $BaCl_2$, $MgCl_2$, $MnCl_2$ and $LaCl_3$ respectively. Calcium-depleted solutions were made by simply omitting $CaCl_2$ from the composition of the Ringer solution and in some experiments by addition of 10^{-4} M ethylene glycol tetraacetic acid (EGTA) to the calcium-depleted solution. The composition of the internal perfusing solution was (mM); K aspartate 120, ATP 5, phosphocreatine 5, $MgCl_2$ 2, HEPES buffer 5, pH 7.2 (Pater & Sauviat, 1987).

PTX was dissolved in distilled water at a concentration of 3.7×10^{-5} M and kept at 4°C. The stock solution was appropriately diluted just before use.

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Results

Characterization of the effect of palytoxin upon membrane resting potential and current and the effect of the external Na *concentration*

Figure 1a illustrates the effect of PTX on the membrane resting potential of an intact muscle. The toxin induced a slow and irreversible depolarization which reached a plateau. The resting membrane potential was decreased from -81.6 ± 1.7 mV in the control Ringer solution to -51.2 ± 5.2 mV in the presence of the toxin ($n = 8$). The depolarization induced by PTX was abolished when Na^+ ions were removed from the control solution.

Under steady-state voltage clamp conditions PTX (4×10^{-8} M) induced a marked inward resting current (I_{rest}) which did not inactivate and was abolished by the removal of Na^+ from the toxin-containing solution (Figure 1b). The addition of 25% of normal Na^+ to the sodium-free medium re-established an inward I_{rest} of smaller amplitude than control which was also suppressed upon return to sodium-free medium. Figure 1c shows that the inward I_{rest} induced by pre-washing the fibre with PTX was a function of $[Na^+]_o$. I_{rest} increased monotonically upon the addition of Na^+ and reached a plateau whatever the Na^+ concentration. In Figure 1d, the open circles represent a plot of I_{rest} induced by PTX against the logarithm of $[Na^+]_o$. It can be seen that I_{rest} shows no $[Na^+]_o$-dependent saturation in the concentration-range studied. The representation of the logarithm of I_{rest} as a function of the logarithm of $[Na^+]_o$ (Figure 1d) indicated that experimental data are best fitted by a straight line with a slope of unity suggesting a first order relation between Na ions and the effect of PTX on the membrane. The voltage-dependence of the Na conductance induced by the toxin is illustrated in Figure 2b. Experimental data are best fitted by a straight line which intercepted the membrane potential axis at +48 mV value closed to the apparent reversal potential for Na^+. The PTX-induced conductance determined from the slope of the I-V curves was 8.9 ± 1.8 μS ($n = 9$).

Total replacement of Na^+ by guanidinium ions (Gua^+) in the Ringer solution containing PTX generated an inward I_{rest} the amplitude of which was larger ($154 \pm 50\%$, ($n = 4$) at 0 mV) than that recorded in the presence of Na^+ (Figure 2a). The current was abolished after removal of the permeating ion (Na^+ or Gua^+) from the perfusing solution. Figure 2b shows that experimental data obtained with Gua^+ are best fitted by a straight line which crossed the abscissae at +76 mV. With Li^+ instead of Na^+ in the control solution, the inward current induced by PTX was of smaller amplitude and the apparent reversal potential shifted by 9 mV towards less positive membrane potentials. Total replacement of Na^+ by K^+ in the Ringer solution also shows that K^+ are able to carry the inward resting current in the presence of PTX with an apparent reversal potential of 0 mV. Results are summarized in Table 1 and suggest the following permeability sequence $K^+ < Li^+ < Na^+ < Gua^+$. Table 1 also shows that the conductance induced by the toxin, smaller in the presence of Li^+, was of similar magnitude when the current was carried by either Na^+, K^+ or Gua^+ and suggested the following conductance sequence $Li^+ < Gua^+ < K^+ < Na^+$. In three experiments performed in a mannitol-containing Na^+-free solution in the absence and in the presence of 2 mM Ca^{2+}, Ca^{2+} was found to carry an inward current in the presence of PTX with a conductance of 1.2 ± 0.2 μS and an apparent reversal potential of $+38 \pm 4$ mV.

Effect of tetrodotoxin and amiloride on palytoxin induced conductance

Figure 3a shows that TTX (5.7×10^{-7} M) did not inhibit the depolarization of the resting membrane potential induced by PTX. Under voltage clamp conditions, 5.7×10^{-7} M TTX

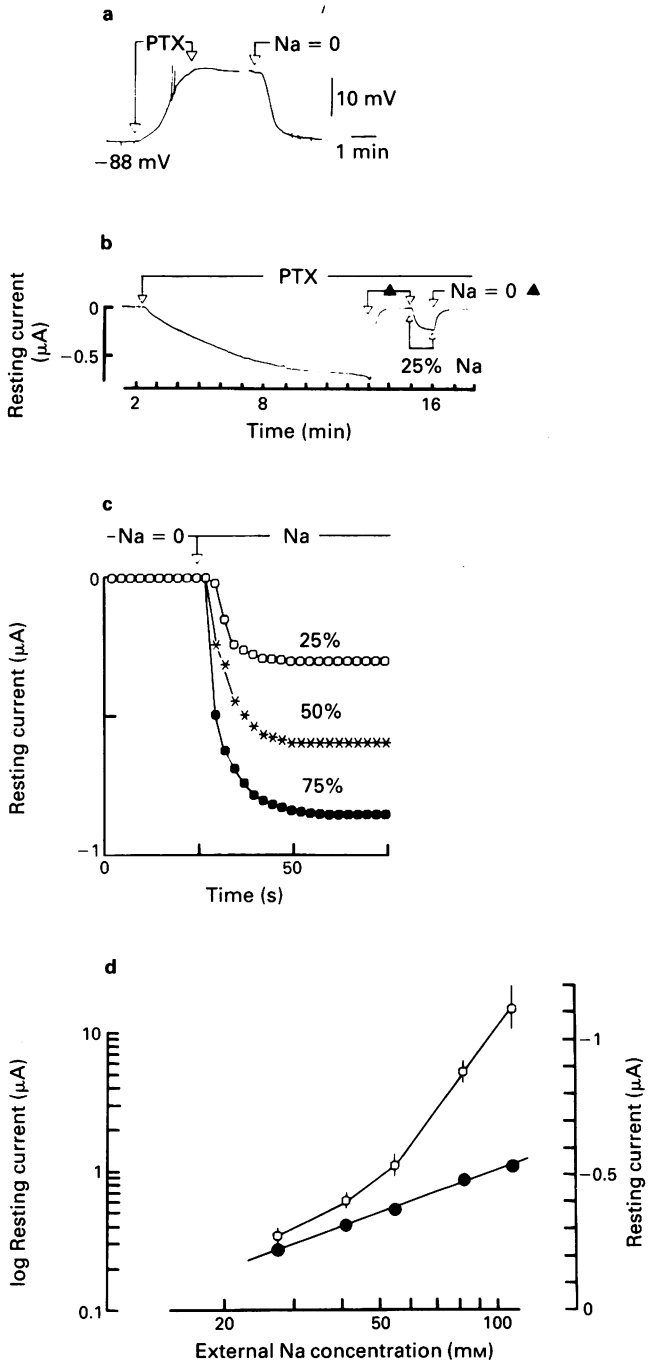


Figure 1 Effect of palytoxin (PTX) on the resting membrane potential and resting current (I_{rest}). (a) PTX (2×10^{-7} M) in Ringer solution irreversibly depolarized the resting membrane potential recorded in a whole cleaned muscle with an intracellular microelectrode. The depolarization induced by the toxin was reversed when external Na was replaced by mannitol (Na = 0). Interruption of the trace: 3 min of continuous record have been omitted. (b) PTX (4×10^{-8} M) induced an inward resting current which was suppressed when Na^+ was removed from the Ringer solution. A subsequent application of Na^+ (25%) induced an inward current of smaller magnitude than in normal Ringer solution. (▲) Na-free Ringer solution. The membrane potential was held at −80 mV 2 min before the toxin application. (c) Effect of variation of extracellular Na concentration ($[Na^+]_o$) on the inward resting current induced by PTX (4×10^{-8} M). I_{rest} induced by PTX was recorded during the application of: (○) 25%; (*) 50%; (●) 75% of normal $[Na^+]_o$ from a Na-free solution (Na = 0). Points corresponded to a digitization of the original records every 2.5 s. (d) Upper curve (○): I_{rest} induced by PTX as a function of the external Na concentration (ordinates, right scale). Points and vertical bars represent the mean and s.e. of I_{rest} ($n = 6$). Lower curve (●): logarithm of I_{rest} induced by PTX as a function of the logarithm of the external Na concentration (ordinates, left scale). The holding potential was −80 mV in b, c and d.

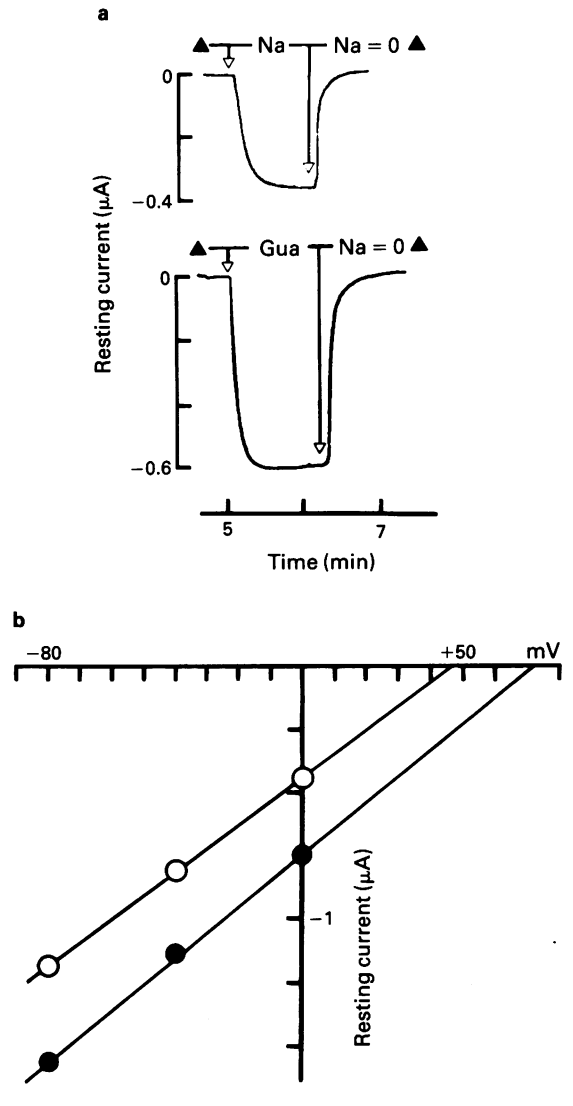


Figure 2 Selectivity of inward resting current induced by palytoxin (PTX, 4×10^{-8} M). (a) Effect of the addition of 110 mM Na (upper traces) or 110 mM guanidinium (Gua; lower traces) to the Na-free Ringer solution (Na = 0; ▲) containing the toxin, applied 5 min before each run, on the inward resting current induced by PTX recorded at 0 mV holding potential. (b) Current-voltage relationships for the inward current recorded as in (a). (○) Ringer solution; (●) solution containing guanidine.

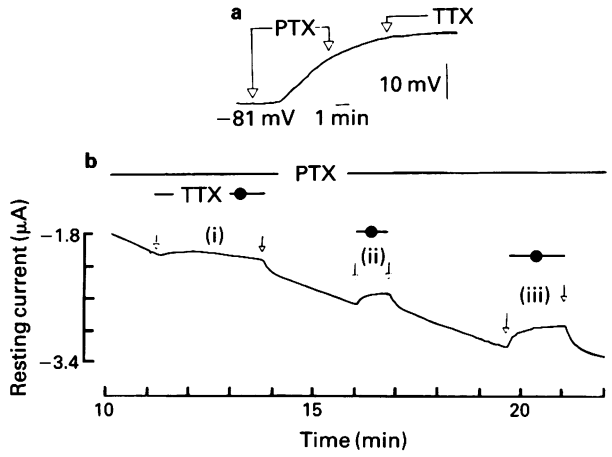


Figure 3 Effect of tetrodotoxin (TTX) on the palytoxin (PTX)-induced resting potential and inward resting current (I_{rest}) recorded in the normal Ringer solution. (a) TTX (6×10^{-7} M) did not suppress the depolarization induced by PTX (2×10^{-7} M). (b) Effect of successive (i, ii, iii) application of TTX (10^{-5} M; ●) on the PTX (4×10^{-8} M)-induced inward I_{rest} recorded at −80 mV holding potential. PTX was first applied 10 min before starting the experiment.

slowed the development of the inward I_{rest} induced by PTX (not shown). At a higher concentration, TTX (1×10^{-5} M) induced a small and reversible block of the inward I_{rest} (Figure 3b).

The addition of amiloride to the Ringer solution containing PTX rapidly and dose-dependently inhibited the inward I_{rest} induced by the toxin (Figure 4). After amiloride washout, the current recovered.

Effect of external pH on palytoxin-induced conductance

Fibres were first perfused with a sodium-free Ringer solution containing PTX and then solution containing Na was applied for each pH studied. Figure 5a shows that an inward I_{rest} developed monotonically and reached a plateau whatever the pH tested in the presence of Na^+. After sodium removal, the current rapidly recovered its initial value. Lowering the pH in the range 7.2 to 6.3 decreased the amplitude of the inward I_{rest} while increasing the pH in the range 7.2 to 8 increased it. The dose-response curve plotted for effect of external pH upon I_{rest} indicated that the amplitude of the current, taken as 100% at pH 7.2, is reduced by 42% at pH 5.5 and increased by 40% at pH 9.8 (Figure 5b). Figure 5b also shows that I_{rest} recorded in the absence of toxin depends weakly upon the external pH.

Table 1 Selectivity of the palytoxin (PTX, 4×10^{-8} M)-induced inward resting current in frog skeletal muscle fibres

Ion		*Conductance* (μS)	E_{rev} (mV)	P_x/P_{Na}	*Voltage-dependent Na channels* P_x/P_{Na}	P_x/P_{Na}	*Ionic radius* (Å)
Na	($n = 9$)	8.9 ± 1.8	+50.0 ± 6	1.00	1.00	1.00	0.95
Li	($n = 6$)	5.0 ± 1.2	+41.0 ± 3	0.69	0.96	0.87	0.60
K	($n = 5$)	7.9 ± 1.0	+1.6 ± 1	0.019	0.086	1.11	1.33
Gua	($n = 5$)	7.0 ± 3.0	+68.0 ± 6	2.09	0.093 (1) (frog muscle)	1.59 (2) (end plate)	2.40

The resting inward current induced by PTX was measured at different holding potentials, the slope conductance and the apparent reversal potential were determined as in Figure 2. The permeability ratio (P_x/P_{Na}) was calculated according to the Goldman-Hodgkin-Katz equation (see Methods).

The permeability ratio (1) and (2) are taken from Hille (1984). The ionic radius are given by Edwards (1982) and Hille (1984).

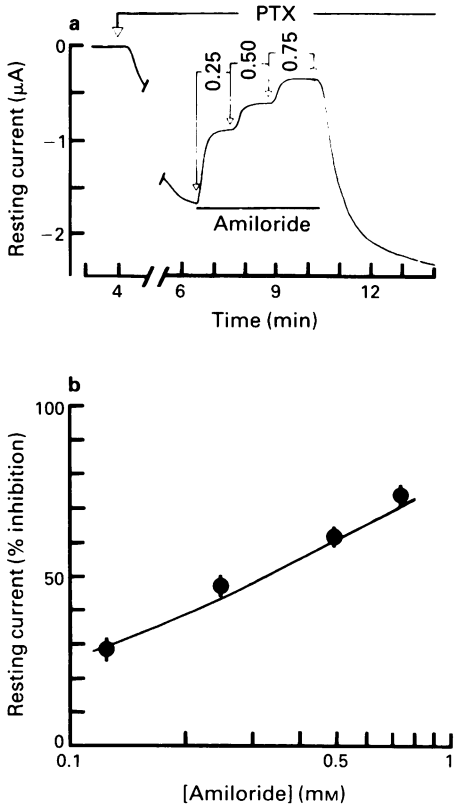


Figure 4 Effect of amiloride on the inward resting current induced by palytoxin (PTX, 4×10^{-8} M) at −80 mV holding potential. (a) Effect of successive applications of amiloride (0.25 mM; 0.5 mM; 0.75 mM). (b) Dose-response curve for the inhibition of the PTX-induced I_{rest} by amiloride. The line fitted to the data was estimated from the Hill equation with K_D of 0.34 mM and a slope of 1.02. Vertical lines represent s.e.mean of $n = 6$.

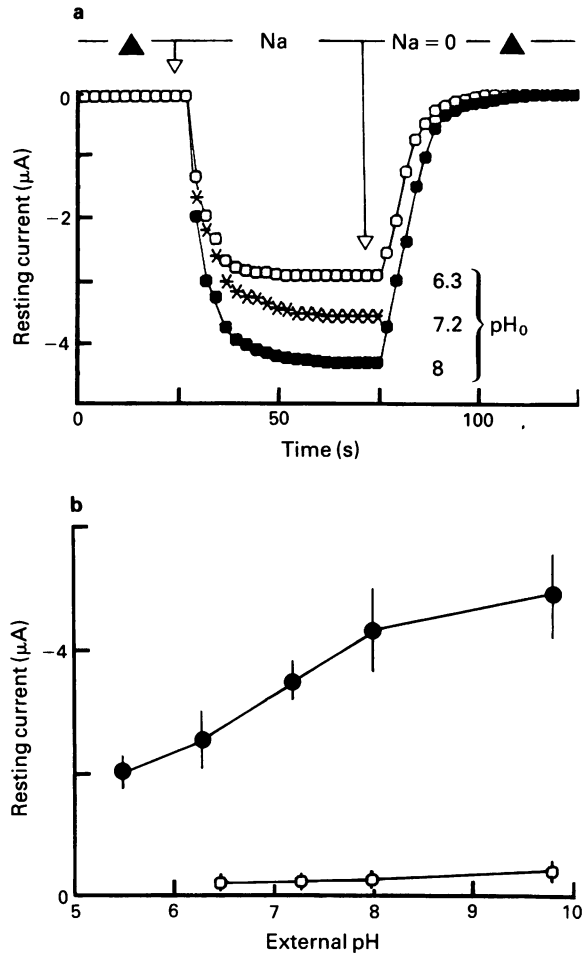


Figure 5 Effect of external pH on the inward current induced by palytoxin (PTX, 4×10^{-8} M) recorded at −80 mV holding potential. (a) The resting current induced by PTX was elicited by addition, for each pH tested, of 110 mM Na to the Na-free Ringer solution containing PTX at the chosen pH (Na = 0; ▲): (○) pH 6.3; (*) pH 7.2; (●) pH 8. The recovery phase of pH 7.2 has not been included for clarity since it lies between the other two curves. Points were digitized from the original records every 2.5 s. (b) The resting current magnitude recorded in the Ringer solution before (○) and after (●) PTX treatment as a function of the external pH. Vertical lines represent the s.e.mean of $n = 6$.

The logarithmic representation of I_{rest} as a function of the external pH was best fitted by a straight line with a slope of 0.03 in the absence and 0.45 in the presence of PTX.

Effect of external cations on palytoxin-induced conductance

Figure 6a shows that the addition of 20 mM Mn^{2+} to the Ringer solution containing PTX induced a moderate ($11.8 \pm 6.1\%$; $n = 4$) transient and reversible reduction of the inward I_{rest}. Increasing the external Ca^{2+}, Ba^{2+} or Mg^{2+} concentrations to 20 mM in the solution containing the toxin only transiently reduced the inward I_{rest} by $14 \pm 8\%$ ($n = 4$), 16.6 ± 1.4 ($n = 3$) and $3.6 \pm 1\%$ ($n = 3$) respectively. The addition of 6 mM Cd^{2+} to the Ringer solution containing PTX markedly and durably inhibited I_{rest} (Figure 6a). The inhibitory effect of Cd^{2+} was dose-dependent (Figure 6b) and saturated at concentrations larger than 10 mM. The inhibition reached 50% at about 3 mM Cd^{2+}. Figure 6c shows that the addition of 2 mM La^{3+} to the solution containing PTX also inhibited ($52.5 \pm 1.4\%$; $n = 4$) the inward I_{rest} which recovered rapidly after La^{3+} removal.

Figure 6d shows that a pretreatment of the fibre with Cd^{2+} did not prevent the effect of PTX though the inward I_{rest} was of smaller amplitude than that induced by the toxin in the absence of Cd^{2+} and the rate of development of the current in the presence of Cd^{2+} (1.0 ± 0.3 nA s^{-1}; $n = 3$) was decreased compared with the value obtained in the absence of Cd^{2+} (4.4 ± 0.2 nA s^{-1}; $n = 10$). Figure 6d also shows that removal of Cd^{2+} from the solution containing PTX led to an immediate increase of the amplitude of I_{rest} which was inhibited again when Cd^{2+} was reapplied.

Effect of Ca depletion on the development of the PTX-induced resting conductance

Figure 7a shows that the inward I_{rest} induced by the toxin in the calcium-free solution was of much smaller amplitude than the current recorded in the presence of Ca^{2+} (see Figure 1b) and the rate of development was slowed to 0.29 ± 0.07 nA s^{-1} ($n = 4$). In Figure 7b, the fibre was successively perfused with different solutions to reduce external membrane Ca^{2+} and to replace Ca^{2+} bound at the membrane surface by La^{3+} in order to study the effect of PTX on a calcium-depleted membrane (Figure 7b(i)). Figure 7b(ii) shows that the inward I_{rest} subsequently evoked by PTX was of very small amplitude compared with the current recorded in Ringer solution (Figure 1b) and the rate of the current development was even slower (0.16 ± 0.07 nA s^{-1}; $n = 3$) than that recorded in calcium-free solution.

Discussion

The present study shows that PTX depolarized the membrane in frog skeletal muscle by inducing an inward resting current (I_{rest}) which presented at least five properties. It was: (i) a func-

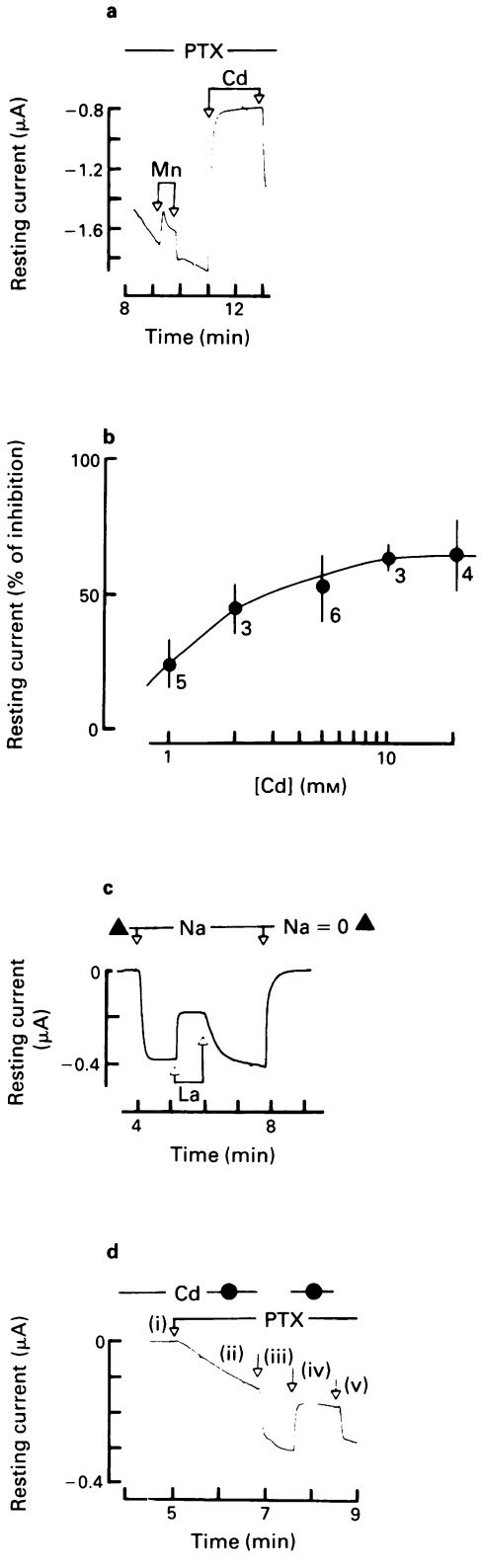


Figure 6 Effect of external cations on the inward resting current (I_{rest}) induced by palytoxin (PTX, 4×10^{-8} M). (a) Inhibitory effect of manganese (Mn; 20 mM) and cadmium (Cd; 6 mM) on I_{rest} induced by 8 min of PTX application. (b) Dose-response relation for the inhibitory effect of cadmium (Cd) on the inward I_{rest} induced by PTX. The line was fitted by eye. (c) Reversible blockade of the inward I_{rest} recorded by the addition of 110 mM Na in the Na-free Ringer solution containing PTX (Na = 0; ▲) by lanthanum (La; 2 mM). After recovery I_{rest} was suppressed by Na^+ removal from the Ringer solution. (d) Effect of PTX on the inward current in the presence of Cd^{2+} (20 mM; ●). (i) The membrane was bathed in a Ringer solution containing Cd^{2+} (20 mM). (ii) PTX was added to the control solution. (iii) Cd^{2+} was removed from the solution containing PTX, added again in (iv) and removed in (v). Holding potential was −80 mV in a, b, c, d.

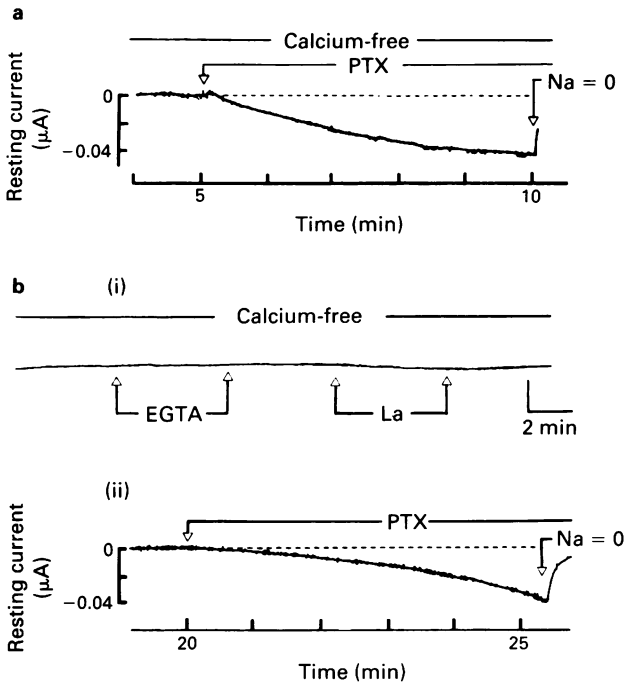


Figure 7 Effect of palytoxin (PTX, 4×10^{-8} M) on the resting current recorded at −80 mV holding potential after external Ca depletion. (a) Effect of PTX in Ca-free solution. The fibre had been bathed for 5 min in a Ca-free Ringer solution before PTX was added. (b) Effect of PTX on a Ca^{2+}-free and La^{3+}-treated membrane. (i) The membrane was bathed for 5 min each in a Ca^{2+}-free Ringer solution, Ca^{2+}-free solution containing 10^{-4} M ethylene glycol tetraacetic acid (EGTA), Ca^{2+}-free solution, Ca^{2+}-free solution containing La^{3+} (2 mM), Ca^{2+}-free solution and finally Ca^{2+}-free solution containing PTX. (ii) Continuation of trace (i) showing the development of the PTX current in Ca^{2+}-free solution and its suppression by removal of Na^+ from the solution containing the toxin. Dashed lines represent the zero current in (a) and (b).

tion of $[Na^+]_o$; (ii) highly permeable to guanidine and partially blocked by tetrodotoxin and amiloride; (iii) sensitive to external pH; (iv) reversibly inhibited by the cations Cd^{2+} and La^{3+}; (v) markedly reduced by external calcium depletion.

The inward I_{rest} induced by PTX was a direct function of $[Na^+]_o$ and suggested that PTX either forms a channel by which Na^+ enters the cells as suggested in frog heart muscle (Sauviat *et al.*, 1987) and mammalian cardiac myocytes (Muramatsu *et al.*, 1988; Ikeda *et al.*, 1988) or activates an existing, inactive Na^+ channel.

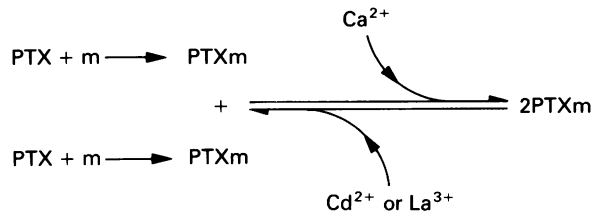
Li^+, considered to be a fairly good substitute for Na^+ as far as the TTX-sensitive Na channel is concerned (P_{Li^+}/P_{Na^+} = 0.96 in frog muscle according to Campbell, 1976), was less permeant (permeability ratio of 0.69) than Na^+ through the PTX-induced channel (see Table 1). A similar result has been reported in squid giant axon (P_{Li^+}/P_{Na^+} = 0.62) according to Muramatsu *et al.* (1984) and the frog heart (Sauviat *et al.*, 1987). Guanidinium ions which are considered as only weakly permeant through the voltage-dependent Na channel (Hille, 1975; Benoit & Dubois, 1987), are able to generate an inward current in the PTX-treated fibres. The permeability ratio P_{Gua^+}/P_{Na^+} of 2.09 was larger than the ratio reported for the voltage-dependent Na channel of the skeletal muscle of the frog (0.093; Campbell, 1976) or mammal (0.16; Pappone, 1980). It should be noted that a high permeability ratio for guanidine (1.59) has already been reported for the endplate Na^+ channel of the frog (Dwyer *et al.*, 1980). However, the endplate sodium channel allows Na^+ and K^+ to cross the membrane with nearly equal ease (Adams *et al.*, 1980) while, in the PTX-induced one, K^+ are only weakly permeant (Table 1). This suggests that the PTX-induced conductance did not use the known voltage-dependent Na channel but that PTX

formed its own channel. From the present results, the following selectivity sequence is proposed for the PTX-induced conductance: $K^+ < Li^+ < Na^+ < Gua^+$. This allows us to suggest that the size of the PTX-induced pore should thus be at least as large as the diameter of Gua^+, estimated to be 4.8 Å (Edwards, 1982). Such a pore size might account for the permeability of large organic cations (hydroxymethylamine, methylhydroxylamine, methylamine, ammonium) and Cs^+ reported by Castle & Strichartz, (1988) in frog sciatic nerve and Ca^{2+} in the present work. However, pore size does not directly determine the conductance which is mainly determined by binding of ions to the channel. The conductance sequence ($Li^+ < Gua^+ < K^+ < Na^+$) is different from the permeability sequence ($K^+ < Li^+ < Na^+ < Gua^+$) indicating that permeating ions bind to the channel. This observation strongly suggested the existence of binding sites in the channel which attract ions when they pass from one side of the membrane to the other.

Due to the existence of a guanidinium group in tetrodotoxin and amiloride, it is tempting to think that this is responsible for the effect of these molecules on the PTX-induced conductance. Tetrodotoxin, at about hundred times the concentration required to inhibit entirely voltage-dependent Na channels in frog muscle (Pater & Sauviat, 1987), only reduced the PTX-induced current. The rapid onset of inhibition and the rapid reversal suggested that amiloride blocked the inward I_{rest} induced by PTX by acting from the outside of the membrane. The unity stoichiometry of the reaction amiloride-I_{rest} indicated a relation between one amiloride molecule and one PTX pore. Amiloride perhaps plugs the PTX formed channel by means of its guanidine group which interacts with the Na^+ site, as is generally proposed for the blocking action of the molecule on the Na channel in epithelial membranes (Sariban-Sohraby & Benos, 1986; Eaton & Hamilton, 1988). We should emphasise that the selectivity of the amiloride-blockable Na^+ channel is different from that induced by PTX in the sense that Li^+ could traverse the channel more easily than Na^+ while ammonium, hydroxylammonium, hydrazinium and Cs^+ are essentially impermeant (Garty & Benos, 1988). The rapid inhibition and recovery of the PTX-induced current by TTX and amiloride suggested that both molecules do not bind to a specific site or receptor at the PTX channel input but behave mainly as simple cationic molecules. The results do point out the existence of an antagonism between PTX and TTX in frog muscle as well as in the giant axon of the cockroach (Sauviat & Pichon, 1977; Pichon, 1982). Moreover, Castle & Strichartz, (1988) have shown that saxitoxin, an analogue of TTX, reduced the depolarizing effect of PTX in frog nerve.

Acidic pH solution is known to inhibit the voltage-dependent Na conductance in heart (Chesnais *et al.*, 1975) and skeletal muscle (Campbell & Hille, 1976). Although no apparent effect of pH in the range 6.2 to 8.4 on the PTX-induced depolarization was found in frog nerve (Castle & Strichartz, 1988). We observed that acidic pH solution blocked the PTX-induced current whereas, basic pH facilitated the conducting property of the PTX pathway in skeletal muscle. According to Frelin *et al.* (1990), PTX increased or decreased the intracellular pH depending on the value of extracellular pH in chick embryonic ventricular cells suggesting that PTX opened a pre-existing H^+ conductive pathway. In the present study, PTX modifies by a factor of about ten the slope of the relation between the resting current and the external pH indicating that the PTX-induced conductance decreased with the proton activity. This might suggest that the toxin activates the Na^+/H^+ antiporter whose sodium flux is known to be amiloride – sensitive. Although the antiporter can be activated by the toxin via a mechanism that is distinct from that of phorbol ester (Frelin *et al.*, 1990), it cannot be considered as the only process responsible for the Na influx induced by PTX since the ionic selectivity of the PTX-induced conductance differed from the selectivity of the Na^+/H^+ exchanger; Li^+ is a good substitute for Na^+ and Cs^+ presents no appreciable affinity for the exchanger (Mahnensmith & Aronson, 1985), whereas, concerning the PTX-induced conductance, Li^+ is a relatively poor substitute for Na (this work) and Cs^+ is almost as permeable as Na^+ (Castle & Strichartz, 1988). On the other hand, the perfect reversibility of the effect of external pH changes also suggest that pH might (i) induce changes in the molecular configuration around a pKa value, (ii) alter the insertion of the molecule into the membrane, (iii) modify the degree of availability of negative surface charges and (iv) alter the amount of Ca^{2+} bound to the external side of the membrane. Low pH is known to displace Ca^{2+} from the phospholipids phosphate and carboxyl groups (Seimiya & Ohki, 1973; Hille *et al.*, 1975).

Divalent cations inhibited the PTX-induced Na current. The following sequence for the inhibitory effect of the divalent cations can be deduced: $Mg^{2+} < Ca^{2+} = Ba^{2+} = Mn^{2+} < Cd^{2+}$; La^{3+} was found to be more effective than Cd^{2+}. Cd^{2+} did not protect the membrane against PTX since the toxin-induced inward current developed immediately after Cd^{2+} removal, an observation suggesting that Cd effect occurred in the vicinity of the external surface of the membrane. According to Sorensen *et al.* (1985), Cd^{2+} might be able to occupy anionic sites on the phospholipid head group of the membrane which would otherwise be occupied by Ca^{2+}. La^{3+} is known to replace Ca^{2+} and to bind firmly and almost irreversibly to the membrane (Langer & Franck, 1972). Experiments in which the membrane has previously been depleted of Ca^{2+} and bathed in a solution containing La^{3+}, show that the effect of PTX is markedly delayed and smaller than in the presence of Ca^{2+} and suggested that PTX binds to the membrane even when it has been depleted of Ca^{2+} and when negative surfaces charges have been occupied by Cd^{2+} or La^{3+}. This suggests that external membrane Ca^{2+} plays an important role in the action of PTX and might appear as a cofactor. It could be suggested that Ca^{2+} either activated an unidentified subunit of the toxin or facilitated the formation of a dimer, since two molecules of PTX are involved in the resting Na^+ conductance development of the frog heart muscle (Sauviat *et al.*, 1987) and in neuroblastoma cells (Rouzaire-Dubois & Dubois, 1990). On the basis of the present results, the following model might be proposed to account for the mode of action of PTX:



PTX first binds either to the membrane phospholipids or an unidentified membrane receptor called (m) and forms a complex (PTXm) which is responsible for the poor conducting state observed in calcium-depleted membranes (Figure 7). Then two nearby PTXm are associated or activated by external Ca^{2+} which behave as a cofactor and lead to the development of the high conducting state (2PTXm). This high conducting state can be deactivated by external Cd^{2+} or La^{3+}, and drive back to the low conduction state (PTXm).

The authors thank Dr I. Findlay for helpful discussion and reading of the manuscript. Part of this work was supported by a grant from the Association Française contre les Myopathies.

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(Received June 22, 1990
Revised September 3, 1990
Accepted September 26, 1990)

Br. J. Pharmacol. (1991), **102**, 530–532 © Macmillan Press Ltd, 1991

Influence of lorcainide on microsomal Na^+, K^+-ATPase in guinea-pig isolated heart preparations

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1 The effects of lorcainide on the myocardial Mg^{2+}-dependent, Na^+ and K^+-activated adenosine triphosphatase (Na^+, K^+-ATPase) were compared in guinea-pig heart preparations with those of ouabain, a specific inhibitor of the enzyme activity.

2 Both ouabain and lorcainide inhibited the microsomal Na^+, K^+-ATPase activity in a concentration-dependent fashion. Their inhibitory effective ranges were 0.05–100 μM and 0.15–125 μM respectively, and the concentrations for half maximal inhibition (IC_{50} values) were 2.1 ± 0.3 and 33.5 ± 7.3 μM, respectively.

3 In a second series of experiments, the combined effects of the two drugs on the enzyme activity were studied. In these experiments, lorcainide produced a concentration-dependent potentiation of the inhibitory effects of ouabain on Na^+, K^+-ATPase activity.

4 The present study demonstrates that lorcainide is a potent inhibitor of myocardial Na^+, K^+-ATPase.

Introduction

Lorcainide is an acetanilide derivative which possesses both local anaesthetic and antiarrhythmic properties. In experimental animals, it elevates the ventricular fibrillation threshold and protects against ouabain-induced arrhythmias (Carmeliet *et al.*, 1978; Almotrefi & Baker, 1981). Clinically, it is gaining an important place in the therapy of recurrent premature ventricular contractions (Mead *et al.*, 1985), recurrent ventricular tachycardia and repetitive atrial tachycardias (Bär *et al.*, 1981; Saksena *et al.* 1983; Jaillon & Drici, 1989). Like other class 1 antiarrhythmic drugs, the primary electrophysiological effect of lorcainide is the reduction of the rate of rise of action potential during phase O (Carmeliet *et al.*, 1978). Because of the slow recovery kinetics of the sodium ion channel, it has been classified as a class IC antiarrhythmic agent (Campbell, 1983; Vaughan Williams, 1984; Harrison, 1986). Accordingly, it probably exerts its antiarrhythmic drug actions primarily by inhibiting the sodium channels in a voltage- and frequency-dependent fashion (Catterall, 1987; Sheldon *et al.*, 1987; 1989).

Although the electrophysiological actions of lorcainide certainly justify its place in this subclassification, very little information is currently available on its interactions with the sodium channels or any other membrane-bound protein that may be involved in the generation of the action potential. On the other hand, it has become increasingly evident that many antiarrhythmic agents have multiple ionic effects that may contribute to their antiarrhythmic actions (Bigger, 1984a). Thus, because of the diversity in the mechanisms responsible for the genesis of ventricular arrhythmias in particular, the antiarrhythmic drug actions of a single class or an individual agent may occur by more than one mode of interaction. Such possibilities include direct interactions with certain receptors, channels and exchange proteins (Ijzerman & Soudijn, 1989). In particular, because of its function in transmembrane active transport of monovalent cations, Na^+-K^+-ATPase appears to be a potential target for some of the antiarrhythmic drug actions, especially in combating digitalis-induced arrhythmias.

In view of this, we found it appropriate to investigate the effects of lorcainide on myocardial Na^+, K^+-ATPase and compare them with those of ouabain, a specific inhibitor of the enzyme activity, in an attempt to understand its mechanism of action.

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Methods

Enzyme preparation

Guinea-pigs of either sex weighing 0.6 – 1.1 kg were killed by a blow on the head and bled. The heart was removed rapidly and placed in ice-cooled isotonic sucrose buffer (180 mM). Myocardial Na^+, K^+-ATPase was prepared according to a modified method of Fricke & Klaus (1974). Four guinea-pig hearts were minced in a bio-homogenizer (Biospec Products Inc., USA) and homogenized with Stedfast Stirrer (Fisher Scientific) for 10 min in the sucrose buffer to give a 15% suspension.

The homogenate was centrifuged for 15 min at 14,000 *g* (Sorvall Instruments, Model RC5C Centrifuge). The supernatant was then filtered through a Millipore filter (type SC 8.0 μm, Millipore Corporation, Bedford, Mass., U.S.A.) and applied to a TSK Toyopearl Gel HW-55F column (Pierce Chemicals, U.S.A.) equilibrated with potassium chloride buffer (KCl 500 mM, imidazole 10 mM, Na_2 EDTA 1 mM, pH 7.4). Ascending elution of the enzyme was carried out at a flow of 0.7 ml min^{-1} maintained by means of a peristaltic pump (LKB Bromma). The fractions were collected and dialysed in a Visking dialysis membrane (type 20/32, Serva Finebiochemica, U.S.A.) three times, each time for at least 6 h with twice 10 mM first, and then once with 100 mM imidazole buffer, pH 7.4. All steps were carried out at 4°C. The enzyme was stored at the same temperature and could be used for up to five days without appreciable loss of activity. The purity of the enzyme so obtained has been checked and found to contain mitochondria-free particles.

Determination of enzyme activity

Enzyme assays were run at 37°C in the presence of different concentrations of the drug under test. The drug was pre-incubated together with 10–12 μg protein for 20 min in 100 mM imidazole buffer containing (in mM): Mg^{2+} 5, Na^+ 100, K^+ 5 and Na_2/EDTA 1, pH 7.4. The reaction was then initiated by adding ATP, the final concentration being 2 mM. The inorganic phosphate was determined spectrophotometrically after 20 min at 660 nm by the method of Eibl & Lands (1969), each assay being performed in duplicate. The Na^+, K^+-stimulated ATPase activity was calculated as the difference between the total activity and that of the Mg^{2+} and Na^+-dependent activity. All results were corrected for blank values without enzyme. The concentrations for half maximal inhibition (IC_{50})

of the Na^+, K^+-ATPase were calculated from individual concentration-effect curves as proposed by Hafner *et al.* (1977). The protein concentration of the enzyme preparation was determined with the Coomassie protein assay reagent (Pierce Chemicals, U.S.A.).

Drugs used were lorcainide hydrochloride (Janssen) and ouabain (Fluka AG). All the other reagents were of analytical grade. Values are given as mean ± s.e.mean, or mean and 95% confidence limits according to the suggestion of Fleming *et al.* (1972). Statistical significance was calculated with the Statgraphics software package version 3.0 (Graphic Software Systems, Inc., 1988). Probability levels of less than 0.05 were taken as indicating significant differences.

Results

Total inhibition of the Mg^{2+}-dependent Na^+, K^+-ATPase activity of the guinea-pig heart was achieved with 5.0 mM ouabain. The value obtained at this concentration was employed as control for the ouabain inhibitable activity of the enzyme. The concentration-response relationships of the actions of both ouabain and lorcainide on the ATP-hydrolysis by the Na^+, K^+-ATPase system are depicted in Figure 1. The inhibitory actions of the drugs are given as percentage decrease in enzyme activity (i.e. the activity of the Na^+, K^+-ATPase without the drug) and plotted as a function of the drug concentration. The specific activity of the enzyme was 331 ± 94 nmol Pi mg^{-1} protein min^{-1}.

Ouabain inhibited the Na^+-K^+-ATPase activity in a concentration-dependent fashion. Its inhibitory effective range, defined as the concentrations at which the drug inhibits the enzyme activity by 5–95% was 0.05–100 μM, exhibiting an IC_{50} value of 2.1 ± 0.3 μM. Similarly, lorcainide also exhibited concentration-dependent inhibitory effects on the enzyme activity in the range of 0.15–125 μM, with an IC_{50} value of 33.5 ± 7.3 μM. At this concentration-range, lorcainide showed no significant inhibitory action on the Mg^{2+}/Na^+ activity of the enzyme. These results are summarized in Table 1.

Concentrations of 2.5 and 5.0 μM ouabain inhibited the Na^+, K^+-ATPase activity by approximately 55 and 65% respectively. As shown in Figure 2, assays of lorcainide in the presence of either of these two ouabain concentrations resulted in a concentration-dependent potentiation of the inhibitory effects of ouabain.

Discussion

Ouabain is a cardiotonic steroid known to inhibit the catalytic hydrolysis of adenosine triphosphate by the myocardial Na^+, K^+-ATPase in a specific fashion (Smith *et al.*, 1984). The present observations with regard to its inhibitory actions are in complete agreement with those of other previous studies (Dzimiri *et al.*, 1987). These activities can therefore appropriately be used for a comparative assessment of the effects of lorcainide on this enzyme system.

The results of the present study demonstrate that lorcainide inhibits the myocardial Na^+, K^+-ATPase activity *in vitro* in a manner similar to that of ouabain. Its IC_{50} value also falls within pharmacologically relevant concentrations. The potency of lorcainide is in fact comparable to that of the dihydro-derivatives of cardiac glycosides (Dzimiri *et al.*, 1987), which are regarded as weak cardiotonic steroids. Although such interactions have been suggested for a number of other antiarrhythmic agents, only a few drugs such as amiodarone (Broekhuysen *et al.*, 1972; Prasada Rao *et al.*, 1986; Aomine, 1989) and bretylium (Dzimiri & Almotrefi, 1990) have actually been shown to interact with the Na^+, K^+-ATPase system at pharmacologically relevant concentrations.

The effective inhibitory concentration-range for lorcainide is relatively higher than its therapeutic levels. However, this is probably not a determinant factor in evaluating the potential pharmacological relevance of these effects. This argument is based on recent findings which demonstrate that several pharmacological effects associated with antiarrhythmic drug actions, particularly those of the local anaesthetic type, such as the inhibition of the Na^+ current, occur at concentrations much higher (1 μM–10 mM) than the therapeutic concentrations (Catterall, 1987).

Our studies have also demonstrated that lorcainide potentiates the inhibitory effects of ouabain on the enzyme activity.

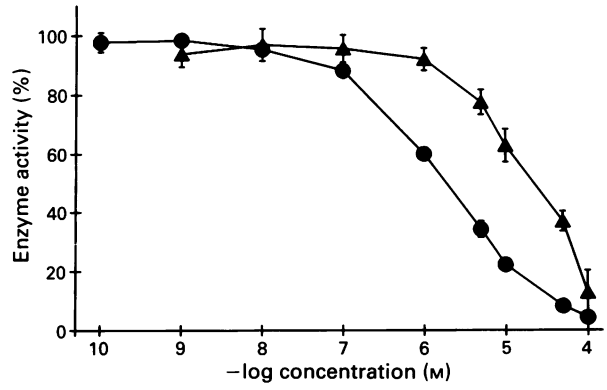


Figure 1 Influence of increasing concentrations of ouabain (●) and lorcainide (▲) on the activity of guinea-pig myocardial Na^+, K^+-ATPase. The total activity (100%) of the enzyme preparations was 24.7 ± 5.4 μmol Pi mg^{-1} protein h^{-1}. Plotted are the mean values of 7 and 8 individual determinations; s.e.mean shown by vertical bars.

Table 1 The effects of ouabain and lorcainide on the Mg^{2+}-dependent, Na^+, K^+-ATPase activity of the guinea-pig heart

Drug	n	*IE-range** (μM)	IC_{50}** (μM)
Ouabain	7	0.05–100	2.1 (1.9 – 2.3)
Lorcainide	8	0.15–125	33.5 (28.5 – 38.4)

* The inhibitory effective range, defined as the concentration-range at which the drug inhibits 5–95% of the enzyme activity.

** The median inhibitory concentration, defined as the concentration required to inhibit the enzyme activity by 50%. IC_{50} values are expressed as means (and 95% confidence limits).

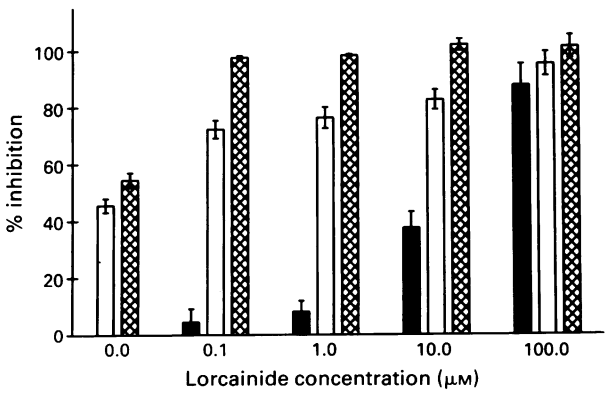


Figure 2 Influence of different concentrations of lorcainide alone (solid columns), lorcainide + 2.5 μM ouabain (open columns) and lorcainide + 5.0 μM ouabain (hatched columns) on guinea-pig myocardial Na^+, K^+-ATPase activity. The first points represent the percentage inhibition by 2.5 μM and 5.0 μM ouabain prior to the addition of lorcainide. The average baseline activity of the enzyme preparations was 26.4 ± 7.6 μmol Pi mg^{-1} protein h^{-1}. Plotted are the mean values of 6–8 individual determinations; vertical bars show s.e.mean.

This finding may indicate that the interaction between lorcainide and the Na^+, K^+-ATPase probably involves the same receptor sites that are responsible for the inhibition by ouabain. Receptor binding studies should furnish supportive data in order to determine whether these interactions are competitive in nature or not. On the other hand, further studies are obviously necessary to determine the implications of the present findings with regard to the antiarrhythmic actions of lorcainide. Assuming that lorcainide may have some different component from ouabain in its Na^+, K^+-ATPase inhibitory action, it seems reasonable to suggest at this stage that these inhibitory effects of lorcainide may be of some importance with regard to the possible mechanism involved in combating digitalis toxicity. The inhibition of the Na^+, K^+-ATPase by cardiotonic steroids is believed to be the mechanism underlying digitalis toxicity (Smith *et al.*, 1984). This is also associated with the production of the transient inward current (Kass *et al.*, 1978). Since this current is probably carried by a pathway that is permeable to both sodium and potassium ions, and its inhibitors are known to exert important effects on digitalis-induced arrhythmias (Kass *et al.*, 1978), a mechanism could be envisaged whereby lorcainide may indirectly impede it by its direct actions on the Na^+, K^+-ATPase activity.

Furthermore, according to the argument recently advanced by Ijzerman & Soudijn (1989), any interaction with membrane-bound proteins such as receptors, channels and exchange proteins involved in the generation of action potential can result in pronounced changes in its shape. It also appears from electrophysiological data that most antiarrhythmic agents are not specific for any one of such proteins and may exert their effects by more than just one mechanism (Bigger, 1984b; Ijzerman & Soudijn, 1989). For example, both local anaesthetic types of antiarrhythmic agents and β-adrenoceptor blockers are thought to exert membrane stabilizing effects by interacting with the lipid bilayer and structural proteins. These effects probably contribute considerably towards the mechanism for their antiarrhythmic actions, particularly those of β-adrenoceptor blockers (Ijzerman & Soudijn, 1989).

We may conclude, therefore, that pharmacological concentrations of lorcainide do inhibit myocardial ouabain-sensitive ATP-hydrolysis by Na^+, K^+-ATPase. These actions are probably due to direct interactions with the enzyme system itself. However, further studies are necessary to establish the pertinence of these inhibitory actions particularly with regard to its observed electrophysiological effects.

We would like to thank Dr L. Tritsmans (Janssen Research Foundation) for the generous gift of lorcainide hydrochloride. Our thanks also go to Ms Shirley Aglibut for her skilled technical assistance in carrying out the experiments.

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(Received April 25, 1990
Revised July 18, 1990
Accepted September 26, 1990)

Br. J. Pharmacol. (1991), **102**, 533–539 © Macmillan Press Ltd, 1991

Chemosensitive cardiopulmonary afferents and the haemodynamic response to simulated haemorrhage in conscious rabbits

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1 We set out to test whether the signal from the heart that initiates the decompensatory phase of acute central hypovolaemia in conscious rabbits is conveyed by chemosensitive afferents.

2 Haemorrhage was simulated by inflating an inferior vena caval cuff so that cardiac output fell at a constant rate of 8% of its baseline level per min. After sham or vehicle treatments the haemodynamic response had two phases. In the first, sympathoexcitatory, phase systemic vascular conductance fell in proportion to cardiac output so that mean arterial pressure fell by only 13 mmHg. When cardiac output had fallen by ~50% a second, sympathoinhibitory, phase supervened. There was an abrupt rise of systemic vascular conductance and an abrupt fall of mean arterial pressure, to ~40 mmHg.

3 The sympathoinhibitory phase was prevented by injection of the δ-opioid antagonist ICI 174864 (100–300 nmol) or the μ-opioid agonist H-Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAMGO) (100–300 pmol) into the fourth cerebral ventricle.

4 5-HT_3 receptors on myocardial or pulmonary afferents were excited by injection of ascending doses of phenylbiguanide (6.25–400 μg) into the left or right atrium respectively. Neuronal-type nicotinic cholinoceptors in the epicardium were excited by injecting ascending doses of nicotine bitartrate (6.25–400 μg) into the pericardial sac. Each of these treatment regimens caused a reproducible, dose-dependent, fall in mean arterial pressure. Intravenous injection of the 5-HT_3 antagonist MDL 72222 (1.0 mg kg^{-1}) markedly attenuated the responses to phenylbiguanide. Intrapericardial injection of the neuronal-type nicotinic cholinoceptor antagonist mecamylamine HCl (0.1 mg kg^{-1}) abolished the effects of intrapericardial nicotine. Neither of these treatments affected the haemodynamic response to simulated haemorrhage.

5 Injection into the fourth ventricle of ICI 174864 (100–300 nmol) or DAMGO (100–300 pmol) had no effects on the dose-response relationships for phenylbiguanide or nicotine.

6 We conclude that the cardiac afferents responsible for initiating the sympathoinhibitory phase of simulated haemorrhage in conscious rabbits do not correspond to the populations of phenylbiguanide-sensitive cardiopulmonary afferents, nor to the population of nicotine-sensitive epicardial afferents. We also conclude that the reflex haemodynamic responses to atrial phenylbiguanide and intrapericardial nicotine do not depend on an endogenous δ-opioid receptor mechanism in the brainstem, and are not affected by exposure of the brainstem to exogeneous DAMGO.

Introduction

When phenylbiguanide is injected into the left or right artrium it excites two anatomically distinct sets of afferents that originate, respectively, in the left side of the heart and close to the pulmonary vasculature (Evans *et al.*, 1990a). The effect of phenylbiguanide is mediated by pharmacologically-specific 5-HT_3 receptors (Wright & Angus, 1988; Evans *et al.*, 1990a). The afferents run mainly, though not exclusively, in the vagus nerves. When nicotine bitartrate is injected into the pericardial sac it excites afferents that originate in the epicardium (Evans *et al.*, 1990b). These afferents are not sensitive to phenylbiguanide. The effect of nicotine is mediated by pharmacologically-specific neuronal-type nicotinic cholinoceptors. The afferents travel exclusively in the vagus nerves. The reflex hypotension resulting from excitation of these afferents is chiefly due to a fall in peripheral resistance, presumably due to sympathoinhibition (Evans *et al.*, 1990b; Evans & Ludbrook, unpublished observations).

Sympathoinhibition resulting from the excitation of cardiac afferents is also a feature of one phase of the response to acute haemorrhage, or simulated haemorrhage, in unanaesthetized rabbits. At first, peripheral resistance increases along with the fall of cardiac output, so that mean arterial pressure is maintained at a near-normal level (Ludbrook & Graham, 1984; Schadt *et al.*, 1984; Ludbrook & Rutter, 1988; Ludbrook *et al.*, 1988). The maintenance of blood pressure is associated with a progressive rise in sympathetic drive (Burke & Dorward, 1988; Morita *et al.*, 1988) and plasma noradrenaline concentration (Schadt & Gaddis, 1985), so that this first phase can be described as sympathoexcitatory. When about 30% of blood volume has been withdrawn (Schadt *et al.*, 1984; Burke & Dorward, 1988; Ludbrook & Rutter, 1988), or when cardiac output has fallen by about 50% (Evans *et al.*, 1989a, b), a second phase begins in which there is an abrupt decrease in sympathetic activity, peripheral resistance and mean arterial pressure. This sympathoinhibition is initiated by a signal from the heart (Burke & Dorward, 1988; Evans *et al.*, 1989a) and depends on activation of a δ-opioid receptor mechanism within the central nervous system (Evans *et al.*, 1989b). The sympathoinhibition of Phase 2 can also be prevented by injecting a specific μ-opioid receptor agonist into the 4th ventricle (Evans & Ludbrook, 1990).

The properties of the cardiac afferents responsible for the second, sympathoinhibitory, phase of acute hypovolaemia in rabbits are not known, but in rats there is circumstantial evidence that they may be sensitive to phenylbiguanide. Both at the onset of the hypotensive phase of haemorrhage (Victor *et al.*, 1989) and after injection of phenylbiguanide (Higuchi *et al.*, 1988) there is a sharp decline of renal sympathetic nerve activity but an increase of adrenal sympathetic nerve activity. As is the case in rabbits, an endogenous opioid mechanism appears to mediate the severe hypotension resulting from haemorrhage in rats (Faden & Holaday, 1979).

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We have set out to determine whether one or more of the populations of chemosensitive afferents described above might be responsible for the sympathoinhibitory phase of hypovolaemia, by testing the effects of a δ-opioid receptor antagonist and a μ-opioid receptor agonist on the chemoreflexes. In addition it is unclear whether 5-hydroxytryptaminergic or cholinergic mechanisms are essential to the putative physiological function of these chemosensitive cardiopulmonary afferents. We therefore tested the effects of blockade of 5-HT_3 receptors and of epicardial neuronal-type nicotinic cholinoceptors on the haemodynamic response to simulated haemorrhage.

Methods

Six New Zealand White rabbits were used, weighing 2.40–2.71 kg (mean 2.57). Each rabbit was studied 4–6 times, at intervals of 2–5 days. The experiment was conducted in accordance with the Statement on Animal Experimentation by the National Health and Medical Research Council of Australia, and was approved by the Animal Ethics Committee of the Amalgamated Melbourne and Essendon Hospitals.

Surgical preparation

The methods used to prepare the rabbits have been described in detail previously (Evans *et al.*, 1989a,b; 1990a; Evans & Ludbrook 1990). In brief, they were as follows.

The rabbits were anaesthetized with a halothane/oxygen mixture after induction with i.v. thiopentone sodium (25 mg kg^{-1}) (Pentothal, Abbott) and endotracheal intubation. Buprenorphine HCl (60 μg s.c.) (Reckitt & Colman) was administered post-operatively as an analgesic. Each rabbit underwent three separate operations. During the first operation an inflatable cuff was placed round the thoracic inferior vena cava (caval cuff). Two to three weeks later an electromagnetic flow probe (Biotronex BL5050) was placed round the ascending aorta and an inflatable cuff round the descending thoracic aorta (aortic cuff). At the same operation a catheter was either placed in the pericardial sac (3 rabbits), or inserted into the left atrium through the tip of its appendage (3 rabbits). Two weeks later a catheter was introduced through the atlanto-occipital ligament so that its tip lay in the 4th ventricle. The first study was done 10 days later.

Preparations for studies

These were done under local analgesia with 2% procaine HCl (David Bull Laboratories). The rabbit was placed in a 15 × 40 cm box fitted with a wire mesh lid, 60 min before the beginning of the study. The ends of the catheters, the tubes leading to the cuffs, and connecting plug for the flow probe were retrieved from their subcutaneous positions. A catheter was introduced into the central ear artery and advanced to the root of the ear to measure arterial pressure. A catheter was also introduced into a marginal ear vein and advanced to the right atrium for drug administration. In some studies, a catheter was introduced into the contralateral ear artery to collect arterial blood samples.

Measurement of haemodynamic and respiratory variables

The ear artery catheter was connected to a Statham P23Dc strain gauge zeroed 50 mm above the floor of the rabbit's box. The flow probe was connected to a Biotronex BL-613 meter to measure ascending aortic flow (cardiac output). Heart rate was measured by a tachometer actuated by the flow pulse. A mercury-in-rubber strain gauge was placed around the thorax and connected to a bridge-circuit (Parks Electronics Laboratory) to monitor respiration and other movements. Signals were amplified and recorded on a Grass Model 7 Polygraph.

The output from the Grass Polygraph was sent to an Olivetti M24 computer with an analogue-to-digital converter which provided 1 or 10 s mean values of arterial pressure (MAP, mmHg), cardiac output (MCO, ml min^{-1}) and heart rate (MHR, beats min^{-1}). The computer also calculated mean values for cardiac index (MCI = MCO/body weight in kg) and systemic vascular conductance index (MSVCI = 10^2 MCI/MAP).

Analysis of blood samples

Duplicate measurements of haematocrit (Hawksley) were made before each day's study on 0.2 ml samples of ear vein blood. The haematocrit at the beginning of the first and last studies was 35.8 ± 1.2% and 34.0 ± 0.8% respectively. Po_2 and Pco_2 were measured on 0.6 ml samples of ear artery blood (Radiometer ABL_4 acid-base analyser).

Simulated haemorrhage

The caval cuff was inflated gradually by means of a micrometer syringe so that MCI fell at a constant rate of 13.32 ± 0.45 ml kg^{-1} min^{-1} per min (8.29 ± 0.15% of the baseline level per min). The cuff was released when MAP had fallen to ~40 mmHg or when MCI had fallen to 39.7 ± 1.5% of its resting level.

Baroreceptor-heart rate reflex

This was elicited by inflating the aortic and caval cuffs so that MAP rose or fell at 1–2 mmHg s^{-1} (Ludbrook, 1984).

Drugs

Unless otherwise indicated, these were made up in 154 mM NaCl (saline) on the day of a study. All doses were calculated as the salt. Sham treatments consisted of the equivalent volume of vehicle.

Cardiopulmonary chemoreceptor excitants Nicotine bitartrate (TCI) and 1-phenylbiguanide (Aldrich) were administered as successive bolus doses of 6.25, 25, 100 and 400 μg in 200 μl. Phenylbiguanide was injected into the left or right atrium at 10 min intervals. Nicotine was injected into the pericardial sac at 30 min intervals. These doses cover the full range of the dose-response relationship (Evans *et al.*, 1990a,b).

Cardiopulmonary chemoreceptor antagonists These were the 5-HT_3 receptor antagonist MDL 72222 (1αH, 3α, 5αH-tropan-3yl-3,5-dichlorobenzoate) (Fozard, 1984a, b) (Research Biochemicals Inc.) and the neuronal-type nicotinic cholinoceptor antagonist mecamylamine HCl (Van Rossum, 1962) (Sigma). MDL 72222 (1.0 mg kg^{-1}) was injected i.v. in a volume of ~2.5 ml over 1 min followed by an infusion at 0.05 mg kg^{-1} min^{-1}. Mecamylamine (0.1 mg kg^{-1}) was given intrapericardially in a volume of 200 μl, repeated at 60 min intervals. We have shown that these regimens shift the dose-response curves for phenylbiguanide and nicotine approximately 16 fold to the right (Evans *et al.*, 1990a, b).

Intracerebroventricular drug administration The μ-agonist H-Try-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAMGO) (Handa *et al.*, 1981) (Sigma) was made up as 300 pmol per 15 μl in saline. The δ-antagonist ICI 174864 (N,N-diallyl-Tyr-(α-aminoisobutyric acid)$_2$-Phe-Leu-Oh) (Cotton *et al.*, 1984) (Cambridge Research Biochemicals) was made up as 50 nmol per 15 μl of alkalinized saline (0.1 M NaOH in 154 mM NaCl). Immediately prior to 4th ventricular administration this solution was brought to pH 7–8 by addition of 1.0 M HCl. The doses of DAMGO and ICI 174864 were respectively 100 or 300 pmol and 100, 200 or 300 nmol, administered at 30 min intervals.

Experimental protocols

First, we set out to test whether 4th ventricular administration of the δ-antagonist ICI 174864 or the μ-agonist DAMGO, in doses that prevented the occurrence of Phase 2 of simulated haemorrhage, would also attenuate the hypotensive responses to ascending doses of the chemoreflex excitants phenylbiguanide (left and right atrial) and nicotine (intrapericardial). Secondly, we tested the effects on the haemodynamic response to simulated haemorrhage of the 5-HT_3 antagonist MDL 72222 (i.v.) and the neuronal-type nicotinic cholinoceptor antagonist mecamylamine (intrapericardial), in doses that markedly antagonized the corresponding chemoreflexes.

The steps in each study followed were similar. (1) Vehicle was injected by the appropriate route, then a simulated haemorrhage was performed. (2) Thirty min later the dose-response relationship for intrapericardial nicotine, or left or right atrial phenylbiguanide was constructed. (3) Thirty min later the active treatment was given, and simulated haemorrhage was repeated. (4) Thirty min later, the active treatment was given by the appropriate route and the chemoreflex dose-response relationship was again constructed. (5) When the active treatment was 4th ventricular ICI 174864 or DAMGO, it was repeated 30 min later and a further simulated haemorrhage was performed.

Step (3) was sometimes varied in the case of 4th ventricular ICI 174864 and DAMGO, since it was necessary to establish a dose that prevented Phase 2 of simulated haemorrhage. If the first dose did not do so, the higher dose was tested after a 30 min recovery period.

When the active treatment was 4th ventricular DAMGO, arterial blood gases were measured before, and at 30 min intervals during steps 3–5 to test for respiratory depression.

When the active treatment was intrapericardial mecamylamine, the baroreceptor-heart rate reflex was elicited before and after Step 3, to test for cardiac vagal ganglionic blockade.

Order of studies

In the 3 rabbits with pericardial catheters the effects of treatment with intrapericardial mecamylamine, 4th ventricular ICI 174864 and 4th ventricular DAMGO on simulated haemorrhage and the dose-response relationship for intrapericardial nicotine were tested in randomized order (3 × 3 Latin square). Then a fourth study was done, in which none of these treatments were given, to serve as a time control.

In the 3 rabbits with left atrial catheters the effects of treatment with i.v. MDL 72222 and 4th ventricular ICI 174864 and DAMGO on simulated haemorrhage, and the dose-response relationship for left atrial phenylbiguanide were tested in randomized order. Then a fourth, time control, study was done in which none of the treatments were given.

In 2 of the rabbits with pericardial catheters and 1 with a left atrial catheter the effects of treatment with 4th ventricular ICI 174864 and DAMGO on simulated haemorrhage and the dose-response relationship for right atrial phenylbiguanide were tested in randomized order. The effects of MDL 72222 were not retested, nor was the time control study repeated.

Autopsy

At the conclusion of the experiments the rabbits were killed with i.v. pentobarbitone sodium (200 mg) (Nembutal, Ceva Chemicals). Autopsies were performed on all rabbits to confirm that the catheters were properly placed and that there were no signs of infection or adhesions in the 4th ventricle or pericardial sac.

Analysis of results

Data were analysed as described previously (Evans *et al.*, 1989a,b; 1990a; Evans & Ludbrook, 1990).

Two- or three-way analyses of variance (ANOVA) were used to evaluate effects of treatments (drugs or vehicles) on the resting levels of the haemodynamic and respiratory variables. If specific contrasts were made within the ANOVA the critical value of F was adjusted by the Bonferroni method.

Regression analysis confirmed that MCI fell linearly with time during the simulated haemorrhages ($r = 0.983 \pm 0.001$). As we have found previously (Evans *et al.*, 1989a), there was a near-linear relationship of MAP, MHR and MSVCI to MCI during simulated haemorrhage, up to the onset of Phase 2 when MSVCI rose abruptly. As before (Evans *et al.*, 1989a,b) we used a graphical method for analysing the effects of treatments on the responses of MSVCI, MAP and MHR to simulated haemorrhage. The relationships of MAP, MHR and MSVCI to MCI were characterized by three sets of co-ordinates: (a) Post-treatment levels, immediately before the onset of simulated haemorrhage. (b) The point at which MSVCI reached a minimum at the end of Phase 1, before rising abruptly. (c) The final observation before the caval cuff was deflated. These co-ordinates were averaged, first within and then between rabbits, so as to distinguish the effects of the different treatments. ANOVA was used to detect effects of treatments on the levels of the haemodynamic variables immediately before caval cuff deflation.

Mean log dose-response curves were constructed for the effects of intrapericardial nicotine and left and right atrial phenylbiguanide on MAP. ANOVA was used to detect effects of treatments (treatment × dose interaction).

Unless otherwise indicated, the levels of the variables are tabulated as between rabbit means ± 1 s.e.mean. The s.e.mean for grand means across rabbits, studies and treatments was calculated from the error sum of squares in ANOVA.

Results

The baseline levels of the haemodynamic variables were unaffected by the injection of vehicle by any route ($F \leqslant 1.3$; d.f. 3,

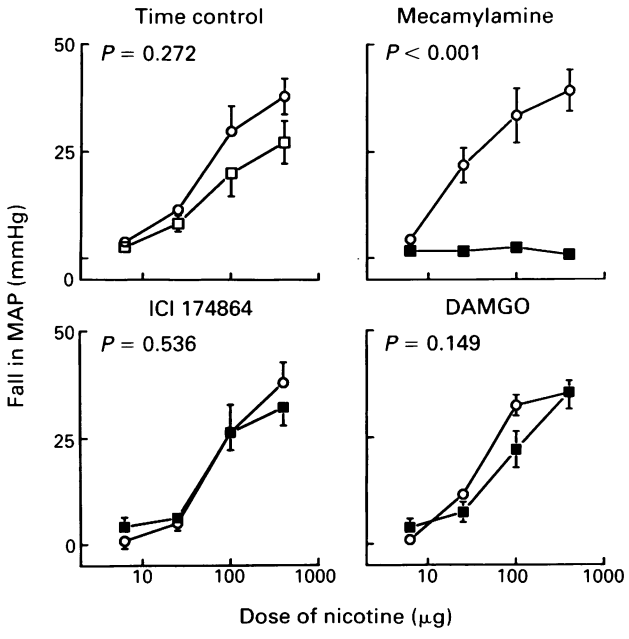


Figure 1 Dose-response relationships for intrapericardial nicotine according to the 4 studies, and treatments within studies. MAP, mean arterial pressure. Mean values for 3 rabbits. Vertical bars indicate 1 s.e.mean. Treatments are indicated by symbols: (○), no treatment (time control study) or vehicle (remaining studies), (□), no treatment (time control study), (■), intrapericardial mecamylamine (0.1 mg kg^{-1}), 4th ventricular ICI 174864 (100–300 nmol), or 4th ventricular H-Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAMGO, 300 pmol). P values attributable to dose × treatment interaction within ANOVA.

14; $P \geqslant 0.33$). Their grand mean values, across rabbits and studies, were: MCI, 164 ± 9 ml kg^{-1} min^{-1}; MSVCI, $194 \pm 11\,10^2$ ml kg^{-1} min^{-1} mmHg^{-1}; MAP, 85 ± 3 mmHg; MHR, 234 ± 13 beats min^{-1}.

In the time-control studies within the three protocols the pattern of the response to simulated haemorrhage, and the dose-response relations for atrial phenylbiguanide and intrapericardial nicotine, were usually unaffected by repetition ($F \leqslant 1.4$; d.f. 3, 14; $P \geqslant 0.27$). The exception was that the rabbits became supersensitive to the effects of left atrial phenylbiguanide (Figure 3). Across studies, the patterns of the responses to simulated haemorrhage after vehicle treatment were indistinguishable (Figures 2, 4 and 6). During Phase 1 MCI fell by 80 ± 6 ml kg^{-1} min^{-1}, MSVCI by $78 \pm 5\,10^2$ ml kg^{-1} min^{-1}, and MAP by 13 ± 2 mmHg. MHR rose by 61 ± 8 beats min^{-1}. When the caval cuff was deflated at the end of Phase 2, the corresponding changes from the baseline levels were 99 ± 6 ml kg^{-1} min^{-1}, $27 \pm 8\,10^2$ ml kg^{-1} min^{-1} mmHg^{-1}, 47 ± 2 mmHg and 37 ± 9 beats min^{-1}.

Intrapericardial mecamylamine and i.v. MDL 72222 had no effects on the haemodynamic variables. In the phenylbiguanide protocols 4th ventricular ICI 174864 and DAMGO had no effect, but in the intrapericardial nicotine protocol they lowered MHR by 28 ± 14 and 36 ± 20 beats min^{-1} respectively ($F \geqslant 7.1$; d.f. 1, 6; $P \leqslant 0.04$). Fourth ventricular DAMGO, in doses that prevented Phase 2 of simulated haemorrhage (100–300 pmol), lowered respiratory rate and caused a steady fall of Po_2 over 180 min from 88 ± 2 to 75 ± 3 mmHg and a steady rise of Pco_2 from 34 ± 1 to 46 ± 2 mmHg.

Intrapericardial nicotine protocol

Intrapericardial mecamylamine (0.1 mg kg^{-1}) did not affect baseline MHR. It did reduce the range of MHR in the baroreceptor-heart rate reflex from 137 ± 7 to 102 ± 18 beats min^{-1}, suggesting that there may have been a degree of cardiac vagal ganglionic blockade, though this effect was not consistent ($F = 3.2$; d.f. 1, 2; $P = 0.27$). It completely abolished the responses of MAP to intrapericardial nicotine (Figure 1), but had no effect on the responses to simulated haemorrhage (Figure 2).

Fourth ventricular ICI 174864 (100–300 nmol) and DAMGO (300 pmol) prevented the occurrence of Phase 2 of simulated haemorrhage (Figure 2), but had no effect on the responses of MAP to intrapericardial nicotine (Figure 1).

Left atrial phenylbiguanide protocol

MDL 72222 (1.0 mg kg^{-1}) markedly attenuated the responses of MAP to left atrial phenylbiguanide (Figure 3), but had no effect on the responses to simulated haemorrhage (Figure 4).

Fourth ventricular ICI 174864 (100 nmol) and DAMGO (100–300 pmol) prevented the occurrence of Phase 2 of simu-

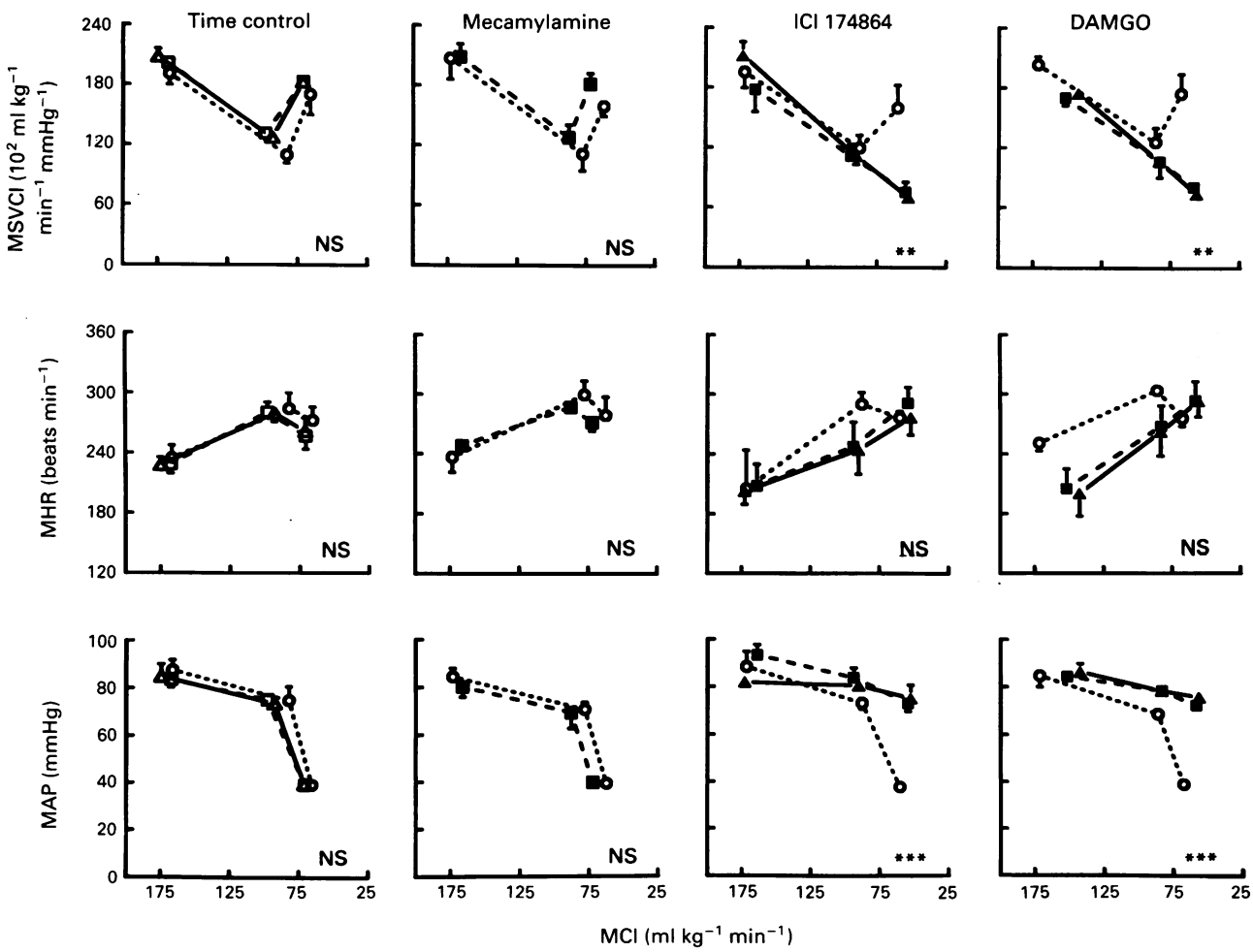


Figure 2 Haemodynamic effects of simulated haemorrhage in intrapericardial nicotine protocol according to the 4 studies, and treatments within studies. MSCVI, mean systemic vascular conductance index. MAP, mean arterial pressure. MHR, mean heart rate. MCI, mean cardiac index. Symbols indicate mean coordinates for 3 rabbits. Vertical bars indicate 1 s.e.mean. Treatments are indicated by symbols: (○) no treatment (time control study No. 1) or vehicle treatment (remaining studies); (□) no treatment (time control study No. 2); (△) no treatment (time control study No. 3); (■) intrapericardial mecamylamine (0.1 mg kg^{-1}), 4th ventricular ICI 174864 (100–300 nmol), or 4th ventricular H-Tyr-D-Ala-Gly-MePhe-NH(CH$_2$)$_2$OH (DAMGO, 300 pmol); (▲) second treatment with ICI 174864 or DAMGO. Comparisons between levels at end of simulated haemorrhage were made by ANOVA. NS, $P \geqslant 0.05$; *$0.01 \leqslant P < 0.05$; **$0.001 \leqslant P < 0.01$; ***$P < 0.001$.

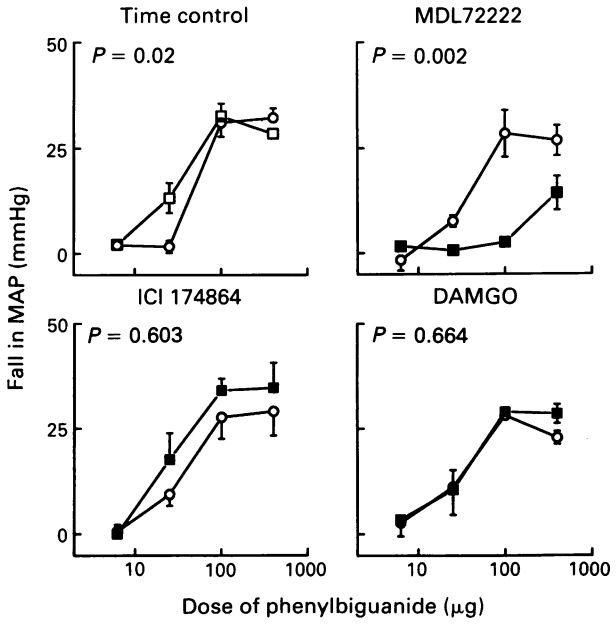


Figure 3 Dose-response relationships for left atrial phenylbiguanide according to the 4 studies, and treatments within studies. MAP, mean arterial pressure. Mean values for 3 rabbits. Vertical bars indicate 1 s.e.mean. Treatments are indicated by symbols: (○) no treatment (time control study 1) or vehicle (remaining studies). (□) No treatment (time control study 2); (■) i.v. MDL 72222 (1.0 mg kg^{-1}), 4th ventricular ICI 174864 (100 nmol), or 4th ventricular H-Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAMGO, 100–300 pmol). *P* values attributable to dose × treatment interaction within ANOVA.

lated haemorrhage (Figure 4), but had no effects on the pattern of the responses of MAP to left atrial phenylbiguanide (Figure 3).

Right atrial phenylbiguanide protocol

Fourth ventricular ICI 174864 (100–300 nmol) and DAMGO (300 pmol) prevented the occurrence of Phase 2 of simulated haemorrhage (Figure 6), but had no effects on the pattern of the responses of MAP to right atrial phenylbiguanide (Figure 5).

Discussion

So far as we can ascertain, this experiment is the first to be described in which an attempt has been made to attribute a physiological phenomenon in a conscious animal to a signal transmitted by chemosensitive cardiac afferents. Our results are negative, in the sense that we have found no evidence that the cardiopulmanory afferents responsible for the signal that initiates the sympathoinhibitory phase of simulated haemorrhage in unanaesthetized rabbits correspond to those excited by atrial injection of phenylbiguanide or intrapericardial injection of nicotine.

We have confirmed our previous findings that, in conscious rabbits the sympathoinhibitory phase of simulated haemorrhage depends on a δ-opioid receptor mechanism within the central nervous system, and its expression can also be prevented by activating central μ-opioid receptors (Figures 2, 4 and 6) (Evans *et al.*, 1989b; Evans & Ludbrook, 1990). We have now shown that the reflex hypotension which follows excitation of

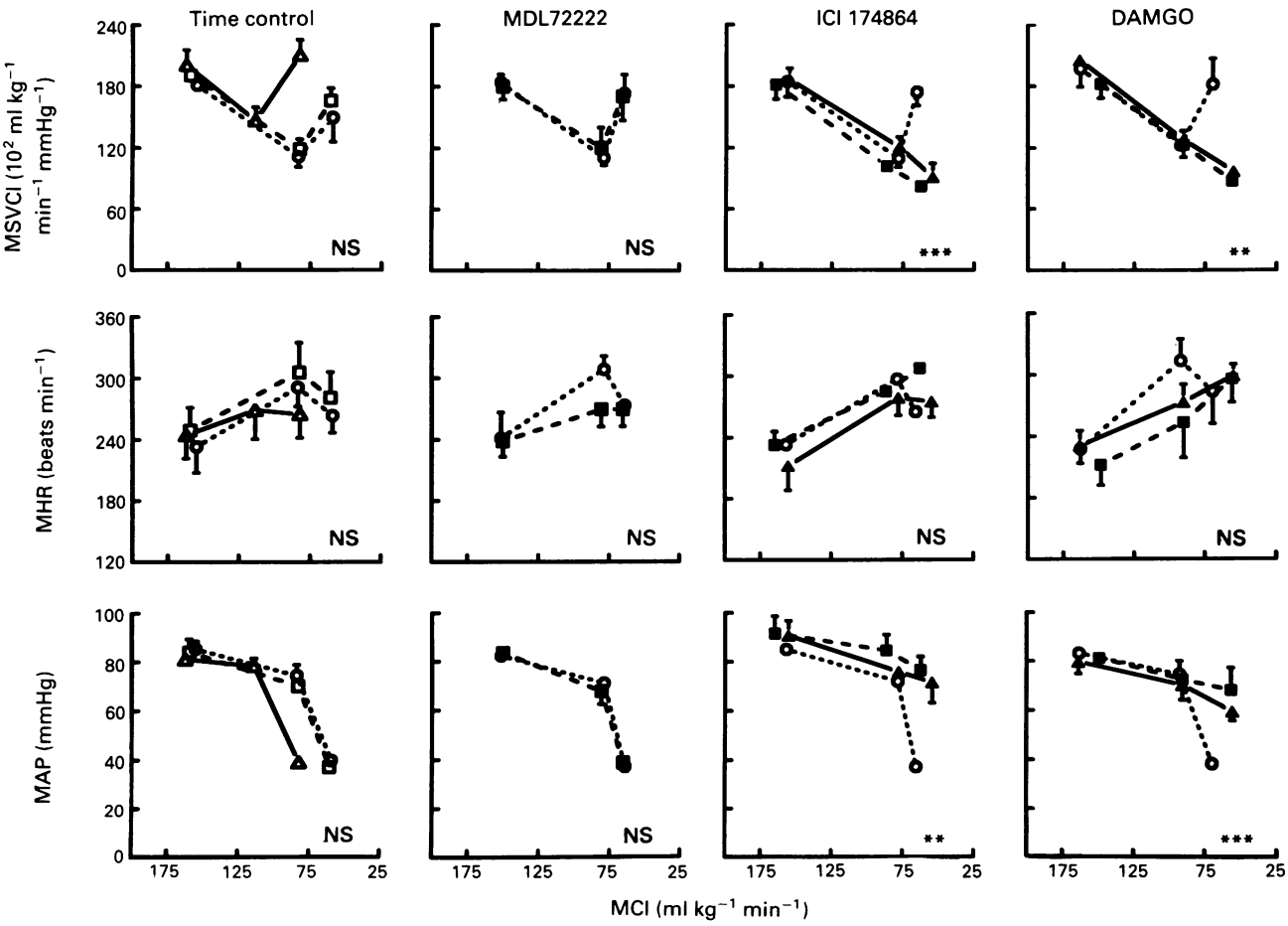


Figure 4 Haemodynamic effects of simulated haemorrhage in left atrial phenylbiguanide protocol according to studies within protocol and treatments within studies. Haemodynamic variables as in Figure 2. Symbols indicate mean coordinates for 3 rabbits. Vertical bars indicate 1 s.e.mean. Treatments are indicated by symbols: (○) no treatment (time control study No. 1) or vehicle treatment (remaining studies); (□) no treatment (time control study No. 2); (△) no treatment (time control study No 3); (■) i.v. MDL 72222 (1.0 mg kg^{-1}), 4th ventricular ICI 174864 (100 nmol), or 4th ventricular H-Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAMGO, (100–300 pmol)); (▲) second treatment with ICI 174864 or DAMGO. Comparisons between levels at end of simulated haemorrhage were made by ANOVA. NS, $P \geqslant 0.05$; *$0.01 \leqslant P < 0.05$; **$0.001 \leqslant P < 0.01$; ***$P < 0.001$.

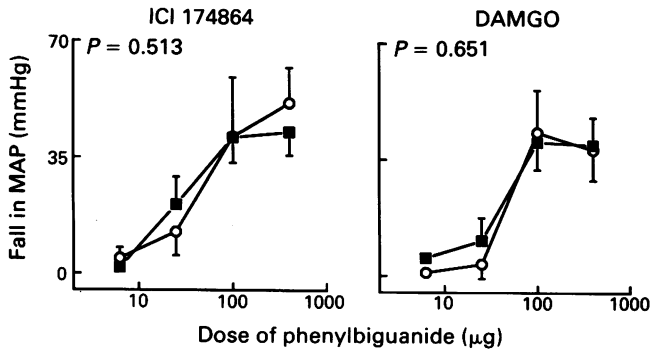


Figure 5 Dose-response relationships for right atrial phenylbiguanide according to the 2 studies, and treatments within studies. MAP, mean arterial pressure. Mean values for 3 rabbits. Vertical bars indicate 1 s.e.mean. Treatments are indicated by symbols: (○) saline treatment; (■) 4th ventricular ICI 174864 (100–300 nmol), or 4th ventricular H-Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAMGO, 300 pmol). *P* values attributable to dose × treatment interaction within ANOVA.

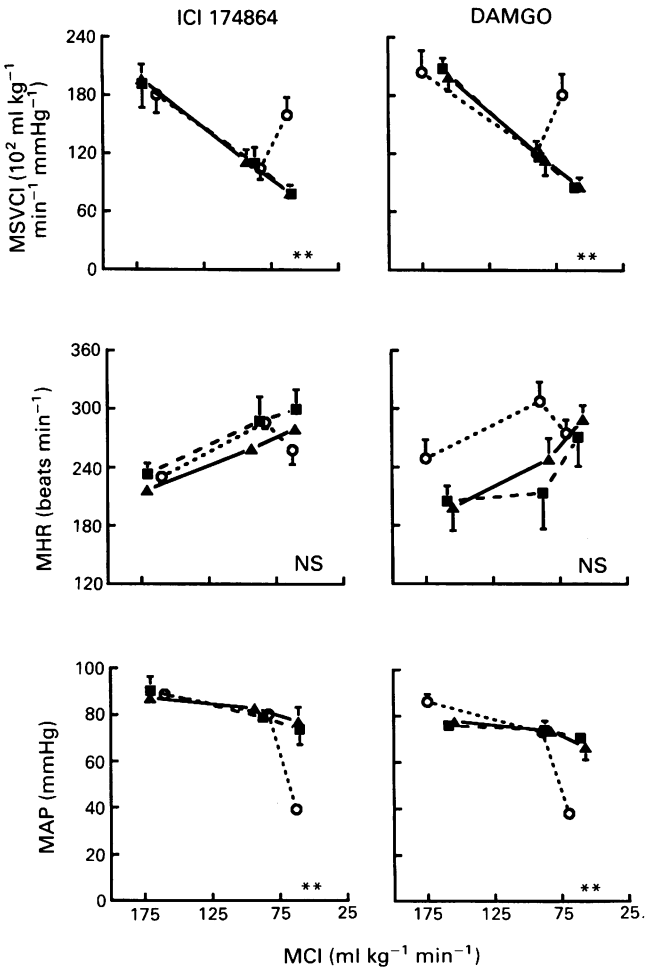


Figure 6 Haemodynamic effects of simulated haemorrhage in right atrial phenylbiguanide protocol according to the 2 studies, and treatments within studies Haemodynamic variables as in Figure 2. Symbols indicate mean coordinates for 3 rabbits. Vertical bars indicate 1 s.e.mean. Treatments are indicated by symbols: (○) vehicle treatment; (■) 4th ventricular ICI 174864 (100–300 nmol) or 4th ventricular H-Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAMGO, 300 pmol); (▲) second treatment with ICI 174864 or DAMGO. Comparisons between levels at end of simulated haemorrhage were made by ANOVA. NS, $P \geqslant 0.05$; *$0.01 \leqslant P < 0.05$; **$0.001 \leqslant P < 0.01$.

the chemosensitive afferents does not depend on a δ-opioid receptor mechanism and is not modulated by central μ-opioid receptors (Figures 1, 3 and 5). In view of this finding, it is not surprising that the sympathoinhibitory phase of simulated haemorrhage was not prevented, delayed or attenuated by antagonizing the 5-HT_3 receptors responsible for the sensitivity of cardiac afferents to phenylbiguanide (Figure 4), or by antagonizing the neuronal-type nicotinic cholinoceptors responsible for the sensitivity of epicardial afferents to nicotine (Figure 2).

There are two possible criticisms of our conclusions. The first is a matter of the doses of the δ-antagonist and μ-agonist that we used as markers of the pathway followed by the input from cardiac afferents which is responsible for initiating the sympathoinhibitory phase of simulated haemorrhage. We are confident that the biophase levels of ICI 174864 and DAMGO were adequate at the time that the dose-response relationships for phenylbiguanide and nicotine were constructed, since we showed that the sympathoinhibitory phase was absent in a subsequent simulated haemorrhage (Figures 2, 4 and 6). Likewise, the effects of the 5-HT_3 antagonist MDL 72222 and the neuronal-type nicotinic cholinoceptor antagonist mecamylamine on simulated haemorrhage were tested before the chemoreflex dose-response relations were constructed. The dose-response relationships showed that the effects of phenylbiguanide and nicotine were still markedly antagonized (Figures 1 and 3).

The second possible criticism is as follows. Though we can be confident that the afferents responsible for the sympathoinhibitory phase of simulated haemorrhage do not correspond to the whole population of phenylbiguanide-sensitive cardiopulmonary afferents, or to the whole population of nicotine-sensitive epicardial afferents, we cannot exclude the possibility that they constitute a small sub-population of one or the other. If this were indeed so, effects of ICI 174864 or DAMGO on the pathway followed by the small sub-population might be concealed by lack of effects on the pathway followed by the larger sub-population.

We have already referred to the circumstantial evidence that phenylbiguanide-sensitive cardiopulmonary afferents might be responsible for the sympathoinhibitory phase of simulated haemorrhage (see Introduction). There is another piece of evidence that obliquely supports this hypothesis. Whereas cardiac afferents in rabbits (Burke & Dorward, 1988; Evans *et al.*, 1989a), and vagal afferents in rats (Skoog *et al.*, 1985), convey the signal responsible for the sympathoinhibitory phase of haemorrhage or simulated haemorrhage, in dogs this signal does not appear to travel in cardiac afferents (Shen *et al.*, 1990), and in primates the source of the signal is unknown (*see* Schadt & Ludbrook, 1990). In rabbits (Evans *et al.*, 1990a) and rats (Willette *et al.*, 1982) phenylbiguanide-sensitive cardiopulmonary afferents are prominent, whereas they appear to be absent in dogs (Dawes *et al.*, 1952) and man (Jain *et al.*, 1972). On the basis of these pieces of circumstantial evidence we had expected to find that in conscious rabbits the signal that initiates the sympathoinhibitory phase of simulated haemorrhage was conveyed by phenylbiguanide-sensitive cardiac afferents, but we have not been able to demonstrate that this is so. The nicotine-sensitive epicardial afferents are also mechanosensitive (Sleight & Widdicombe, 1965), and the sympathoinhibitory phase of haemorrhage has been attributed to paradoxical excitation of left ventricular mechanoreceptors (Thorén, 1979). Though the latter may be true, at least in rats and rabbits, we have been unable to demonstrate that these correspond to the nicotine-sensitive epicardial receptors.

We are grateful to Louise Gallagher and Giannina Legudi for their technical assistance with these experiments. This work was supported by the National Health and Medical Research Council of Australia and the National Heart Foundation of Australia.

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(Received June 21, 1990
Revised September 3, 1990
Accepted October 4, 1990)

Br. J. Pharmacol. (1991), **102**, 540–544 © Macmillan Press Ltd, 1991

Sympathetic co-transmission to the cauda epididymis of the rat: characterization of postjunctional adrenoceptors and purinoceptors

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1 Electrical field stimulation (10 Hz, 60 V, 1 ms, for 10 s) produced monophasic contractions of isolated preparations of rat cauda epididymis which could be abolished by guanethidine, and attenuated by prazosin and α,β-methylene ATP.

2 The rank order of potency of adrenoceptor agonists in causing contraction of the preparation in the presence of the neuronal uptake blocker, nisoxetine (0.1 μM) was: adrenaline $\geqslant$ phenylephrine $\geqslant$ noradrenaline > clonidine > methoxamine > metaraminol > dopamine $\geqslant$ isoprenaline > xylazine.

3 Responses to the agonists were blocked by prazosin but not by propranolol or idazoxan.

4 The rank order of potency of purinoceptor agonists in causing contraction of the cauda epididymis was: α,β-methylene ATP > β,γ-methylene ATP $\geqslant$ 2-methylthioATP > ATP > ADP. AMP and adenosine did not cause contractions.

5 Contractile responses to the purine nucleotide analogues were blocked by repeated application of α,β-methylene ATP.

6 It is concluded that both ATP and noradrenaline may act as co-transmitters in the sympathetic nerves supplying the smooth muscle of the rat cauda epididymis, and that α_1-adrenoceptors and P_{2X}-purinoceptors are present postjunctionally.

Introduction

The epididymis is a single highly convoluted tube which transports sperm from the testis to the vas deferens. The last section, the cauda epididymis is an important site of sperm storage. It is continuous with the vas deferens and is the only section of the epididymis which contains innervated smooth muscle. It is innervated by the same branch of the hypogastric nerve that supplies the vas deferens (Mitchell, 1935).

It is well documented that the contractions of the rat vas deferens evoked by nerve stimulation are mediated through the release of noradrenaline and of adenosine 5'-triphosphate (ATP) which act post-junctionally at α_1-adrenoceptors (van Rossum, 1965; Leedham & Pennefather, 1986) and P_{2X}-purinoceptors (Burnstock & Kennedy, 1985) respectively. Little research has been carried out on neurotransmission to the cauda epididymis. There have been reports that α-adrenoceptors and muscarinic receptors mediating contractile effects are present on the smooth muscle (Laitinen & Talo, 1981; Pholpramool & Triphrom, 1984). Previous studies from this laboratory have also shown that isolated preparations of rat cauda epididymis contract in response to exogenously applied purine nucleotide analogues (Ventura *et al.*, 1990).

The present series of experiments was undertaken in order to characterize the excitatory adrenoceptors and purinoceptors present on the smooth muscle of the rat cauda epididymis and to investigate the possibility that as in the vas deferens, noradrenaline and ATP act as co-transmitters.

A preliminary report on the presence of excitatory postjunctional P_{2X}-purinoceptors has previously been presented (Ventura *et al.*, 1989a).

Methods

Isolated organ bath preparations

Male Long-Evans Hooded rats weighing 200–300 g were killed by cervical dislocation. The abdomen was opened and the cauda epididymides from each side of the animal were removed. With the aid of an Olympus S071 stereo dissecting microscope, the proximal part of each cauda epididymis (i.e. that closest to the vas deferens-cauda epididymis junction) was carefully unravelled and 4–6 cm sections were taken. Cauda epididymis sections were then cut into two 2–3 cm sections, giving four preparations from each animal.

In order to study the effects of adrenoceptor agonists and of nerve stimulation the sections were mounted in separate 30 ml organ baths. For studies of the effects of the purinoceptor agonists, 5 ml organ baths were used. Organ baths contained Krebs-Henseleit solution of the following composition (mM): NaCl 118.1, KCl 4.7, $MgSO_4$ 0.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.7 and $CaCl_2$ 2.5. This solution was warmed to 35°C and bubbled with 5% CO_2 in 95% O_2. Preparations in 30 ml baths were attached by a thread at one end to a fixed platinum electrode in the tissue holder. The tissues mounted in 5 ml baths had one end of each section attached to small wire holders. The other end of each preparation was attached by another thread to an FT03 transducer and isometric contractions were displayed on a Grass polygraph (Model 79D). Tissues were positioned so that the longitudinal muscle fibres were oriented vertically under approximately 0.2 g resting force. These were allowed to equilibrate for 60 min, before the effects of agonists or of field stimulation were investigated.

Responses to field stimulation

Intramural nerve terminals were field stimulated by pulses applied from a Grass S88 stimulator via two platinum electrodes (10 mm apart) incorporated in the tissue holder. The stimulation parameters were 60 V and 1.0 ms duration at a frequency of 10 Hz for 10 s every 100 s. Antagonist drugs were added to the isolated organ baths once the contractile responses to field stimulation had stabilized. Following drug administration, when the electrically evoked contractions had again stabilized, the responses were measured and compared with those obtained before administration of the drug. The magnitude of contraction in the presence of antagonist drugs was then compared with the contraction remaining in control tissues over the same time course.

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Effects of agonists

Discrete concentration-response curves to agonists of unstimulated preparations of rat cauda epididymis were constructed. When the response to each concentration of an agonist had reached a plateau, the tissue was washed with three to five times the bath volume and was allowed 15 min to recover before the next concentration was applied. Only one concentration-response curve was obtained from each tissue and only one agonist was applied to any one tissue.

Inhibitors of uptake and antagonist drugs

Experiments conducted in the presence of α,β-methylene ATP; the adrenoceptor antagonists, prazosin, idazoxan or propranolol; or the inhibitors of neuronal and extraneuronal uptake of noradrenaline namely, nisoxetine or β-oestradiol, were carried out with the drug present in the bathing solution for the entire duration of the experiment.

Statistical analysis

Statistical analysis was performed by use of the standard formulae for calculating mean and s.e.mean. Student's non-paired t test was used to evaluate the significance of differences between mean values or, if more than two groups were compared one-way analysis of variance was used. In all cases, the criterion of statistical significance was taken as $P < 0.05$.

Mean log concentration-response curves were constructed by pooling data from individual log concentration-response curves constructed from tissues from 6 rats and linear regression analysis was undertaken to examine whether or not pairs of pooled lines were parallel, linear and coincident (Documenta Geigy, 1970).

Estimates of potency ratios for each agonist compared to noradrenaline or ATP were obtained by use of least squares regression lines fitted to the central portions obtained from each log concentration-response curve (Documenta Geigy, 1970).

Drugs

The following drugs were used: adenosine (Sigma); adenosine 5′ diphosphate (lithium salt) (ADP, Sigma); adenosine 5′ monophosphate (sodium salt) (AMP, Sigma); adenosine 5′ triphosphate (disodium salt) (ATP, Sigma); (−)-arterenol bitartrate (noradrenaline, Sigma); atropine sulphate (Sigma); clonidine hydrochloride (Boehringer Ingelheim); (−)-epinephrine bitartrate (adrenaline, ICN); guanethidine sulphate (Ciba-Geigy); 3-hydroxytyramine hydrochloride (dopamine, Sigma); idazoxan (Reckitt & Colman); (−)-isoproterenol bitartrate (isoprenaline, Sigma); metaraminol bitartrate (Merck, Sharp & Dhome); methoxamine hydrochloride (Burroughs Wellcome); α,β-methylene adenosine 5′ triphosphate (lithium salt) (α,β-methylene ATP, Sigma); β,γ-methylene adenosine 5′ triphosphate (sodium salt) (β,γ-methylene ATP, Sigma); 2-methylthioadenosine 5′ triphosphate (sodium salt · $4H_2O$) (2-methylthioATP, RBI); nisoxetine hydrochloride (Eli Lilly); β-oestradiol (Sigma); (−)-phenylephrine hydrochloride (Sigma); pirenzipine dihydrochloride (Boehringer); prazosin (Pfizer); propranolol hydrochloride (ICI); xylazine hydrochloride (Sigma).

Adrenaline, dopamine, isoprenaline, metaraminol, noradrenaline and phenylephrine were dissolved and diluted to required concentrations in a catecholamine diluent (mM: NaCl 154.0, NaH_2PO_4 1.2, ascorbic acid 0.2). Clonidine and xylazine were initially dissolved in 0.1 ml of 0.1 M HCl and then diluted to the required concentration in distilled water. β-Oestradiol was dissolved in ethanol. A stock solution of prazosin was made by initially dissolving it in glycerol and then making it up to volume with a 5% w/v dextrose solution (see Leedham & Pennefather, 1986). Dilutions to the required concentration were made with distilled water. All other drugs were dissolved and diluted to the required concentrations in distilled water.

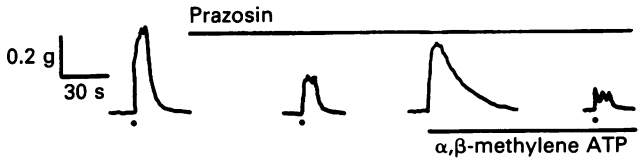


Figure 1 Typical trace showing the effects of prazosin (0.1 μM) and α,β-methylene ATP (10 μM) on responses to field stimulation (●) (10 Hz, 1 ms, 60 V, for 10 s every 100 s) of isolated preparations of the rat cauda epididymis. Note that application of α,β-methylene ATP caused a contraction of the tissue.

Results

Responses to field stimulation

Field stimulation (10 Hz, 1 ms, 60 V, for 10 s every 100 s) caused monophasic contractions of the isolated preparations of rat cauda epididymides (Figure 1). Responses were abolished by incubation of cauda epididymides in guanethidine (1 μM) ($n = 6$) (see Figure 2).

Prazosin and α,β-methylene ATP significantly attenuated the response to electrical nerve stimulation (Figure 1). Prazosin (0.1 μM) reduced the mean height of contraction to 45.0 ± 10.4% of control (Figure 2). Increasing the concentration of prazosin did not cause any further reduction in the size of the response. α,β-Methylene ATP (10 μM) reduced the height of the electrically evoked contractions to 63.9 ± 3.8% of control (Figure 2). The effects of these concentrations of the

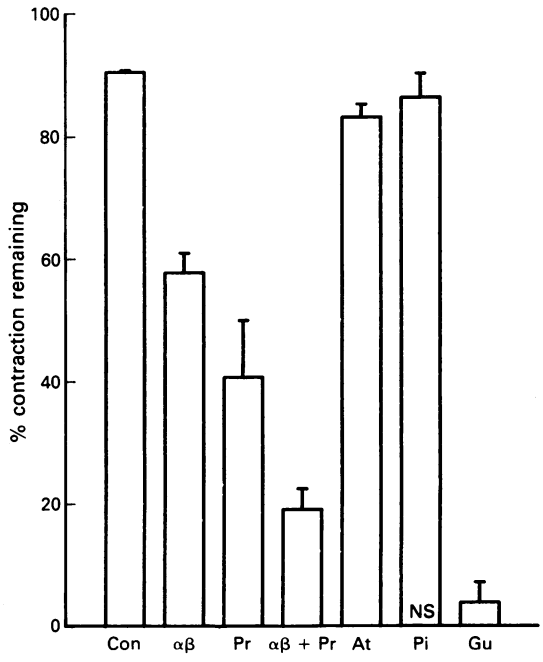


Figure 2 Mean percentage contractile responses remaining to field stimulation (10 Hz, 1 ms, 60 V, for 10 s every 100 s) following administration of: (Con) no drug, (αβ) α,β-methylene ATP (10 μM), (Pr) prazosin (0.1 μM), (αβ + Pr) α,β-methylene ATP (10 μM) and prazosin (0.1 μM), (At) atropine (1 μM), (Pi) pirenzipine (1 μM), (Gu) guanethidine (10 μM). Each column is the mean of estimates from 4–10 experiments and the vertical bars represent s.e.mean. Note that responses were significantly less than the drug-free time control for each drug treatment ($P < 0.05$, $n = 4$–10, unpaired t tests) except pirenzipine (Pi) ($P > 0.05$, $n = 4$, unpaired t test).

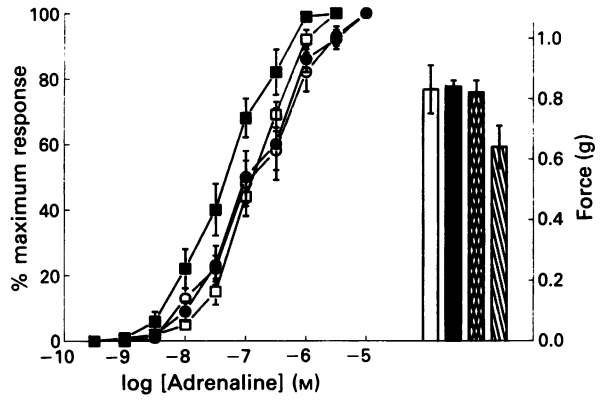


Figure 3 Mean log concentration-response curves for: adrenaline applied sequentially to isolated cauda epididymis segments (●) in control tissues, (○) in the presence of propranolol (1 μM), (■) in the presence of nisoxetine (0.1 μM) and (□) in the presence of β-oestradiol (10 μM). Points represent the means of estimates from six experiments and vertical bars represent the s.e.mean. The histogram columns represent the mean maximum force developed by (open) control tissues, (closed) in the presence of propranolol, (cross-hatched) in the presence of nisoxetine and (hatched) in the presence of β-oestradiol. Vertical bars represent s.e.mean.

two drugs were additive, such that in combination they reduced the mean size of contractions to 21.1 ± 3.8% of control (Figure 2).

The muscarinic receptor antagonist, atropine, caused a very small but significant attenuation of the electrically evoked contractions of the cauda epididymides. Thus atropine (1 μM) reduced the size of contraction to 91.9 ± 2.7% of control (Figure 2). Addition of atropine (1 μM), in the presence of both prazosin (0.1 μM) and α,β-methylene ATP (10 μM) did not cause any further reduction in the size of the response. The muscarinic M_1-receptor antagonist pirenzipine did not significantly reduce the size of contractions (Figure 2). Contractions in the presence of pirenzipine (1 μM) were 95.0 ± 4.3% of control.

Effects of inhibitors of uptake and propranolol on concentration-response curves to adrenaline

Adrenaline produced concentration-dependent tonic contractions of isolated preparations of the rat cauda epididymis. The mean negative log molar EC_{50} (i.e. the mean negative log molar concentration of agonist which gave a response which was 50% of the maximum response obtained to that agonist) in control preparations was 6.88 ± 0.12. Propranolol (1 μM) and β-oestradiol (10 μM) did not significantly affect the mean concentration-response curve to adrenaline; however nisoxetine (0.1 μM) shifted the adrenaline concentration-response curve significantly to the left (Figure 3) (potency ratio = 2.7, 95% confidence limits = 1.2–6.6, d.f. = 29). Maximum responses to adrenaline in the absence and in the presence of the various blocking drugs were not significantly different ($P > 0.05$, d.f. = 23, analysis of variance).

Adrenoceptor classification

Since nisoxetine but not β-oestradiol nor propranolol influenced responses to adrenaline, rank order of adrenoceptor agonist potency experiments were established in the presence of nisoxetine (0.1 μM) only. The catecholamines and all of the other sympathomimetic drugs tested produced concentration-related contractions of the cauda epididymides.

The mean maximum force developed in response to each agonist is shown in Table 1. Although analysis of variance showed a significant heterogeneity in the magnitudes of maximum responses to the agonists, none of the agonists produced mean maximal responses which differed significantly from that to noradrenaline ($P > 0.05$, $n = 6$ for all experiments, unpaired t tests).

Table 1 Mean negative log EC_{50} values, mean maximum force developed and potency ratios at α-adrenoceptors on the rat cauda epididymis[1]

Agonist	*−log EC_{50} values* (mean ± s.e.mean)	*Mean maximum tension developed* (g; mean ± s.e.mean)	*Potency ratio*
Adrenaline	7.32 ± 0.15	0.82 ± 0.04	1.6
Phenylephrine	7.17 ± 0.12	0.85 ± 0.04	1.1
Noradrenaline	7.12 ± 0.12	0.86 ± 0.08	1.0
Clonidine	6.61 ± 0.16	0.83 ± 0.07	0.31
Methoxamine	6.41 ± 0.12	0.81 ± 0.05	0.19
Metaraminol	5.94 ± 0.10	1.04 ± 0.04	0.07
Dopamine	5.06 ± 0.10	1.07 ± 0.07	0.009
Isoprenaline	4.97 ± 0.06	0.90 ± 0.04	0.007
Xylazine	4.88 ± 0.11	0.79 ± 0.06	0.006

[1] Nisoxetine (0.1 μM) present.
$n = 6$ animals for each experimental group.
Potency ratio = antilog ((neg log EC_{50} value for agonist) − (neg log EC_{50} value for noradrenaline)).

The mean log concentration-response curves for each of the agonists were parallel to that for noradrenaline. The mean negative log EC_{50} values determined from fitted regression lines and potencies relative to noradrenaline are shown in Table 1.

The order of potency for the nine adrenoceptor agonists was: adrenaline ≥ phenylephrine ≥ noradrenaline > clonidine > methoxamine > metaraminol > dopamine ≥ isoprenaline > xylazine.

Contractile responses evoked by methoxamine, isoprenaline and xylazine were antagonized by prazosin (0.1 μM); however, neither idazoxan (1 μM) nor propranolol (1 μM) ($n = 4$–5) inhibited these responses.

Purinoceptor classification

ATP, α,β-methylene ATP, β,γ-methylene ATP, 2-methylthioATP and ADP applied exogenously, each produced concentration-dependent, transient contractions of the rat cauda epididymides. AMP and adenosine were inactive. The potencies of ATP, β,γ-methylene ATP, 2-methylthioATP and ADP were low, and at 3 mM (the highest concentration of these agonists tested) maximum responses had not been attained (see Figure 4). α,β-Methylene ATP produced a maximal response at a concentration of approximately 30 μM. The mean log concentration-response curves for each of the active agonists were parallel to that for ATP (Figure 4).

The order of potency of these purines in producing contractions of the tissue was: α,β-methylene ATP > β,γ-methylene

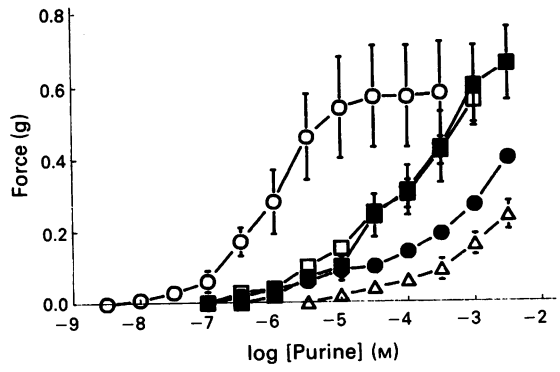


Figure 4 Mean log concentration-response curves for: (●) ATP, (○) α,β-methylene ATP, (■) β,γ-methylene ATP, (□) 2-methylthioATP and (△) ADP on control preparations of cauda epididymis. Points represent the means of estimates from six experiments. Vertical bars represent the s.e.mean.

ATP = 2-methylthioATP > ATP > ADP (Figure 4). ADP was 4.7 (95% confidence limits = 2.5–12.5, d.f. = 33) times less potent than ATP. α,β-Methylene ATP was 1150 (95% confidence limits = 483–2588, d.f. = 30) times more potent than ATP in producing contraction of the tissue. There was no significant difference in the potencies of β,γ-methylene ATP and 2-methylthioATP but these agonists were respectively 17.3 (95% confidence limits = 8.2–38.6, d.f. = 43) and 14.0 (95% confidence limits = 7.5–31.6, d.f. = 23) times more potent than ATP.

The contractions caused by these purine analogues could be almost totally abolished by desensitization of the receptor by prior exposure to α,β-methylene ATP (10 μM) ($n = 4$, for each agonist).

Discussion

The results of this study indicate that α_1-adrenoceptors and P_{2X}-purinoceptors mediating excitatory effects are present on the smooth muscle of the rat cauda epididymis, and that both noradrenaline and ATP may be released from sympathetic nerve terminals within the cauda epididymides in response to field stimulation.

The rank order of agonist potencies in causing contractions of preparations of rat cauda epididymides (i.e. adrenaline $\geqslant$ phenylephrine $\geqslant$ noradrenaline > clonidine > methoxamine > metaraminol > dopamine $\geqslant$ isoprenaline > xylazine) was substantially similar to that found in an earlier study from this laboratory using preparations of the epididymal segment of the vas deferens of this species (Leedham & Pennefather, 1986). The order of potency found in the present study also conforms to that considered by Wikberg (1978) to indicate the presence of α_1-adrenoceptors. In this investigation, as in our earlier study with vas deferens, phenylephrine was approximately three times more potent than clonidine. This estimate conforms to one of the criteria proposed by Starke in 1981 for subclassification of α-adrenoceptors as α_1. One difference, however, was that the potency of phenylephrine relative to that of noradrenaline was greater than expected. Thus it was found to be approximately equipotent with adrenaline and noradrenaline. Further evidence suggesting that the α-adrenoceptors were of the α_1-subtype has come from the observations that the effects of methoxamine and xylazine, which have high selectivity for α_1- and α_2-adrenoceptors respectively, were both blocked by prazosin but were unaffected by idazoxan. Evidence that isoprenaline was also acting at α_1-adrenoceptors in this preparation came from the fact that its effects, like those of methoxamine and xylazine, were blocked by prazosin, but not by either idazoxan or propranolol.

Since neither propranolol nor β-oestradiol enhanced the potency of adrenaline, and propranolol did not enhance the effects of isoprenaline, it can be assumed that postjunctional β-adrenoceptors and extraneuronal uptake play little or no functional role in this tissue. Despite the omission of propranolol in the present experiments, the EC_{50} obtained for isoprenaline in this study was similar to that obtained by Starke (1981) and Leedham & Pennefather (1986) both of whom included propranolol in their incubation media.

The rank order of potency of the naturally occurring purines i.e. ATP > ADP $\gg$ AMP, adenosine in producing contractions of the cauda epididymis is consistent with actions at P_2 rather than at P_1-purinoceptors (Burnstock, 1978). The relative order of potency of ATP and its methyl phosphate modified isosteres and 2-methylthioATP in causing contraction of the tissue, namely, α,β-methylene ATP $\gg$ β,γ-methylene ATP = 2-methylthioATP > ATP is broadly consistent with the subclassification of the P_2-purinoceptors mediating these effects as the P_{2X}-subtype (Burnstock & Kennedy, 1985). Indeed the relative potencies of these compounds on the cauda epididymis are very similar to those observed by Burnstock & Warland (1987), who compared the effects of these purines in causing contraction of preparations of mesenteric artery from rabbits. They found that α,β-Methylene ATP was over 1500 times more potent than ATP. β,γ-methylene ATP and 2-methylthioATP which were equipotent were approximately 30 times more potent than ATP.

The finding that the rank order of potency of ATP analogues obtained by Burnstock & Warland (1987) and that observed by us differs from that originally outlined by Burnstock & Kennedy (1985) may reflect 'between tissue' differences in the rate of catabolism of ATP. Until potent and selective inhibitors of ATP become available, P_2-purinoceptor subclassification based on agonist potencies alone will remain tentative. However, the relative agonist order of potency of the four agonists used in this study and in that of Burnstock & Warland (1987) on the rabbit mesenteric artery may prove to be a better description of the order of potency of purine nucleotide analogues at the P_{2X}-purinoceptor, since none of the studies reviewed by Burnstock & Kennedy (1985) actually employed all four of these agonists.

Further evidence for the classification of the purinoceptors in the cauda epididymis as being of the P_{2X}-subtype was the finding that responses to the agonists were blocked by prior application of α,β-methylene ATP. This acts selectively to desensitize P_{2X}-purinoceptors (Burnstock & Kennedy, 1985).

Investigations of the hypothesis that both noradrenaline and ATP are released from sympathetic nerve terminals within the rat vas deferens were prompted, in part, by observations that the response of this tissue to field stimulation are biphasic. The fast or 'purinergic' component of the response is prominent at the prostatic end of the tissue, whereas the slow or 'adrenergic' component of the response is more prominent at the epididymal end of the tissue (McGrath, 1978; Brown *et al.*, 1979; Rohde *et al.*, 1986). In the present study we have found that the contractions evoked by field stimulation of the cauda epididymis are monophasic and relatively slow. Nevertheless studies, using selective antagonists for α_1-adrenoceptors, P_2-purinoceptors and muscarinic cholinoceptors, were undertaken to deduce whether, as in the vas deferens, field stimulation of preparations of cauda epididymis led to release of substances other than noradrenaline from intrinsic sympathetic nerve terminals. Evidence that the field stimulation applied stimulated sympathetic nerve terminals was obtained in experiments using guanethidine which reduced stimulation-induced contractions by over 90%. Furthermore, although muscarinic agonists have been shown to cause contraction of preparations of cauda epididymides (Laitinen & Talo, 1981; Pholpramol & Triphrom, 1984; Ventura *et al.*, 1989b) neither pirenzipine nor atropine had any major effect on responses to field stimulation. In contrast, prazosin and α,β-methylene ATP both partially inhibited the contractile response to field stimulation, moreover, the effects of these two antagonists were additive. These findings taken together suggest that field stimulation of preparations of the rat cauda epididymis leads to the release of ATP as well as noradrenaline from sympathetic nerve terminals within the tissue.

The residual response to electrical stimulation in the presence of α,β-methylene ATP and prazosin may be due to direct smooth muscle stimulation, since there was also a small guanethidine-resistant residual response to electrical stimulation; or to the use of insufficient amounts of prazosin to block all of the postjunctional excitatory adrenoceptors present in the preparation. However, a third transmitter may be involved. Neuropeptide Y is perhaps the best candidate since it has previously been suggested that there is a dense innervation by neuropeptide Y-containing nerve fibres in the male reproductive tract of rodents. This peptide has been reported to induce a rise in resting tension of isolated preparations of mouse vas deferens by a postjunctional effect (Stjärne *et al.*, 1986).

In conclusion, the major findings of this study are that electrical stimulation of the nerve terminals present in prep-

arations of the cauda epididymis leads to contractions which can be inhibited by either α,β-methylene ATP or prazosin indicating that both ATP and noradrenaline act as cotransmitters. Secondly we have demonstrated that the smooth muscle of this tissue contains postjunctional α_1-adrenoceptors and P_{2X}-purinoceptors.

This work was supported in part by a grant to J.N.P. from the Australian Research Council.

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(Received August 10, 1990
Revised October 9, 1990
Accepted October 10, 1990)

Br. J. Pharmacol. (1991), **102**, 545–549 © Macmillan Press Ltd, 1991

Modulation by endothelium of the responses induced by endothelin-1 and by some of its analogues in rat isolated aorta

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1 The contractile effects of endothelin-1 and various analogues were studied in rat isolated aortic rings. The potency order of the peptides studied was endothelin-1 > sarafotoxin S6b > [$Ala^{3,11}$]endothelin-1 > [$Ala^{1,15}$]endothelin-1. [$Ala^{1,3,11,15}$]endothelin-1 was neither agonist nor antagonist.

2 The concentration of endothelin-1 required to induce contractions equal to 50% of those induced by 1 μM noradrenaline was reduced from 5.8 nM when the vascular endothelium was present to 1.4 nM after it had been mechanically removed.

3 Contractions elicited by [$Ala^{3,11}$]endothelin-1, [$Ala^{1,15}$]endothelin-1 and sarafotoxin S6b were not modulated by the endothelium.

4 Endothelin-1 increased the tissue content of guanosine 3',5'-cyclic monophosphate (cyclic GMP) in rat aortic segments with endothelium by about 4 fold, suggesting that it increased the release of endothelium-derived relaxing factor. Sarafotoxin S6b did not reduce, or significantly increase, tissue cyclic GMP levels and therefore had little effect on EDRF release.

5 The concentration of sodium nitroprusside required to relax half-maximally aortae denuded of endothelium was 430 nM if the aortae had been precontracted with 10 nM endothelin-1 and 35 nM if 10 nM sarafotoxin S6b was used as the spasmogen. This indicates that differential sensitivities of the smooth muscle to cyclic GMP cannot explain differences between responses to endothelin-1 and sarafotoxin S6b in the presence of endothelium.

6 It is concluded that endothelin-1 contractions of rat aorta are modified by the endothelium, probably by enhancing the release of endothelium-derived relaxing factor (EDRF) and not by affecting the sensitivity of the smooth muscle to EDRF. This suggests that a stimulated release of an adequate amount of EDRF is necessary to modulate contractile responses to these peptides.

Introduction

The existence of a contracting factor derived from endothelial cells was first demonstrated about 5 years ago (O'Brien & McMurtry, 1984; Agricola *et al.*, 1984; Hickey *et al.*, 1985) and in 1988 a 21-amino acid peptide, endothelin, was purified from the medium of porcine endothelial cells in culture (Yanagisawa *et al.*, 1988). This peptide has been shown to be one of the most potent vasconstrictors known (Yanagisawa *et al.*, 1988; Tomobe *et al.*, 1988). The endothelin originally characterized was that produced by human and porcine endothelial cells in culture. It is a member of a family of at least three closely related mammalian peptides and has been renamed endothelin-1 (Inoue *et al.*, 1989). Sarafotoxin S6b, a constituent of the venom of the burrowing snake *Atractaspis engaddensis*, is homologous with the endothelins and is also a vasoconstrictor (Lee & Chiapinelli, 1988; Takasaki *et al.*, 1988). Sarafotoxin S6b and endothelin-1 inhibit the specific binding of each other to heart and brain membranes (Ambar *et al.*, 1989) and other active analogues of endothelin-1, such as [$Ala^{3,11}$]- and [$Ala^{1,15}$]-endothelin-1, inhibit [^{125}I]-endothelin-1 specific binding (Hiley *et al.*, 1990) but do not have an identical pharmacological profile to that of endothelin-1 (Randall *et al.*, 1989; Topouzis *et al.*, 1989b; Miller *et al.*, 1990). Endothelin-1 contains two disulphide bonds between residues 3–11 and 1–15, generating a peptide containing two almost equal sized rings (Yanagisawa *et al.*, 1988). In the analogues [$Ala^{3,11}$]- and [$Ala^{1,15}$]-endothelin-1, the cysteines at positions 3–11 and 1–15 respectively have been removed creating one large ring, the [$Ala^{3,11}$] analogue, or one small ring, the [$Ala^{1,15}$] analogue. The cystine residues were replaced with alanines because this substitution retains a chiral amino acid, it is also a neutral residue and does not introduce any new charge at the substituted position and is pseudo-isosteric with cystine.

The vasoconstrictor activity of many compounds is modulated by the presence of endothelium (Miller *et al.*, 1988). In rat aorta the modulatory effect of endothelium is thought to be due to either basal (Martin *et al.*, 1986; MacLeod *et al.*, 1987) or both basal and stimulated (Miller *et al.*, 1984; Bigaud *et al.*, 1984; Malta *et al.*, 1986), release of endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980). In any case this modulation in the presence of endothelium is associated with an increase in tissue levels of guanosine 3',5'-cyclic monophosphate (cyclic GMP) compared to tissues without endothelium.

This study was undertaken to assess the effects of endothelium on responses of rat isolated aorta elicited by endothelin-1, sarafotoxin S6b and three vasoactive analogues of endothelin-1; [$Ala^{3,11}$]-, [$Ala^{1,15}$]- and [$Ala^{1,3,11,15}$]-endothelin-1 (Randall *et al.*, 1989; Topouzis *et al.*, 1989b). The results show that while responses elicited by endothelin-1 were modulated by the presence of endothelium, responses to the other peptides were not affected. From measurements of tissue cyclic GMP content, it is concluded that a stimulation of EDRF release, produced only by endothelin-1, is required to modulate the contractile effects of these peptides in the rat isolated aorta. Preliminary reports of some of these results have been made (Topouzis *et al.*, 1989a; Huggins *et al.*, 1990).

Methods

Male Sprague-Dawley rats weighing 200–250 g were killed and the thoracic aortae removed taking care not to damage the endothelium. Four rings were taken from each aorta and mounted, under a tension of 2 g, in organ baths containing a physiological solution (composition in mM: NaCl 112, KCl 5,

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$NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 1.25, glucose 11.5) equilibrated to pH 7.4 and gassed with a mixture of 95% O_2 and 5% CO_2. Half of the segments were stripped of their endothelium by rubbing with a wooden match stick or with forceps.

Contractile experiments

Artery segments were allowed to equilibrate for 60 min and were then contracted with noradrenaline, 1 μM, a concentration which elicits a maximal response (Godfraind & Miller, 1983). After the response had reached a plateau, acetylcholine, 1 μM, was added to the organ bath to test for the absence or presence of endothelium (Furchgott & Zawadzki, 1980). Tissues that responded to acetylcholine with a relaxation of greater than 50% of the initial noradrenaline-induced tone were considered to be intact, whereas tissues in which acetylcholine elicited no effect were regarded as endothelium-denuded. Tissues were then washed and left to equilibrate for 60 min, during which period the medium was changed 4 times. In some experiments tissues were further incubated for 30 min in the presence of indomethacin, 10 μM, or for 45 min in the presence of [$Ala^{1,3,11,15}$]endothelin-1, 50 nM. After the equilibration period, concentrations of peptides were added to the baths of artery segments in a cumulative fashion. In all cases pairs of rings were studied in parallel, one with and one without endothelium. Only one concentration-effect curve to a single peptide was obtained in each artery ring in the absence or presence of indomethacin or of [$Ala^{1,3,11,15}$]endothelin-1. In other experiments, aortic rings without endothelium were precontracted with endothelin-1, 10 nM, or sarafotoxin S6b and then concentration-effect curves were obtained for the relaxation of the rings by cumulative addition of sodium nitroprusside.

Cyclic GMP determinations

Aortic segments were equilibrated in physiological solution for 60 min at 37°C and then treated with acetylcholine, 1 μM, endothelin-1, 10 nM, or sarafotoxin S6b 10 nM. At various times thereafter the tissues were frozen with clamps cooled in liquid nitrogen and stored at −70°C. Without thawing, each ring was homogenized in 1 ml trichloroacetic acid 0.6 M in a Polytron and subsequently centrifuged at 40000 *g* for 15 min. The pellet was resuspended in NaOH 1 M and assayed for protein content (Lowry *et al.*, 1951). The supernatants were washed four times with 5 volumes of water-saturated diethyl ether and assayed for cyclic GMP with an Amerlex radioimmunoassay kit. Tissue cyclic GMP was expressed relative to protein content in order to normalise for variations in amount of tissue.

Materials

Noradrenaline bitartrate (Boyer, France), acetylcholine HCl (Sigma, France) and sodium nitroprusside (Prolabo, France) solutions were prepared daily in deionised water. Indomethacin (Sigma, France) was dissolved in 0.5% $NaHCO_3$ solution to a concentration of 10 mM and diluted with deionised water as necessary. Endothelin-1, sarafotoxin S6b (both from Peptide Institute, Japan), [$Ala^{3,11}$]endothelin-1, [$Ala^{1,15}$]-endothelin-1 and [$Ala^{1,3,11,15}$]endothelin-1 (synthesized at Merrell Dow Research Institute, Strasbourg, France) were stored at 4°C as 0.1 mM solutions in deionised water and further dilutions were prepared daily. The Amerlex cyclic GMP assay kit was from Amersham International, United Kingdom.

Statistics

Tests of significance were made by Student's *t* test on a Compac 286e computer with RS/1 software (Version 12.10E, BBN Software Products Co). *P* values less than 0.05 were taken to indicate significant differences.

Results

Endothelin-1 (0.3 to 30 nM), [$Ala^{3,11}$]endothelin-1 (2.25 to 225 nM), [$Ala^{1,15}$]endothelin-1 (41 nM to 1.36 μM) and sarafotoxin S6b (0.1 to 30 nM) all contracted rat aortic preparations without endothelium in a concentration-dependent manner (Figure 1). At the highest concentrations tested all peptides induced contractile effects similar to the maximal responses to noradrenaline 1 μM obtained in the same tissues, but the concentrations eliciting 50% of the maximal responses to noradrenaline were different. The potency order was endothelin-1 > sarafotoxin S6b > [$Ala^{3,11}$]endothelin-1 > [$Ala^{1,15}$]- endothelin-1 (Table 1). Higher concentrations of peptides than indicated in Figure 1 were not tested due to their limited availability. In the presence of endothelium, endothelin-1 was significantly less potent than in tissues without endothelium, but maximal responses were not significantly changed (Figure 1a). Indomethacin (10 μM) had no significant effect on contractions to endothelin-1 in the presence or absence of endothelium.

Maximal responses elicited in the presence and absence of endothelium by the highest concentrations tested of [$Ala^{3,11}$]endothelin-1 and [$Ala^{1,15}$]endothelin-1 were also not significantly different (Figure 1b, c) and nor were their potencies as assessed by comparison of concentrations eliciting responses equal to 50% of the effect of noradrenaline 1 μM (Table 1). Maximal contractions were elicited by sarafotoxin S6b and these were not significantly different in the presence or absence of endothelium (Figure 1d). [$Ala^{1,3,11,15}$]endothelin-1 failed to elicit detectable contractions at concentrations of 10 nM to 1 μM in either intact or endothelium-denuded tissues. A 50 nM concentration of this peptide also failed to modulate responses to endothelin-1 or sarafotoxin S6b.

The modulation of vasoconstrictor responses of rat aorta to endothelin-1, but not to [$Ala^{3,11}$]endothelin-1, [$Ala^{1,15}$]endothelin-1 or sarafotoxin S6b in the presence of the endothelium was surprising and was investigated further.

Modulation of agonist responses in intact vessels is often due to release of EDRF by the endothelium and an index of its release is changes in tissue cyclic GMP levels (Miller *et al.*, 1988). When tissues with endothelium were incubated with 10 nM endothelin-1, a significant increase in tissue cyclic GMP, of about 4 fold basal levels, was observed after 1 min. On prolonged incubation, tissue cyclic GMP levels returned to basal values (Figure 2b). Incubation with sarafotoxin S6b 10 nM did not significantly elevate cyclic GMP levels. In tissues without endothelium, neither endothelin-1 nor sarafotoxin S6b had any significant effect on cyclic GMP levels (Figure 2b). One min incubation of endothelium-containing tissues with 1 μM acetylcholine increased tissue content by about 13 fold (Figure 2a).

It has been postulated that the presence of endothelium can be enough to alter agonist-induced responses (Malta *et al.*, 1986; Martin *et al.*, 1986). A reason, therefore, for the lack of effect of endothelium on responses to sarafotoxin S6b, is that sarafotoxin S6b desensitizes aortic smooth muscle to the effects of EDRF and hence of intracellular cyclic GMP. The effects of direct stimulation of soluble guanylate cyclase were assessed in the presence of peptides. In aortic rings without endothelium precontracted with either endothelin-1 (10 nM) or sarafotoxin S6b (10 nM), concentrations that elicited similar responses, sodium nitroprusside elicited concentration-dependent relaxations amounting to about 90% of the initial contraction (Figure 3). In tissues precontracted with endothelin-1, the EC_{50} value for sodium nitroprusside was 430 ± 180 nM ($n = 7$) but when sarafotoxin S6b was the spasmogen the EC_{50} value of 34.6 ± 18.8 nM ($n = 8$) was significantly lower ($P < 0.05$).

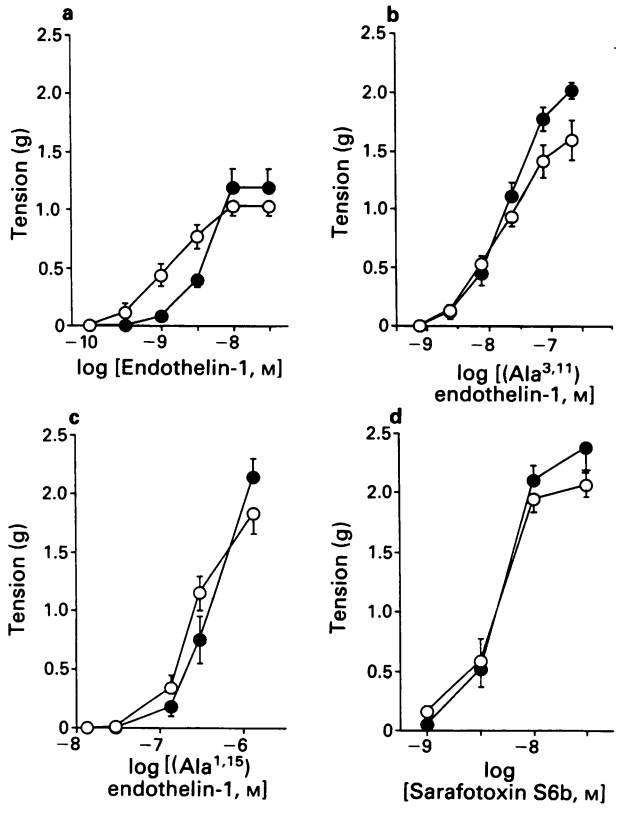


Figure 1 Concentration-effect curves elicited by (a) endothelin-1, (b) [Ala^{3,11}]endothelin-1, (c) [Ala^{1,15}]endothelin-1 and (d) sarafotoxin S6b in rat isolated aorta in the presence (●) and absence (○) of endothelium. Responses are expressed in g developed tension. At the highest concentrations tested, all peptides induced contractile effects similar to the maximal responses to noradrenaline 1 μM obtained in the same tissues. Each point is the mean of 7 to 10 observations. Vertical bars represent s.e.mean.

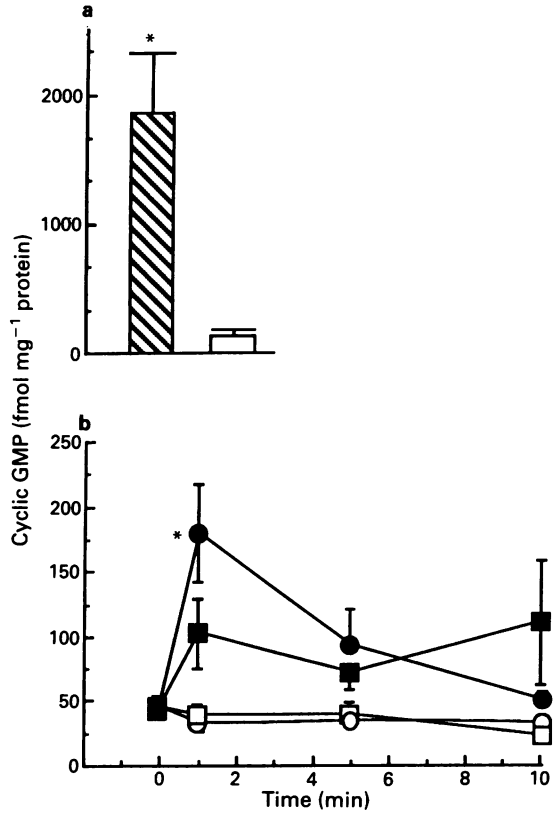


Figure 2 Cyclic GMP levels measured in isolated rat aortic rings. (a) Effect of 1 min exposure to acetylcholine 1 μM with (hatched column) and without (open column) endothelium. (b) Time course of the effects of endothelin-1 (10 nM) (●, ○) and of sarafotoxin S6b (10 nM) (■, □) with (filled symbols) and without (open symbols) endothelium. Each point is the mean of 11 or 12 observations. Vertical lines represent s.e.mean. *indicates significantly different from tissues without endothelium (a) or from time 0 (b).

Discussion

Endothelin-1 as well as two of its analogues, [Ala^{3,11}]- and [Ala^{1,15}]-endothelin-1, that have been shown to compete with [^{125}I]-endothelin-1 for a specific binding site (Hiley *et al.*, 1990), contracted rat isolated aorta in a concentration-dependent manner. Sarafotoxin S6b interacts with specific binding sites for [^{125}I]-endothelin-1 (Ambar *et al.*, 1989) and elicits contractions (Figure 1). The order of potency of the peptides is the same as that reported for rat mesenteric vessels and guinea-pig trachea (Randall *et al.*, 1989; Pelton & Miller, 1990) and is the same as the order of binding affinities to rat cerebellar membranes (Hiley *et al.*, 1990). A third analogue, [Ala^{1,3,11,15}]endothelin-1, also competes with [^{125}I]endothelin-1 for specific binding sites in central nervous tissue (Hiley *et al.*, 1990). This analogue constricts the blood perfused rat mesenteric vasculature (Randall *et al.*, 1989) and guinea-pig isolated trachea (Pelton & Miller, 1990), but failed either to contract rat aorta or to antagonize contractions elicited by endothelin-1 or sarafotoxin S6b. These observations, and other evidence from binding (Badr *et al.*, 1989) and functional studies (Randall *et al.*, 1989) seem to indicate the existence of more than one endothelin receptor.

Contractile responses to endothelin-1 in rat isolated aortic segments were modulated in the presence of endothelium, as compared to responses in its absence, by a displacement to the right of concentration-effect curves by a factor of about 4, without a significant effect on maximal responses (Figure 1a). This sort of modulation is similar to that of many other contractile agonists such as 5-hydroxytryptamine, prostaglandin $F_{2\alpha}$ and full α-adrenoceptor agonists in this and other arterial preparations (Miller *et al.*, 1988). However, all contractile agonists whose responses are modulated by the endothelium do not stimulate the release of EDRF (Miller *et al.*, 1988). The modulation of endothelin-1 responses by endothelium was not mediated by cyclo-oxygenase products since they were unaf-

Table 1 Concentrations of peptides eliciting 50% of the responses to noradrenaline (NA), 1 μM, (EC_{NA50}) in the absence (−endo) and presence (+endo) of endothelium in rat isolated aorta and maximal responses of peptides in the absence of endothelium (E_{max}, expressed as % of responses to noradrenaline 1 μM) obtained at the maximal concentrations tested (EC_{max})

Agonist	EC_{NA50}(nM) −endo	+endo	E_{max}(%)	EC_{max}(nM)
Endothelin-1	1.4 ± 0.4	5.8 ± 1.0*	109.2 ± 22.3	10
[Ala^{3,11}]endothelin-1	18.7 ± 4.6	14.1 ± 4.2	101.1 ± 18.6	225
[Ala^{1,15}]endothelin-1	269.0 ± 64.0	418.0 ± 50.0	125.2 ± 7.4	1360
Sarafotoxin S6b	3.9 ± 0.6	3.8 ± 0.3	114.8 ± 8.0	30

Each value is the mean (± s.e.mean) of 7–10 observations.
* significantly different from value obtained −endo (*t* test, $P = 0.002$).

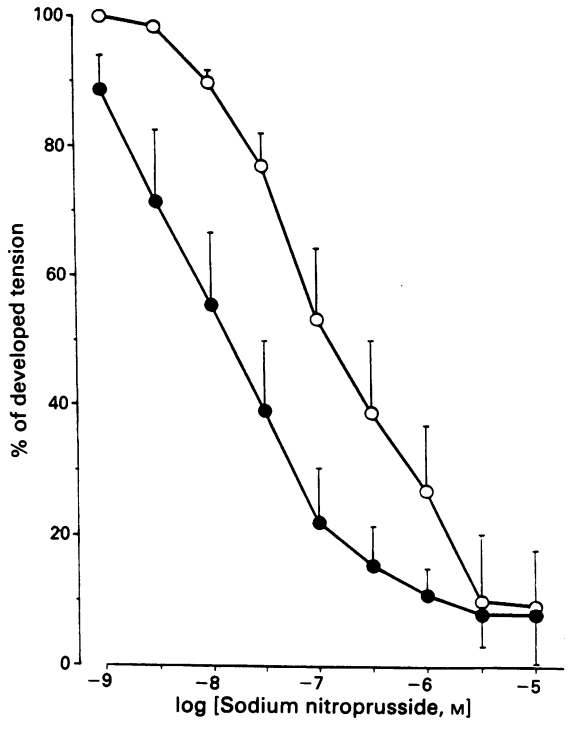


Figure 3 Concentration-effect curves elicited by sodium nitroprusside in tissues precontracted with endothelin-1 (10 nM) (○) or with sarafotoxin S6b (10 nM) (●) in rat isolated aortic segments without endothelium. Each point is the mean of 7 or 8 observations. Vertical bars represent s.e.mean.

fected by indomethacin. Endothelin-1 responses have also been shown to be modulated by endothelium in the perfused mesenteric bed of the rat *in vitro* (Randall *et al.*, 1989).

Acetylcholine stimulated an increase in tissue cyclic GMP levels of about 13 fold in tissues with endothelium compared to those without (Figure 2a). This degree of stimulation is at least comparable with that already reported in vascular tissues with endothelium (Holzmann, 1982; Martin *et al.*, 1986; Schoeffter & Miller, 1986). Endothelin-1 stimulated an increase of cyclic GMP levels of about 4 fold only in tissues with endothelium, demonstrating a stimulation of EDRF release. Such a stimulation of EDRF release has been reported in the rat mesenteric bed (De Nucci *et al.*, 1988). The magnitude of the stimulation of cyclic GMP levels in rat aortic tissues with endothelium is very similar to that observed with contractile agonists such as phenylephrine, B-HT 920 and clonidine, that elicit responses sensitive to the presence of the endothelium (Miller *et al.*, 1984; Bigaud *et al.*, 1984). An alternative explanation, that endothelin-1 raised cyclic GMP levels within endothelial cells is unlikely, given the very small proportion which these cells contribute to aortic tissue volume (Schini *et al.*, 1989).

Contractile responses to [Ala^{3,11}]endothelin-1, [Ala^{1,15}]endothelin-1 and sarafotoxin S6b were not modulated by the presence of an intact endothelium (Figures 1b-d). This surprising result differentiates these three compounds from all other contractile agonists so far studied in this preparation (Miller *et al.*, 1988). The stimulation by sarafotoxin S6b of cyclic GMP levels was small (Figure 2) with an effect at 1 min bordering on significance ($P = 0.059$). Three testable explanations for the differences between these agonists and endothelin-1 involving the influence of EDRF are: that the analogues, but not endothelin-1, might inhibit basal EDRF release; or that sarafotoxin S6b but not endothelin-1 desensitizes aortic smooth muscle to EDRF; alternatively, that basal release of EDRF is not sufficient to modify contractile responses to any of the peptides and that only endothelin-1 stimulates release of EDRF. Basal release of EDRF does not seem to be inhibited by sarafotoxin S6b. In fact it seemed, if anything, to increase tissue cyclic GMP content (Figure 2). Desensitization does not seem to be an explanation either, since responses to sodium nitroprusside, an activator of the same guanylate cyclase stimulated by EDRF (Waldman & Murad, 1987), were potentiated in the presence of sarafotoxin S6b (Figure 3). This interesting observation remains to be explained. The remaining possibility, that only endothelin-1 stimulates EDRF release and tissue cyclic GMP levels sufficiently to modify contractile responses, seems to be a reasonable explanation for the lack of effect of the endothelium on responses to sarafotoxin S6b (Figure 1). This could indicate the presence of different receptors for endothelins on endothelium and smooth muscle, or that sarafotoxin S6b is less efficacious than endothelin-1 in the endothelium.

Overall, this work shows differences in the effects of endothelium, depending on whether endothelin-1 or various analogues were used to contract the aortic smooth muscle. Some of the results suggest that for endothelin-1 and sarafotoxin S6b these differences are likely to be caused by agonist-specific differences in altering EDRF release and not by differences in smooth muscle sensitivity to cyclic GMP. It has been suggested that modulation of contractile responses to spasmogens in vascular tissue may be due to basal (Martin *et al.*, 1986; MacLeod *et al.*, 1987) or agonist-stimulated release of EDRF (Miller *et al.*, 1984) or both (Malta *et al.*, 1986). However the differences between endothelin-1 and its analogues suggests that a stimulated release of an adequate amount of EDRF is necessary to modulate contractile responses to these peptides.

We thank Mrs C. Berg and Mr M. Poirot for technical assistance with the radioimmunoassay.

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(Received May 2, 1990
Revised August 24, 1990
Accepted October 8, 1990)

British Journal of Pharmacology

VOLUME 102 (2) FEBRUARY 1991

PAPERS

M.G. López, P. Michelena, L. Gandía & A.G. García. (+)-Isradipine but not (−)-Bay-K-8644 exhibits voltage-dependent effects on cat adrenal catecholamine release 289

F. Lembeck, T. Griesbacher, M. Eckhardt, S. Henke, G. Breipohl & J. Knolle. New, long-acting, potent bradykinin antagonists 297

G.A. Higgins, C.C. Jordan & M. Skingle. Evidence that the unilateral activation of 5-HT_{1D} receptors in the substantia nigra of the guinea-pig elicits contralateral rotation 305

S.R. O'Donnell, J.C. Wanstall, C.S. Kay & X.-p. Zeng. Tissue selectivity and spasmogen selectivity of relaxant drugs in airway and pulmonary vascular smooth muscle contracted by $PGF_{2\alpha}$ or endothelin 311

G.H. Dreteler, W. Wouters, P.R. Saxena & A.G. Ramage. Pressor effects following microinjection of 5-HT_{1A} receptor agonists into the raphe obscurus of the anaesthetized rat 317

M.O. den Boer, C.M. Villalón, J.P.C. Heiligers, P.P.A. Humphrey & P.R. Saxena. Role of 5-HT_{1A}-like receptors in the reduction of porcine cranial arteriovenous anastomotic shunting by sumatriptan 323

G. Chaudhuri, G.M. Buga, M.E. Gold, K.S. Wood & L.J. Ignarro. Characterization and actions of human umbilical endothelium derived relaxing factor 331

K.M. Bray, A.H. Weston, S. Duty, D.T. Newgreen, J. Longmore, G. Edwards & T.J. Brown. Differences between the effects of cromakalim and nifedipine on agonist-induced responses in rabbit aorta 337

P. Palatini, G. Viola, E. Bigon, A.M. Menegus & A. Bruni. Pharmacokinetic characterization of phosphatidylserine lipsomes in the rat 345

J.-P. Girolami, J.-L. Bascands, C. Pécher, M. Berlan, J.-L. Montastruc & P. Montastruc. Yohimbine increases submaxillar kallikrein release into the saliva in dogs: evidence for α_2-adrenoceptor-mediated inhibition of cholinergic pathways 351

B.G. Frenguelli, J.F. Blake, M.W. Brown & G.L. Collingridge. Electrogenic uptake contributes a major component of the depolarizing action of L-glutamate in rat hippocampal slices 355

M.G. Giovannini, F. Casamenti, A. Nistri, F. Paoli & G. Pepeu. Effect of thyrotropin releasing hormone (TRH) on acetylcholine release from different brain areas investigated by microdialysis 363

M. Grealy & J.M. O'Donnell. Secretion of growth hormone elicited by intravenous disipramine in the conscious, unrestrained rat 369

F. Brunner & W.R. Kukovetz. Radioligand binding to muscarinic receptors of bovine aortic endothelial cells 373

T. Takeuchi, M. Okuda & O. Yagasaki. The differential contribution of endogenous prostaglandins to the release of acetylcholine from the myenteric plexus of the guinea-pig ileum 381

I. Sahin-Erdemli, D. Hoyer, A. Stoll, M.P. Seiler & P. Schoeffter. 5-HT_1-like receptors mediate 5-hydroxytryptamine-induced contraction of guinea-pig isolated iliac artery 386

M. D'Amato, I.F. Stamford & A. Bennett. Studies of three non-peptide cholecystokinin antagonists (devazepide, lorglumide and loxiglumide) in human isolated alimentary muscle and guinea-pig ileum 391

S.C. Parija, V. Raviprakash & S.K. Mishra. Adenosine- and α,β-methylene ATP-induced differential inhibition of cholinergic and non-cholinergic neurogenic responses in rat urinary bladder 396

C. Szabó, J.E. Hardebo & C. Owman. An amplifying effect of exogenous and neurally stored 5-hydroxytryptamine on the neurogenic contraction in rat tail artery 401

M.A. Barrand & B.A. Callingham. Evidence for regulatory control of iron uptake from ferric maltol across the small intestine of the rat 408

A.H. Clark & C.J. Garland. 5-Hydroxytryptamine-stimulated accumulation of 1,2-diacylglycerol in the rabbit basilar artery: a role for protein kinase C in smooth muscle contraction 415

K.O. McKay, J.L. Black & C.L. Armour. The mechanism of action of endothelin in human lung 422

A.A.-B. Badawy & C.J. Morgan. Effects of acute paroxetine administration on tryptophan metabolism and disposition in the rat 429

G.E. Boeckxstaens, P.A. Pelckmans, H. Bult, J.G. De Man, A.G. Herman & Y.M. Van Maercke. Evidence for nitric oxide as mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut 434

J. Sallés & A. Badia. Mechanisms underlying the differential sensitivity to α_1-adrenoceptor activation in the bisected rat vas deferens 439

H. Glossmann, C. Zech, J. Striessnig, R. Staudinger, L. Hall, R. Greenberg & B.I. Armah. Very high affinity interaction of DPI 201-106 and BDF 8784 enantiomers with the phenylalkylamine-sensitive Ca^{2+}-channel in *Drosophila* head membranes 446

P. Kinnunen, T. Taskinen, M. Järvinen & H. Ruskoaho. Effect of phorbol ester on the release of atrial natriuretic peptide from the hypertrophied rat myocardium 453

S.M. Bryant, K.O. Ryder & G. Hart. Effects of captopril on membrane current and contraction in single ventricular myocytes from guinea-pig 462

N. Morel & T. Godfraind. Characterization in rat aorta of the binding sites responsible for blockade of noradrenaline-evoked calcium entry by nisoldipine 467

C.E. Austin & R. Chess-Williams. Diabetes-induced changes in cardiac β-adrenoceptor responsiveness: effects of aldose reductase inhibition with ponalrestat 478

H. Meiri & Y. Shimoni. Effects of aluminium on electrical and mechanical properties of frog atrial muscle 483

K. Aibara, M. Oonuma & N. Akaike. Strychnine-induced potassium current in isolated dorsal root ganglion cells of the rat 492

I.S. Watts, K.A. Wharton, B.P. White & P. Lumley. Thromboxane (Tx) A_2 receptor blockade and TxA_2 synthase inhibition alone and in combination: comparison of anti-aggregatory efficacy in human platelets 497

A. Franco-Cereceda. Calcitonin gene-related peptide and human epicardial coronary arteries: presence, release and vasodilator effects 506

J.M. Hall & I.K.M. Morton. Novel selective agonists and antagonists confirm neurokinin NK_1 receptors in guinea-pig vas deferens 511

A.H. Mulder, D.M. Burger, G. Wardeh, F. Hogenboom & A.L. Frankhuyzen. Pharmacological profile of various κ-agonists at κ-, μ- and δ-opioid receptors mediating presynaptic inhibition of neurotransmitter release in the rat brain 518

E. Ecault & M.-P. Sauviat. Characterization of the palytoxin-induced sodium conductance in frog skeletal muscle 523

A.A. Almotrefi & N. Dzimiri. Influence of Lorcainide on microsomal Na^+, K^+-ATPase in guinea-pig isolated heart preparations 530

R.G. Evans & J. Ludbrook. Chemosensitive cardiopulmonary afferents and the haemodynamic response to simulated haemorrhage in conscious rabbits 533

S. Ventura & J.N. Pennefather. Sympathetic co-transmission to the cauda epididymis of the rat: characterization of postjunctional adrenoceptors and purinoceptors 540

S. Topouzis, J.P. Huggins, J.T. Pelton & R.C. Miller. Modulation by endothelium of the responses induced by endothelin-1 and by some of its analogues in rat isolated aorta 545

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The *British Journal of Pharmacology* is published monthly by the Scientific & Medical Division, Macmillan Press Ltd.

The journal is covered by *Current Contents*, *Excerpta Medica* and *Index Medicus*.

All business correspondence and reprint requests should be addressed to the Scientific & Medical Division, Macmillan Press Ltd., Houndmills, Basingstoke, Hampshire RG21 2XS, UK. Telephone: (0256) 29242; Fax: (0256) 842754.

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ISSN 0007–1188

0007–1188/91 $3.50 + $0.00

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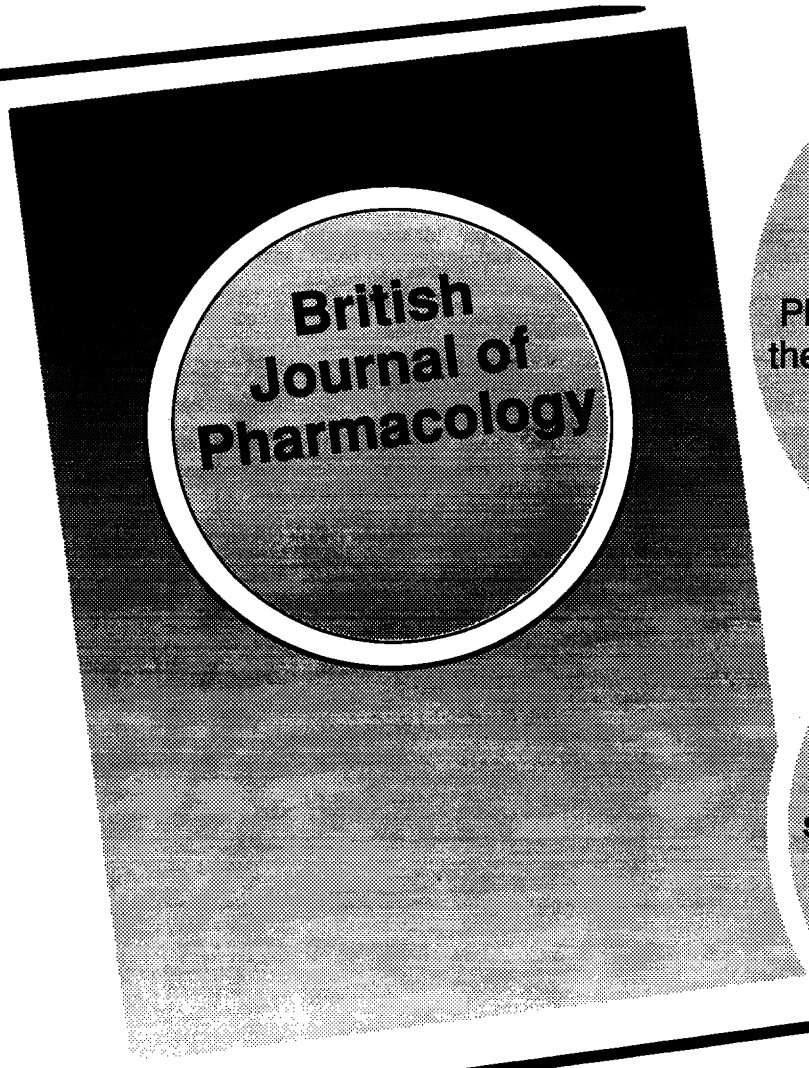
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PAPERS

M.G. López, P. Michelena, L. Gandía & A.G. García. (+)-Isradipine but not (−)-Bay-K-8644 exhibits voltage-dependent effects on cat adrenal catecholamine release 289

F. Lembeck, T. Griesbacher, M. Eckhardt, S. Henke, G. Breipohl & J. Knolle. New, long-acting, potent bradykinin antagonists 297

G.A. Higgins, C.C. Jordan & M. Skingle. Evidence that the unilateral activation of 5-HT_{1D} receptors in the substantia nigra of the guinea-pig elicits contralateral rotation 305

S.R. O'Donnell, J.C. Wanstall, C.S. Kay & X.-p. Zeng. Tissue selectivity and spasmogen selectivity of relaxant drugs in airway and pulmonary vascular smooth muscle contracted by $PGF_{2\alpha}$ or endothelin 311

G.H. Dreteler, W. Wouters, P.R. Saxena & A.G. Ramage. Pressor effects following microinjection of 5-HT_{1A} receptor agonists into the raphe obscurus of the anaesthetized rat 317

M.O. den Boer, C.M. Villalón, J.P.C. Heiligers, P.P.A. Humphrey & P.R. Saxena. Role of 5-HT_{1A}-like receptors in the reduction of porcine cranial arteriovenous anastomotic shunting by sumatriptan 323

G. Chaudhuri, G.M. Buga, M.E. Gold, K.S. Wood & L.J. Ignarro. Characterization and actions of human umbilical endothelium derived relaxing factor 331

K.M. Bray, A.H. Weston, S. Duty, D.T. Newgreen, J. Longmore, G. Edwards & T.J. Brown. Differences between the effects of cromakalim and nifedipine on agonist-induced responses in rabbit aorta 337

P. Palatini, G. Viola, E. Bigon, A.M. Menegus & A. Bruni. Pharmacokinetic characterization of phosphatidylserine lipsomes in the rat 345

J.-P. Girolami, J.-L. Bascands, C. Pécher, M. Berlan, J.-L. Montastruc & P. Montastruc. Yohimbine increases submaxillar kallikrein release into the saliva in dogs: evidence for α_2-adrenoceptor-mediated inhibition of cholinergic pathways 351

B.G. Frenguelli, J.F. Blake, M.W. Brown & G.L. Collingridge. Electrogenic uptake contributes a major component of the depolarizing action of L-glutamate in rat hippocampal slices 355

M.G. Giovannini, F. Casamenti, A. Nistri, F. Paoli & G. Pepeu. Effect of thyrotropin releasing hormone (TRH) on acetylcholine release from different brain areas investigated by microdialysis 363

M. Grealy & J.M. O'Donnell. Secretion of growth hormone elicited by intravenous disipramine in the conscious, unrestrained rat 369

F. Brunner & W.R. Kukovetz. Radioligand binding to muscarinic receptors of bovine aortic endothelial cells 373

T. Takeuchi, M. Okuda & O. Yagasaki. The differential contribution of endogenous prostaglandins to the release of acetylcholine from the myenteric plexus of the guinea-pig ileum 381

I. Sahin-Erdemli, D. Hoyer, A. Stoll, M.P. Seiler & P. Schoeffter. 5-HT_1-like receptors mediate 5-hydroxytryptamine-induced contraction of guinea-pig isolated iliac artery 386

M. D'Amato, I.F. Stamford & A. Bennett. Studies of three non-peptide cholecystokinin antagonists (devazepide, lorglumide and loxiglumide) in human isolated alimentary muscle and guinea-pig ileum 391

S.C. Parija, V. Raviprakash & S.K. Mishra. Adenosine- and α,β-methylene ATP-induced differential inhibition of cholinergic and non-cholinergic neurogenic responses in rat urinary bladder 396

C. Szabó, J.E. Hardebo & C. Owman. An amplifying effect of exogenous and neurally stored 5-hydroxytryptamine on the neurogenic contraction in rat tail artery 401

M.A. Barrand & B.A. Callingham. Evidence for regulatory control of iron uptake from ferric maltol across the small intestine of the rat 408

A.H. Clark & C.J. Garland. 5-Hydroxytryptamine-stimulated accumulation of 1,2-diacylglycerol in the rabbit basilar artery: a role for protein kinase C in smooth muscle contraction 415

K.O. McKay, J.L. Black & C.L. Armour. The mechanism of action of endothelin in human lung 422

A.A.-B. Badawy & C.J. Morgan. Effects of acute paroxetine administration on tryptophan metabolism and disposition in the rat 429

G.E. Boeckxstaens, P.A. Pelckmans, H. Bult, J.G. De Man, A.G. Herman & Y.M. Van Maercke. Evidence for nitric oxide as mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut 434

J. Sallés & A. Badia. Mechanisms underlying the differential sensitivity to α_1-adrenoceptor activation in the bisected rat vas deferens 439

H. Glossmann, C. Zech, J. Striessnig, R. Staudinger, L. Hall, R. Greenberg & B.I. Armah. Very high affinity interaction of DPI 201-106 and BDF 8784 enantiomers with the phenylalkylamine-sensitive Ca^{2+}-channel in *Drosophila* head membranes 446

P. Kinnunen, T. Taskinen, M. Järvinen & H. Ruskoaho. Effect of phorbol ester on the release of atrial natriuretic peptide from the hypertrophied rat myocardium 453

S.M. Bryant, K.O. Ryder & G. Hart. Effects of captopril on membrane current and contraction in single ventricular myocytes from guinea-pig 462

N. Morel & T. Godfraind. Characterization in rat aorta of the binding sites responsible for blockade of noradrenaline-evoked calcium entry by nisoldipine 467

C.E. Austin & R. Chess-Williams. Diabetes-induced changes in cardiac β-adrenoceptor responsiveness: effects of aldose reductase inhibition with ponalrestat 478

H. Meiri & Y. Shimoni. Effects of aluminium on electrical and mechanical properties of frog atrial muscle 483

K. Aibara, M. Oonuma & N. Akaike. Strychnine-induced potassium current in isolated dorsal root ganglion cells of the rat 492

I.S. Watts, K.A. Wharton, B.P. White & P. Lumley. Thromboxane (Tx) A_2 receptor blockade and TxA_2 synthase inhibition alone and in combination: comparison of anti-aggregatory efficacy in human platelets 497

A. Franco-Cereceda. Calcitonin gene-related peptide and human epicardial coronary arteries: presence, release and vasodilator effects 506

J.M. Hall & I.K.M. Morton. Novel selective agonists and antagonists confirm neurokinin NK_1 receptors in guinea-pig vas deferens 511

A.H. Mulder, D.M. Burger, G. Wardeh, F. Hogenboom & A.L. Frankhuyzen. Pharmacological profile of various κ-agonists at κ-, μ- and δ-opioid receptors mediating presynaptic inhibition of neurotransmitter release in the rat brain 518

E. Ecault & M.-P. Sauviat. Characterization of the palytoxin-induced sodium conductance in frog skeletal muscle 523

A.A. Almotrefi & N. Dzimiri. Influence of Lorcainide on microsomal Na^+, K^+-ATPase in guinea-pig isolated heart preparations 530

R.G. Evans & J. Ludbrook. Chemosensitive cardiopulmonary afferents and the haemodynamic response to simulated haemorrhage in conscious rabbits 533

S. Ventura & J.N. Pennefather. Sympathetic co-transmission to the cauda epididymis of the rat: characterization of postjunctional adrenoceptors and purinoceptors 540

S. Topouzis, J.P. Huggins, J.T. Pelton & R.C. Miller. Modulation by endothelium of the responses induced by endothelin-1 and by some of its analogues in rat isolated aorta 545

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