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Pharmacological analysis of the interaction between purinoceptor agonists and antagonists in the guinea-pig taenia caecum

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1 In the absence of adenosine uptake inhibition, adenosine produced a concentration-dependent (threshold 30 μM) relaxation of the 5-methylfurmethide pre-contracted guinea-pig taenia caecum. The relaxation was not blocked by 8-phenyltheophylline (8-PT, 3 μM) or 1,3-dipropyl, 8-cyclopentylxanthine (DPCPX, 30 μM).

2 In the presence of the adenosine uptake inhibitor, dipyridamole (Dip, 3 μM), a biphasic adenosine concentration-effect curve was obtained (threshold 0.3 μM). The time course of the responses to adenosine in the absence of Dip was similar to that of the second phase responses in the presence of Dip and occurred over the same adenosine concentration-range. 5'-(N-ethyl) carboxamido-adenosine (NECA) concentration-effect curves (in the absence of Dip) were also biphasic. Only the first phases of the concentration-effect curves obtained with NECA and adenosine (plus Dip) were inhibited by 8-PT. The pA_2 values for 8-PT of 6.7 and 7.0 versus adenosine and NECA, respectively, were consistent with actions at P_1-purinoceptors. There was a trend towards an increase in the upper asymptote of the first phase of the NECA curve in the presence of increasing concentrations of 8-PT. The A_1-purinoceptor selective antagonist, DPCPX, also blocked only the first phase of the NECA concentration-effect curve and produced a significant increase in the upper asymptote. The pA_2 value (6.8) obtained was consistent with activation of A_2-subtype P_1-purinoceptors by the low concentrations of NECA.

3 There was no correlation between A_1-purinoceptor affinity and the propensity to cause the increase in the upper asymptote of the first phase of the NECA concentration-effect curves amongst a series of 9-methyl adenine analogues, suggesting that the amplification was not due to inhibition of an underlying A_1-purinoceptor-mediated contractile response.

4 DPCPX (10 μM) produced a significant increase in the upper asymptote of the NECA concentration-effect curve, but had no effect on isoprenaline curves whereas the phosphodiesterase inhibitor Ro 20-1724 (30 μM) produced a significant increase in the upper asymptote of both NECA and isoprenaline concentration-effect curves. Therefore the amplification of the first phase responses by DPCPX did not appear to be due to phosphodiesterase inhibition.

5 It was not possible to conclude whether second phase responses to adenosine and NECA were mediated by intracellular or extracellular sites of action. However, if intracellular sites of action were involved then adenosine did not apparently gain access by the Dip-sensitive uptake system.

Keywords: Adenosine; taenia caecum; receptor antagonism; purinoceptors

Introduction

Cell surface purinoceptors were first classified by Burnstock (1978) who proposed that purine receptors could be subdivided into two subtypes, one at which adenosine is most potent (P_1) and the other at which ATP is most potent (P_2). Responses mediated by the P_1-purinoceptor were found to be blocked by theophylline and those mediated by the P_2-purinoceptor were blocked, although not selectively, by high concentrations of quinidine 2-substituted imidazolines, 2'2-pyridyloisatogen or apamin. Subsequently, Van Calker *et al.* (1979) established the existence of two distinct P_1-purinoceptors. Those receptors mediating a decrease in adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels were termed A_1 and those mediating an increase, A_2. In addition to the contrasting effects on cyclic AMP levels, the subtypes could be distinguished by the agonist potency order of adenosine and two analogues, N-ethylcarboxamido-adenosine (NECA) and N^6-phenylisopropyladenosine (PIA). The potency order at the A_1-purinoceptor, PIA > adenosine > NECA, was reversed at the A_2-purinoceptor. The relative selectivity of the R-stereoisomer of PIA over the S-isomer was also noted to be greater at the A_1- than at the A_2-purinoceptor subtype.

It is well established that there are separate receptors for adenosine (P_1) and ATP (P_2) in the guinea-pig taenia caecum, both of which mediate relaxation (Spedding & Weetman, 1976; Brown & Burnstock, 1981; Satchell & Maguire, 1982). Satchell & Maguire (1982) showed that similar structural modifications of ATP and adenosine resulted in divergent effects and Brown & Burnstock (1981) also noted that theophylline, while inhibiting adenosine responses, failed to block the response to ATP and found the reverse to be the case for apamin, which has been classified as a non-specific blocker of ATP responses.

The P_1-purinoceptor present in the taenia caecum was first classified as A_2 on the basis of the potency order of adenosine analogues and the virtual absence of stereoselectivity of PIA (Burnstock *et al.*, 1984). The data presented in that study included the effects of a single concentration of the purinoceptor antagonist, 8-phenyltheophylline (8-PT), on the agonist concentration-effect curves obtained using NECA, 2-chloro-adenosine, L-PIA, D-PIA, cyclohexyladenosine (CHA) and adenosine. The results show that the degree of rightward shift obtained with 8-PT (10 μM) was agonist-dependent. The concentration-ratios ranged from a highly

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significant value of 45 for NECA to non-significant displacements for L-PIA, CHA and adenosine. These results are not consistent with expectations for the competitive antagonism of a homogeneous population of purinoceptors. In an attempt to expose any receptor heterogeneity, we began by performing experiments on the taenia caecum preparation using adenosine and NECA as agonists and a wide range of concentrations of the antagonists, 8-PT and 1,3-dipropyl, 8-cyclopentyl xanthine (DPCPX). The agonist response time-courses and the agonist concentration-effect curves obtained revealed complexity in the actions of both the agonists and antagonists.

Methods

Guinea-pig taenia caecum assay

Lengths of taenia caecum (1.5–2 cm), dissected from male guinea pigs (Dunkin Hartley, 250–350 g), were suspended in 20 ml organ baths containing de Jalons solutions maintained at 31 ± 0.5°C (mM composition: Na^+ 160, K^+ 5.63, Cl^- 160.71, Ca^{2+} 0.54, HCO_3^- 5.95, glucose 2.78). Responses were measured isotonically following addition of a 1.5 g load, a single wash by bath fluid replacement and a 60 min stabilisation period. Test compounds were applied for a 60 min incubation period after the response to a concentration (0.1 μM) of an ACh M-receptor agonist, 5-methylfurmethide (5-mef), reached a plateau. This response was approximately 80% of the maximum response which could be obtained with 5-mef. In preliminary experiments (data not shown) the response to 5-mef was shown to be maintained for at least 180 min which is sufficient time to obtain a fully-defined relaxatory agonist concentration-effect curve. Relaxatory responses were expressed as mm of experimental trace where the recording system amplification was set so that 10 mm was equivalent to 1.3 mm change in tissue length.

Guinea-pig left atrium assay

Purinoceptor-mediated negative inotropic responses were studied under isometric conditions in isolated left atria from male guinea pigs (Dunkin Hartley, 250–350 g). Left atria were rapidly excised and suspended under 1 g tension in 20 ml organ baths containing Krebs-Henseleit solution (mM, composition: Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, $H_2PO_4^-$ 1.2, HCO_3^- 24.9, SO_4^{2-} 1.2, glucose 11) maintained at 37°C and gassed with 95%O_2:5%CO_2. The preparations were electrically stimulated with square wave pulses (1 Hz frequency, 1 ms pulse duration, 120% threshold V) via both a punctate and field platinum electrode. Preparations were washed four times during a 60 min stabilisation period prior to 60 min incubation with test compounds or vehicle. Responses were expressed as % change in basal force (g) of contraction.

Protocols

Single agonist concentration-effect curves were obtained by cumulative dosing in each preparation. Six preparations were used simultaneously and treatments were allocated on a randomised block design so that, as far as possible, each organ bath received each treatment.

Data analysis

Where possible, individual agonist curve data were fitted to the Hill equation,

$$E = \frac{\alpha \cdot [A]^{n_h}}{[A]_{50}{}^{n_h} + [A]^{n_h}}$$

to provide estimates of midpoint slope parameter (n_H), midpoint location ($\log[A]_{50}$) and upper asymptote (α), as described previously (Black & Shankley, 1985). The effect of drug treatment on these parameters was assessed by one-way analysis of variance, paired t test or Bonferroni modified t test for multiple comparisons (Wallenstein *et al.*, 1980), as appropriate. P values of less than 0.05 were considered to be significant. When it was not possible to fit the data to the Hill equation, individual $\log[A]_{50}$ values were estimated by linear interpolation between the data points either side of the half maximal effect level.

When the minimum criteria for competitive antagonism were satisfied, that is the antagonist produced parallel rightward shift of the agonist concentration-effect curves with no change in upper asymptote, pK_B values were obtained by fitting the individual $\log[A]_{50}$ values obtained in the absence and presence of antagonist to a derivation of the Schild equation as described previously (Black *et al.*, 1985). When the criteria were not satisfied or not fully testable, a pA_2 value was estimated from the dose-ratio obtained with the lowest antagonist concentration which produced a significant rightward shift in all the replicate experiments.

Compounds

The following compounds were used: Adenosine, 5'-(N-ethyl)carboxamido adenosine (NECA), 8-phenyltheophylline (8-PT), dipyridamole (Dip), isoprenaline (all from Sigma Chemicals Ltd.), 1,3-dipropyl, 8-cyclopentylxanthine (DPCPX, Cambridge Research Biochemicals Ltd), 5-methylfurmethide (5-Mef, a gift from Wellcome Research Laboratories, Beckenham, Kent) and Ro 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, a gift from Hoffman La Roche, A.G.). The following compounds were supplied by Discovery Therapeutics Inc., Richmond, VA, USA N-0838 (9-methyl adenine), N-0837 (N^6-(3-pentyl), 9-methyl adenine), N-0840 (N^6-cyclopentyl, 9-methyl adenine), N-0861 (N^6-(endo-2-norbornyl), 9-methyl adenine) and N-0946 (N^6-(endo-2-norbornyl)-8-cyclopentyl),9-methyl adenine). NECA, adenosine and 5-Mef were dissolved in water. 8-PT and DPCPX were prepared in 80% ethanol plus 0.2 N NaOH at 20 mM. Dip, Ro 20-1724, N-0838, N-0837, N-0840, N-0946 and N-0861 were dissolved in a minimum volume (< 10 μl)) of absolute ethanol and made up to a concentration of 20 mM with distilled water. Isoprenaline was prepared in stoichiometric ascorbic acid as an antioxidant. Thereafter all dilutions of compounds were made in distilled water. In all experiments a vehicle control was included which corresponded to the highest dose of test compound administered.

Results

Effects of adenosine

In preliminary experiments, it was found that adenosine reduced the basal tone of some, but not all, the taenia caecum preparations examined. Therefore, tissues were precontracted with 5-Mef (0.1 μM). Under these conditions, adenosine, in the absence of adenosine uptake blockade, consistently produced concentration-dependent (30 μM to 3 mM) relaxation. The maximum relaxation ranged between 80 and 180% of the 5-Mef contraction varying considerably between, but not within, experiments. It was not possible to define the upper asymptote of the relaxant concentration-effect curves because of the high concentrations of adenosine involved which were at the limit of its solubility. The responses to adenosine were not blocked by the P_1-receptor non-selective antagonist, 8-PT (3 μM, Figure 1a) or the P_1-purinoceptor, A_1-subtype selective antagonist, DPCPX (30 μM, Figure 1b). These concentrations of the antagonists correspond to approximately 10 and 300 fold their reported

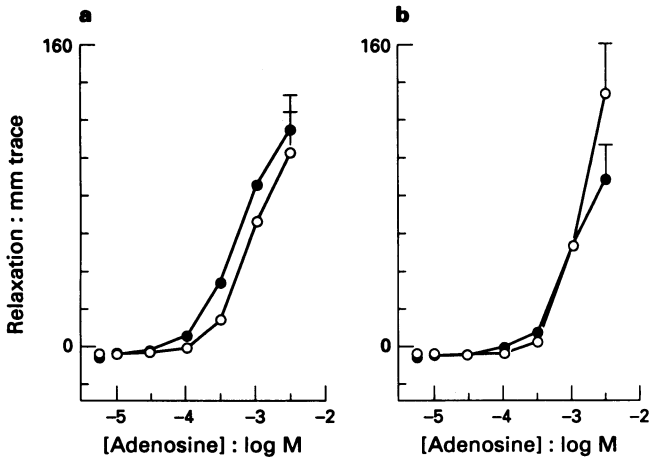

Figure 1 Adenosine concentration-effect curves obtained in the absence (●) and presence (○) of (a) 3 μM 8-PT and (b) 30 μM DPCPX on the guinea-pig taenia caecum assay ($n = 5/6 \pm$ s.e.mean). For abbreviations, see text.

K_B values at A_2-purinoceptors and 10 and 10,000 fold their reported K_B values at A_1-purinoceptors, respectively (Griffith *et al.*, 1981; Martinson *et al.*, 1987). Therefore, the action of adenosine in the absence of uptake blockade was apparently not mediated by A_1- or A_2-purinoceptors.

In the presence of increasing concentrations of the adenosine uptake blocker, dipyridamole (Dip, 0.3–3 μM), adenosine produced concentration-dependent relaxations at progressively lower concentrations (Figure 2a). In the presence of 3 μM Dip, the adenosine concentration-effect curve looked as though it was biphasic and spanned a concentration range of ~4.5 log cycles. Further evidence for the biphasic nature of the curve was provided by the observation that the responses to a concentration (3 μM) of adenosine in the presence of 3 μM Dip, which produced approximately half the first phase maximum, took about 5 min to reach a plateau whereas the second phase response to 1 mM adenosine took about 15 min to reach a plateau. The slow time courses of the individual responses in the second phase of the adenosine curve obtained in the presence of 3 μM Dip appeared to be similar to those obtained throughout the curve obtained in the absence of Dip (Figure 2b). It was not possible to fit the first phase data from all the individual preparations because the upper asymptote was not always sufficiently well-defined. Nevertheless, the $p[A]_{50}$ value of the first phase in the presence of Dip (3 μM) was estimated, by eye, to be around 5.5. Higher concentrations of Dip produced pronounced relaxations of the taenia caecum (data not shown) and so it was not possible to determine the concentration of Dip which would produce no further leftward shift of the adenosine curve and hence, presumably, saturate the uptake process. However, 3 μM Dip may be close to a saturating concentration because it is approximately 190 fold higher than its reported K_I value (16 nM) for the uptake process in guinea-pig left atrium (Kenakin, 1982).

In the presence of Dip (3 μM), 8-PT (0.3–30 μM) produced concentration-dependent rightward shift of the first phase of the adenosine curve although the second phase did not appear to be inhibited (Figure 4a). It was not possible to fit the first phase data to the Hill equation and hence provide an objective estimate of the pK_B value for 8-PT. However, it was possible to obtain an approximate pA_2 value by estimating a dose-ratio by linear interpolation of the mid-region of the adenosine concentration-effect curves obtained in the absence and presence of 3 μM 8-PT. The value obtained (~6.7) is consistent with the value reported for competitive antagonism of P_1-purinoceptors (Griffith *et al.*, 1981).

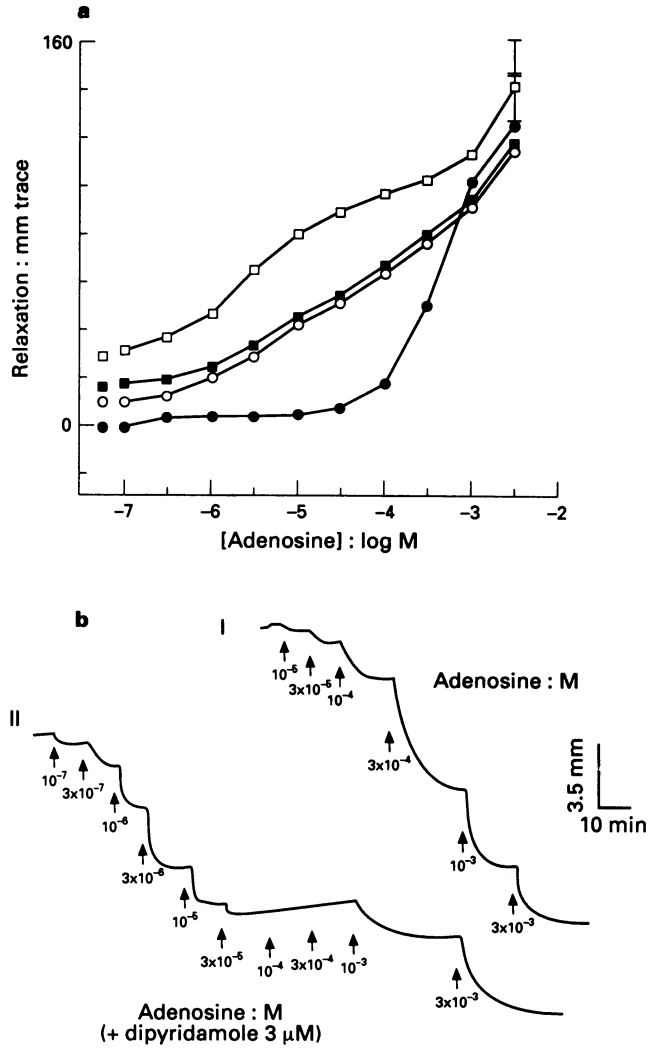

Figure 2 (a) Adenosine concentration-effect curves obtained in the absence (●) and presence of 0.3 (○), 1 (■) and 3 (□) μM dipyridamole on the guinea-pig taenia caecum assay ($n = 6 \pm$ s.e.mean). (b) Examples of experimental traces showing adenosine concentration-effect curves obtained by cumulative dosing in the absence (I) and presence (II) of 3 μM dipyridamole. The scale shown refers to absolute changes in tissue length where 10 mm experimental trace was equivalent to 1.3 mm tissue length.

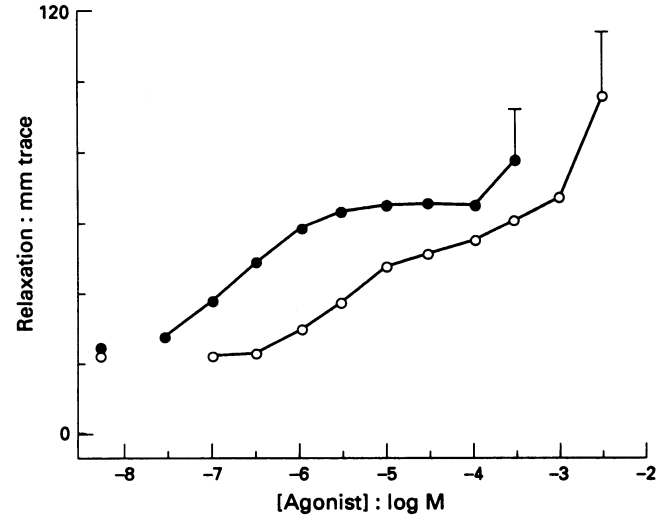

Figure 3 NECA (●) and adenosine (○) concentration-effect curves obtained in the presence of 3 μM dipyridamole on the guinea-pig taenia caecum assay ($n = 3 \pm$ s.e.mean).

Effects of NECA

NECA has been classified as a potent A_2-purinoceptor agonist which is not significantly removed from the receptor compartment by the adenosine uptake process (Collis, 1983; Burnstock *et al.*, 1984). Biphasic concentration-effect curves were obtained with NECA which were similar to those obtained with adenosine in the presence of Dip (3 μM), although both the first and second phase responses took longer than adenosine to reach a plateau. The response to a concentration of NECA (0.1 μM) producing half the maximum first phase response took approximately 15 min to plateau compared to about 5 min for the equivalent adenosine response. The second phase NECA responses took in excess of 60 min to reach a plateau (see Figures 2b and 4d) compared with 15 min estimated for adenosine. Therefore, the responses in both phases of the NECA curve took 3–4 times longer to attain a plateau than the corresponding adenosine responses in the presence of Dip. The presence of 3 μM Dip did not appear to affect the shape or location of

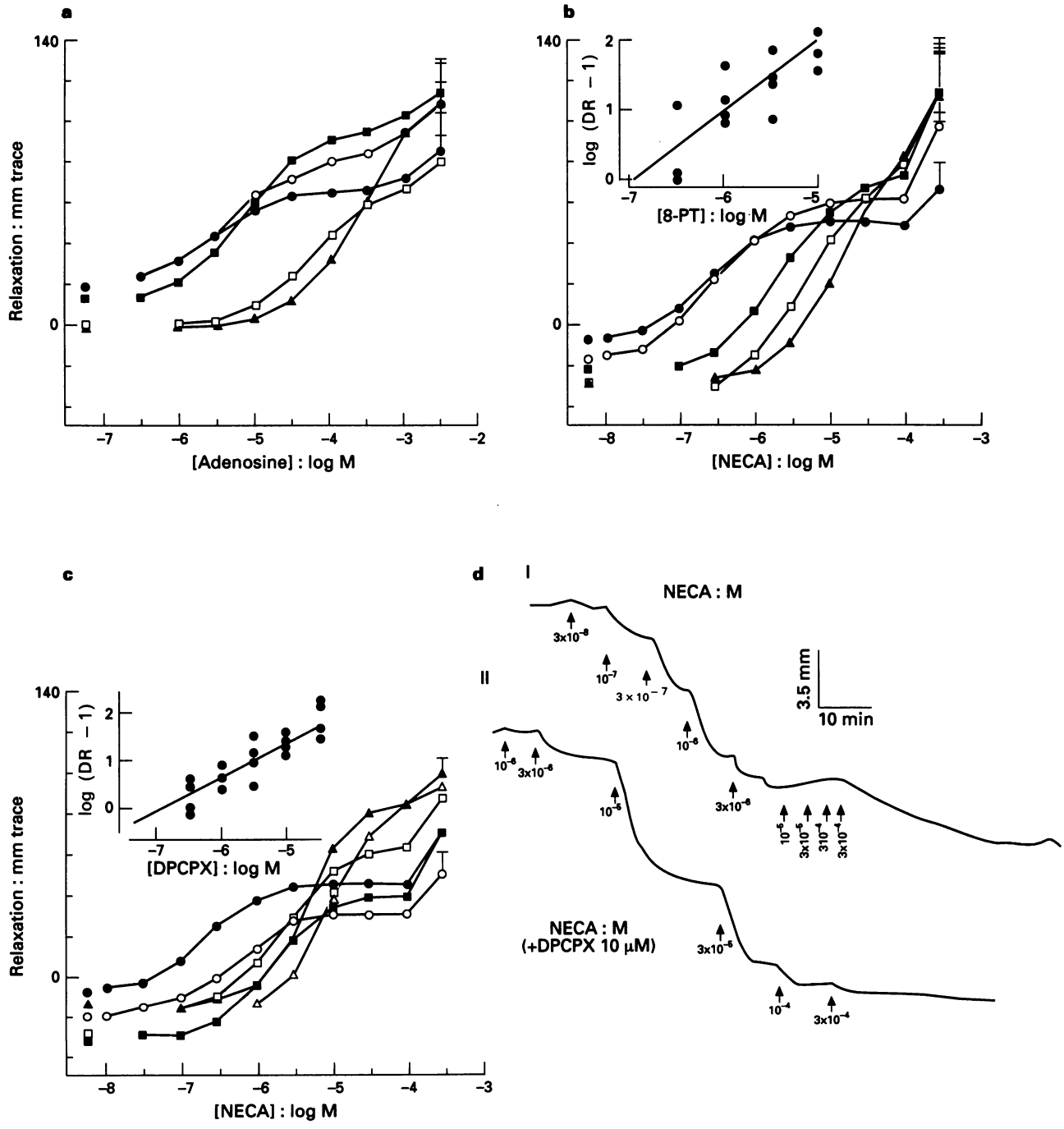

Figure 4 (a) Adenosine concentration-effect curves obtained in the absence (●) and presence of 0.3 (○), 1 (■) 3 (□) and 10 (▲) μM 8-phenyltheophylline with dipyridamole (3 μM) present throughout on the guinea-pig taenia caecum assay ($n = 5/6 \pm$ s.e.mean). (b) NECA concentration-effect curves obtained in the absence (●) and presence of 0.3 (○), 1 (■) 3 (□) and 10 (▲) μM 8-phenyltheophylline (8-PT) on the guinea-pig taenia caecum assay ($n = 4/6 \pm$ s.e.mean). The inset shows the corresponding Schild plot (see text for details). (c) NECA concentration-effect curves obtained in the absence (●) and presence of 0.3 (○), 1 (■) 3 (□) and 10 (▲) and 30 (△) μM DPCPX on the guinea-pig taenia caecum assay ($n = 5/7 \pm$ s.e.mean). The inset shows the corresponding Schild plot (see text for details). (d) Example of experimental trace showing NECA concentration-effect curves obtained by cumulative dosing in the absence (I) and presence (II) of DPCPX (10 μM). The scale shown refers to absolute changes in tissue length where 10 mm experimental trace was equivalent to 1.3 mm tissue length. For abbreviations, see text.

the NECA curve as judged by comparing the NECA curve obtained in the presence of Dip (Figure 3) and the control curves obtained in the absence of Dip (Figures 4b and 4c) albeit in separate experiments.

8-PT and DPCPX (0.3–10 μM) produced concentration-dependent rightward shift of the first phase of the NECA curve without affecting the second phase (Figure 4). Interestingly, in the presence of a higher concentration of DPCPX (30 μM), the curves were monophasic and the concentration-effect profile appeared to change again; all the responses appeared to attain a plateau more rapidly than the first phase control responses. Analysis of the first phase data obtained in the absence and presence of 8-PT (1 μM) gave a pK_B value estimate of 6.97 ± 0.19 (Schild slope parameter, b = 0.99 ± 0.20, d.f. = 19). The Schild slope parameter for DPCPX was significantly different from unity (0.67 ± 0.09, d.f. = 29) but notwithstanding this a pA_2 value of 6.77 ± 0.10 was estimated from the shift obtained in the presence of DPCPX (1 μM). This latter value is possibly consistent with the pK_I value of 7.16 estimated by Martinson et al. (1987) for DPCPX at the A_2-purinoceptors present in human platelets. Therefore, it is likely that the response elicited by low concentrations of NECA and also presumably low concentrations of adenosine in the presence of Dip, are mediated by the A_2-subtype of the P_1-purinoceptor class.

The rightward shifts of the first phase of the NECA curves by DPCPX and 8-PT were associated with other complex changes. The rightward shift obtained with 8-PT was accompanied by a trend towards an increase in the upper asymptote of the first phase of the curve, although the differences in asymptote values were not significant as tested by analysis of variance (Table 1). However, analysis of variance did reveal a significant difference between the DPCPX treatment groups of the NECA curve asymptote values ($F_{(4,20)} = 3.4132$, $P < 0.05$). Subsequent analysis using the Bonferroni modified t test for multiple comparisons indicated that the value in the presence of 10 μM DPCPX was significantly increased from the control value ($t = 2.54$, $P < 0.05$). Furthermore, DPCPX, but not 8-PT, showed significant heterogeneity on analysis of variance in the midpoint slopes of the first phase of the NECA curves (Table 1). However, the steeper slopes at the two highest concentrations of DPCPX were not significantly different from the control as judged by the Bonferroni modified t test.

The finding that an A_1-purinoceptor selective antagonist, DPCPX, increased the upper asymptote of the first phase of the NECA curve suggested that the amplification could be due to NECA activating A_1-purinoceptors coupled to contraction of the guinea-pig taenia caecum. This hypothesis was investigated further with a series of 9-methyl adenine analogues which were shown, by competitive analysis, to exhibit a range of affinity values on the guinea-pig left atrium A_1-purinoceptor assay (Table 2). There was no relationship between the compounds' A_1-purinoceptor affinity and their ability to increase the upper asymptote of the first phase of the NECA curve. Indeed, N-0838 (100 μM), the unsubstituted parent compound, 9-methyl adenine, did not produce a significant shift of the NECA curve on the guinea-pig left atrium or taenia caecum assays although it increased the upper asymptote 2.3 fold in the guinea-pig taenia caecum.

Effects of phosphodiesterase inhibition

Several xanthine based adenosine ligands are also recognised as being inhibitors of phosphodiesterase. Such an action of the antagonists used in this study could, in principle at least, produce the increase in upper asymptote of the first phase of the NECA and adenosine concentration-effect curves. This was investigated by comparing the effects of the antagonists and the selective phosphodiesterase inhibitor, Ro 20-1724 (Bergstrand et al., 1977) on the curves obtained on the 5-Mef pre-contracted taenia caecum assay with NECA and the selective β-adrenoceptor agonist, isoprenaline. Ro 20-1724 (30 μM) produced a significant relaxation of the taenia caecum, equivalent to approximately 20% of the maximum response obtained with NECA under control conditions, and

Table 2 pK_B estimates for a series of 9-substituted adenine derivatives at A_1-purinoceptors on the guinea-pig isolated left atrium assay and their effect, at the concentration shown in parentheses, on the maximum response of the first phase of the adenosine concentration-effect curve obtained on the guinea-pig taenia caecum assay

Ligand	*Guinea-pig left atrium* (pK_B ± s.e.)	α_B/α ± s.e.mean	[*Ligand*]
N-0838	<4	2.32 ± 0.24*	(100 μM)
N-0861	6.28 ± 0.09	2.19 ± 0.22*	(10 μM)
N-0840	6.17 ± 0.11	1.35 ± 0.27	(30 μM)
N-0837	5.26 ± 0.10	2.32 ± 0.23*	(100 μM)
N-0946	5.84 ± 0.06	1.66 ± 0.48	(30 μM)

The pK_B values were estimated according to the methods described in the text using NECA as agonist. In each case the corresponding Schild plot slope parameter was not significantly different from unity. N-0838 (100 μM) did not produce a significant shift of the NECA curve. The effect on the maximum response in the taenia caecum assay (α_B/α) is expressed as the ratio of the first phase maximum responses estimated in the presence and absence of the antagonist at the concentration shown in parentheses. *Significantly greater than unity ($P < 0.05$).

Table 1 The effect of 8-phenyltheophylline (8-PT) and 1,3-dipropyl, 8-cyclopentylxanthine (DPCPX) on estimates of the location ($p[A]_{50}$), midpoint slope and upper asymptote (mm trace) of the first phase of the NECA concentration-effect curves obtained on the guinea-pig taenia caecum assay as shown in Figures 4b and 4c

[8-PT]: μM	0	0.3	1	3	10	
$p[A]_{50}$	6.53	6.26	5.50	5.25	4.81	
(s.e.mean)	(0.16)	(0.22)	(0.15)	(0.13)	(0.06)	
asymptote	49.3	59.8	72.8	84.2	97.2	
(s.e.mean)	(14.0)	(34.6)	(28.5)	(20.3)	(23.6)	
slope	1.32	1.25	1.05	0.98	1.01	
(s.e.mean)	(0.02)	(0.04)	(0.10)	(0.06)	(0.07)	
[DPCPX]: μM	0	0.3	1	3	10	30
$p[A]_{50}$*	6.70	6.20	5.86	5.64	5.34	4.97
(s.e.mean)	(0.10)	(0.10)	(0.07)	(0.14)	(0.07)	(0.14)
asymptote*	45.2	32.3	38.4	64.0	82.0†	92.2
(s.e.mean)	(6.3)	(9.1)	(14.3)	(14.3)	(9.1)	(10.2)
slope*	1.30	1.09	1.28	1.16	1.77	1.45
(s.e.mean)	(0.08)	(0.09)	(0.05)	(0.07)	(0.11)	(0.11)

$n = 4/7$ ± s.e.mean.
*Significant differences between treatment group values ($P < 0.05$) as tested by one-way analysis of variance. †Significant ($P < 0.05$) difference from control value as tested by Bonferroni modified t test.

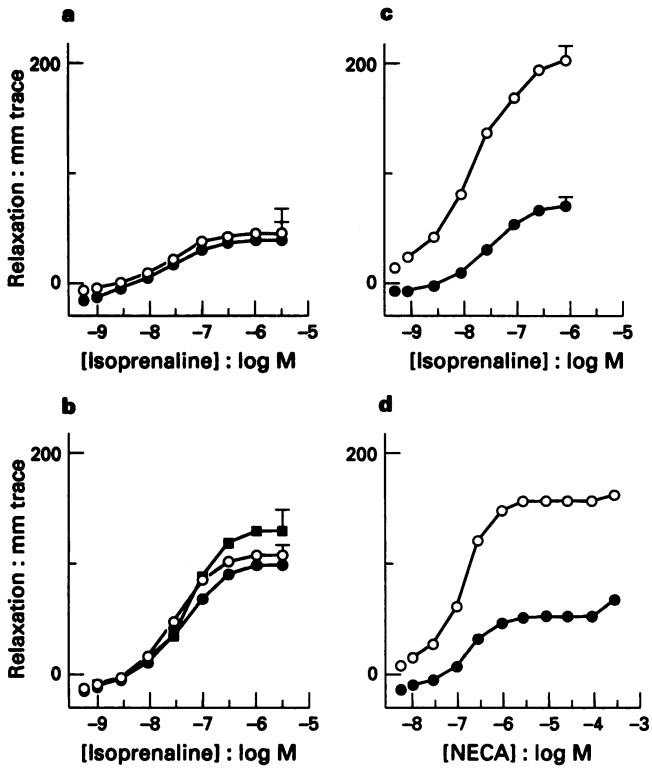

Figure 5 Isoprenaline concentration-effect curves obtained in the absence (●) and presence of (a) 10 μM 8-phenyltheophylline (○); (b) 10 (○) and 30 (■) μM DPCPX and (c) 30 μM of the phosphodiesterase inhibitor Ro 20-1724 (○) on the guinea-pig taenia caecum assay. Panel (d) shows NECA concentration-effect curves obtained in the absence (●) and presence (○) of 30 μM Ro 20-1724. For abbreviations, see text.

produced a significant 250% increase in the upper asymptote of the first phase of the NECA curve (Figure 5a). Similarly, Ro 20-1724 (30 μM) produced a relaxation equivalent to 23% of the upper asymptote of a control concentration-effect curve obtained with isoprenaline and produced a 250% increase in the upper asymptote of the isoprenaline curve (Figure 5b). In contrast, 10 μM 8-PT and 30 μM DPCPX, did not have a significant effect on the upper asymptote of the isoprenaline curves.

Discussion

The finding that adenosine was relatively impotent in the absence of agonist uptake blockade was not surprising because agonist uptake systems are known to be able to lower significantly the concentration of agonists in the receptor compartment of isolated tissue bioassays. However, the difference in the behaviour of adenosine in the absence and presence of uptake blockade could not be accounted for simply by changes in the concentration of adenosine at a homogeneous population of receptors. First, in the presence of adenosine uptake blockade (achieved with Dip), the adenosine concentration-effect curve was biphasic. Second, the selective P_1-purinoceptor antagonist, 8-PT was only effective against the first phase responses exposed by the uptake blockade. Although the criteria for competitive antagonism could not be tested objectively because of the complicating presence of the second phase, the pA_2 value estimated from the dose-ratios obtained in the presence of low concentrations of this antagonist suggested that the first phase was mediated by P_1-purinoceptors. The idea that the second phase responses obtained in the presence of Dip and all of the responses obtained in the absence of Dip were mediated by the same non-P_1-purinoceptor mechanism of action of adenosine was supported by the observation that the time course of the individual responses were similar and significantly slower than those mediated by P_1-purinoceptors.

If the resistant action involved the stimulation of additional purinoceptors present in the same compartment as the P_1-receptors, then uptake blockade, which is assumed to increase the agonist concentration in the receptor compartment, might have been expected to increase the potency of adenosine at both sites to an equal extent, which was not the case. However, this expectation is based on the assumption that the activation of the putative additional receptors occurs over an adenosine concentration-range which can be significantly lowered by the uptake process. In fact, the concentration-range of adenosine over which the second phase responses were obtained is in the region usually considered to saturate the uptake process (~10 μM, Kenakin, 1982; Schrader *et al.*, 1972). Therefore, although at first sight the data might suggest an intracellular rather than an extracellular site of action, it is not possible to draw any unequivocal conclusions regarding the location of the site mediating the resistant action. An intracellular site of action may have been implicated if the second phase responses were inhibited by Dip. In the event, as far as we could ascertain (Figure 1), the second phase responses appeared unchanged in the presence of Dip. This observation, that the second phase responses were independent of the Dip-sensitive adenosine uptake process, was supported by the finding that the agonist NECA, which is reported not to be taken up, also produced, at high concentrations, relatively slow time course responses which were not blocked by P_1-purinoceptor antagonists. Therefore, an explanatory model to account for these data requires that the second phase responses are mediated by high concentrations of adenosine and NECA acting either at an intracellular site which is accessed independently from the Dip-sensitive uptake site or an extracellular site which is activated at concentrations of adenosine above those which saturate the uptake process. If the site of action is intracellular, the high concentration of adenosine and NECA (above 10 μM) required to produce the second phase responses suggest that access to the intracellular compartment could be gained by simple diffusion. Indeed, Schrader *et al.* (1972) found that the Dip-sensitive component of uptake in human erythrocytes was saturated at 10 μM adenosine. At concentrations higher than this adenosine was able to gain access by simple diffusion.

The refractoriness of the adenosine-alone responses to blockade by 8-PT and DPCPX suggests that A_2-receptors are not involved. Burnstock *et al.* (1984) also found that adenosine's relaxation of the taenia caecum was not blocked by 8-PT. There are other reports that adenosine and some of its analogues are able to produce non A_2-purinoceptor-mediated responses in other smooth muscle preparations: Brackett & Daly (1991) noted xanthine P_1-purinoceptor antagonist-resistant relaxations to NECA in the guinea-pig isolated trachea; Martin (1992) and Collis & Brown (1983) noted relaxations to high concentrations of adenosine and analogues in guinea-pig aorta that were refractory to 8-PT: although none of these authors drew attention to the changes in the time courses of the responses. However, the latter group found that these responses were depressed by Dip and thus concluded that they were mediated by an intracellular site. In contrast, we found that the P_1-purinoceptor antagonist resistant responses were neither depressed nor potentiated by Dip and therefore require another mechanism of access to the putative intracellular site such as diffusion. Collis & Brown (1983) postulated that adenosine caused the relaxation in the guinea-pig aorta by inhibiting the enzyme, 5-adenosylhomocysteine hydrolase leading to the accumulation of substrates which in turn caused inhibition of cyclic nucleotide PDE.

The presence in the guinea-pig taenia caecum of the A_3-

receptor which has been reported to mediate responses to adenosine and analogues which are not blocked by 8-PT and DPCPX (Zhou *et al.*, 1992) cannot be ruled out. However, this receptor subtype is possibly not responsible for the second phase relaxant responses observed in this study because it is claimed to be coupled to the inhibition of adenylate cyclase. Inhibition of adenylate cyclase would be expected to produce contraction rather than relaxation of the taenia caecum.

In contrast to our results, Hourani *et al.* (1991) have reported simple competitive blockade of responses to adenosine (in the absence of Dip) by 8-(*p*-sulphophenyl) theophylline in the taenia caecum. However responses were obtained at lower concentrations of adenosine than were required in this study. The reason for this discrepancy is unclear, however, it is possible that there were differences in the activity of the tissue adenosine uptake systems between the two studies. Differences in experimental design might also contribute to the difference in results, for instance, in this study a higher contractile response level was elicited (80% max cf. 50–70% max) which might afford more functional antagonism of the A_2 receptor-mediated relaxant responses. In any event Hourani *et al.* (1991) did not achieve the same high organ bath concentrations of adenosine required to elicit marked second phase responses.

The affinity values estimated for 8-PT and DPCPX suggest that responses to low concentrations of NECA and adenosine (+Dip) are likely to be mediated by the A_2-purinoceptor as originally concluded by Burnstock *et al.* (1984). However, it is apparent that both 8-PT and DPCPX have complex pharmacological profiles in the guinea-pig taenia caecum preparation and are able to amplify NECA concentration-effect curves at the same concentrations as those used to produce blockade of the A_2-purinoceptor. The suggestion that the amplification elicited by the antagonists might be due to the inhibition of a contractile A_1-purinoceptor-mediated response was considered. Although some of the 9-methyl adenosine compounds did produce a significant increase in the upper asymptote of the NECA curves, the data indicate that there was no correlation between their A_1-purinoceptor affinity and propensity to cause amplification within the series of analogues (Table 2). If it can be assumed that this family of antagonists cause amplification by the same mechanism as the two xanthine compounds, then it is unlikely that the amplification observed is due to removal of an A_1-purinoceptor mediated contractile response in the taenia caecum.

If the amplification of the NECA concentration-effect curves were due to phosphodiesterase inhibition by the antagonists, an action previously recognised for some xanthine derivatives, then effects of NECA curves should be mirrored by effects upon the curves obtained with other agonist-receptor systems which are mediated by the production of cyclic AMP. However, in contrast to the phosphodiesterase inhibitor Ro 20-1724, both 8-PT and DPCPX at the highest concentrations tested did not have a significant effect on the upper asymptote of isoprenaline concentration-effect curves. Another possible mechanism was suggested by the work of Ramkumar & Stiles (1988) who reported that the xanthine derivative, 8-(-[[[[(2-amino-ethyl)-aminocarbonyl] methyl]oxy]phenyl]-1,3-dipropylxanthine (XAC) interacts with the inhibitory G protein (Gi), possibly through an allosteric site leading to elevation of adenylate cyclase activity. If this is a general property of xanthine derivatives then it may account for the amplification seen, although once again an effect of DPCPX on isoprenaline responses may have been expected.

In conclusion, it is evident that adenosine and its analogue NECA are able to elicit both A_2 and non-A_2-receptor mediated relaxant responses in the guinea-pig taenia caecum and, under conditions where the adenosine uptake process is functional, non-A_2-receptor mediated responses predominate. It is also clear that in this tissue both 8-PT and DPCPX exhibit complex profiles of activity. Both the response mechanism heterogeneity and the antagonist effects make interpretation of agonist/antagonist interactions difficult and potentially misleading.

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Prevention by blockade of angiotensin subtype$_1$-receptors of the development of genetic hypertension but not its heritability

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1 We determined whether early inhibition of angiotensin II subtype$_1$ (AT_1) receptors by the newly synthesized nonpeptidic antagonist, A-81988, can attenuate the development of hypertension in spontaneously hypertensive rats (SHR) and if the altered blood pressure phenotype can be passed on to the subsequent generation, not exposed to the antagonist.

2 Pairs of SHR were mated while drinking tap water or A-81988 in tap water, and the progeny was maintained on the parental regimen until 14 weeks of age. At this stage, A-81988-treated rats showed lower systolic blood pressure and body weight values (136 ± 5 versus 185 ± 4 mmHg and 247 ± 4 versus 283 ± 4 g in controls, $P < 0.01$), while heart rate was similar. In addition, mean blood pressure was reduced (101 ± 7 versus 170 ± 7 mmHg in controls, $P < 0.01$), and the pressor responses to intravenous or intracerebroventricular angiotensin II were inhibited by 27 and 59%, respectively. Heart/body weight ratio was smaller in A-81988-treated rats (3.2 ± 0.1 versus 3.8 ± 0.1 in controls, $P < 0.01$).

3 The antihypertensive and antihypertrophic effect of A-81988 persisted in rats removed from therapy for 7 weeks (systolic blood pressure: 173 ± 4 versus 220 ± 4 mmHg, heart/body weight ratio: 3.4 ± 0.1 versus 4.1 ± 0.1 in controls at 21 weeks of age, $P < 0.01$ for both comparisons), whereas the cardiovascular hypertensive phenotype was fully expressed in the subsequent generation that was maintained without treatment.

4 These results indicate that chronic blockade of angiotensin AT_1-receptors attenuates the development of hypertension in SHR but it does not prevent the transmission of hypertension to the following generation. Thus, heritability of the SHR's hypertensive trait is not affected by pharmacological manipulation of the cardiovascular phenotype.

Keywords: Renin; angiotensin; blood pressure; genetic hypertension; angiotensin converting enzyme

Introduction

Early phases of life are crucial to the expression of genes encoding for proteins involved in the regulation of cardiovascular and renal function. The finding that the renin-angiotensin system (RAS) is activated in the new-born period, suggests a pivotal role of this system during early developmental phases (Wallace *et al.*, 1980; Gomez *et al.*, 1989; Carbone *et al.*, 1993). Angiotensin II (AII) not only causes vasoconstriction and antinatriuresis but also promotes vascular smooth muscle hypertrophy (Geisterfer *et al.*, 1988), and angiogenesis in the maturing kidney (Robilard *et al.*, 1983; Fogo *et al.*, 1990). In addition, angiotensin-converting enzyme (ACE), a protease that converts AI to AII, is upregulated in the new-born rat as a result of enhanced gene expression at the pretranscriptional level (Yoipiv *et al.*, 1994). The increased ACE activity could influence renal excretory function and haemodynamics in the developing animal by enhancing the rate of AII formation. Therefore, it is not surprising that environmental or pharmacological influences on the activity of the RAS during this early phase of the life can alter the adult blood pressure phenotype (Unger & Retting, 1990). Indeed, lifetime oral administration of ACE inhibitors prevents the development of arterial hypertension in rats genetically predisposed to this disease (Wu & Berecek, 1993), the latter effect persisting even after drug discontinuation (Giudicelli *et al.*, 1980; Harrap *et al.*, 1990). This property seems to be peculiar to ACE inhibitors since the antihypertensive protection exerted by other classes of drugs, such as β-blockers or calcium antagonists, tends to be lost after their discontinuation in young spontaneously hypertensive rats (SHR) (Giudicelli *et al.*, 1980; Nyborg & Mulvany, 1985).

Recently, Wu & Berecek (1993) extended the former concepts by showing that the antihypertensive effect induced by early oral administration of captopril, an ACE inhibitor, is accompanied by a failure in the offspring (that were never exposed to antihypertensive therapy) to express a full hypertensive phenotype. Although these interesting results may be consistent with an alteration of the brain RAS in the progeny of captopril-treated rats (as suggested by a blunted drinking response to intracerebroventricular administration of AI), the exact mechanism resulting in the persistence of the antihypertensive effect in the progeny remains unclear. In addition, as ACE degrades many other endogenous peptides, namely bradykinin, enkephalins and substance P (Erdos, 1974), some of the effects of ACE-inhibitors may also be unrelated to the RAS. Thus, studies using antihypertensive compounds more specific than ACE-inhibitors may help clarify how blood pressure phenotype, once altered by a pharmacological manipulation, can be passed on to subsequent generations.

Recently, nonpeptidic AII receptor antagonists, exemplified by DuP-753 (Timmermans *et al.*, 1992), have been used as a more direct approach to address the role of the RAS in blood pressure regulation, and among AII receptor subtypes recognised so far, the AT_1-receptor subtype is responsible for the majority of the cardiovascular, renal, and central nervous system actions of AII (Duncia *et al.*, 1992).

In the present study, the compound A-81988, a newly synthesized AT_1-receptor antagonist (De *et al.*, 1992), approximately 10 times more potent than DuP-753, was used to test the hypothesis that blockade of the RAS not only prevents the development of genetic hypertension, but also alters the cardiovascular phenotype of the following generation. In particular, we wished to address the following questions: (1) Can early chronic blockade of AT_1 receptors alter the development

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of hypertension and target organ damage in SHR? (2) Is the protective effect associated with blunted central and peripheral responses to AII? (3) Does the antihypertensive effect persist after therapy is stopped? and (4) Can the protective effect be passed on to the progeny despite absence of treatment in these animals?

Methods

Wistar Kyoto rats (WKY) and SHR (Charles River, Milan, Italy) were housed at a constant room temperature (24 ± 1°C) and humidity (60 ± 3%) with a 12 h light/dark cycle. They had free access to rat chow (sodium, 0.12 mmol g^{-1}, Mucedola, Milan, Italy) and tap water for the duration of the experiment. The experimental protocol was approved by the local animal care and use committee. All procedures complied with the standards for the care and use of animals as stated in *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Science, Bethesda, MD, U.S.A.). All surgical procedures (except implantation of cerebroventricular cannula) were performed with rats under ether anaesthesia using disappearance of corneal reflex to adjust the depth of anaesthesia.

Experiment 1 Effect of oral short-term administration of A-81988 on the pressor response to AII in adult rats

Fourteen-week old, male WKY rats and SHR were given a solution containing 0.6 mg A-81988, 2-(N-propyl-*N*(2'-[1H-tetrazol-5-yl]biphenyl-4yl)methyl] amino) pyridine-3-carboxylic acid, (Abbott Company, Abbott Park, IL, U.S.A.) in 100 ml tap water (~1 mg kg^{-1} per day) to drink. Controls were maintained on tap water. Six days later, a polyethylene catheter (PE-10, Clay-Adams, Parsippany, NJ, U.S.A.) was inserted via the left femoral artery and advanced into the abdominal aorta; another polyethylene catheter was inserted into the left femoral vein and advanced into the vena cava. Both catheters were tunnelled under the skin and exteriorized at the back of the neck. On the morning of the following day, the mean blood pressure (MBP) of unanaesthetized A-81988- or vehicle-treated rats was measured by connecting a Statham transducer (Gould, Oxford, CA, U.S.A.) to the femoral artery catheter. After 20 min stabilization, the pressor responses to i.v. boluses of AII (from 100 to 500 ng in 20 μl saline, Sigma Chemical Company) were tested. Doses of AII were given in random order and sufficient time (30 min) was allowed between the injections for MBP to return to basal levels. Each group consisted of 6 rats.

An additional set of rats was used to test the effect of oral administration of A-81988 on the pressor effect induced by intracerebroventricular (i.c.v.) AII. Male SHR and WKY rats were anaesthetized with pentobarbitone (50 mg kg^{-1}, i.p.) and a 22-gauge stainless cannula (15 mm long and bent at 90° angle at midpoint) was implanted stereotaxically into the left lateral cerebral ventricle (1.5 mm lateral and 1.0 posterior to the bregma, and 4.5 mm deep from the skull surface). The cannulae were anchored to the skull with screws embedded in dental acrylic cement. The free end of the cannula was attached to a silicone elastomer tube filled with sterile artificial cerebrospinal fluid (aCSF). The tube was tunnelled under the skin, exteriorized between the scapulae, and occluded with a metal pin. The rats were allowed to recover for three days and then they were instrumented with an arterial femoral catheter as described above. The pressor response to an i.c.v. bolus of AII (500 ng in 5 μl saline) or vehicle was determined in 14-week old male WKY rats and SHR, one week after A-81988 or vehicle was started. Injections were made with a 50 μl Hamilton syringe (Reno, NE, U.S.A.). Each group consisted of 6 rats. At the end of the experiments, the correct placement of the cannula in the lateral cerebral ventricle was tested by injecting 5 μl of 1% fast green dye and then by determining the presence of dye in the cerebroventricular system.

Experiment 2. Effect of early oral administration of A-81988 on the development and heritability of hypertension in SHR

SHR breeders were given a solution containing 0.6 mg A-89188 in 100 ml tap water (~1 mg kg^{-1} per day) to drink. Dams were maintained on A-89188 during pregnancy and lactation. The control group consisted of breeders given tap water. The pups (2nd generation, 2nd G) were weaned at 4 weeks of age and maintained on the same treatment (A-81988 or vehicle) as their parents until the 14th week of age. After weaning, dosage of A-81988 (1 mg kg^{-1} per day) was kept constant throughout the duration of the experiment by adjusting the concentration of the antagonist in the drinking water.

At 14 weeks of age, 12 rats (6 males) given A-89188 had the treatment withdrawn (Off-A-81988 rats). Then, they were mated either immediately or 3 weeks after A-89188 discontinuation. This was done to discover whether duration in the time elapsed from A-81988 withdrawal is crucial in determining the blood pressure phenotype of the following generation. Since no difference was observed among rats conceived either immediately or 3 weeks after antagonist discontinuation, data relevant to these groups were combined (see Results). The progeny (third generation, 3rd G) of A-81988-treated rats was maintained on tap water (without A-81988) throughout the experiment.

Systolic blood pressure (SBP) and heart rate (HR) of rats of the 2nd G (A-81988-treated and control groups, $n = 44$ [22 males] each) and 3rd G ($n = 44$ [22 males]) were measured by tail-cuff plethysmography (Recorder 8002, Ugo Basile, Biological Research Apparatus, Comerio, Italy). Briefly, unanaesthetized rats were warmed for 10 min at 35°C in a thermostatically controlled heating cabinet. Then, measurements were performed with the rat gently wrapped in a cotton hand towel. Each pressure value was obtained by averaging 8 to 10 individual readings. At 14 weeks of age, 24 h urine collections were obtained from rats in individual metabolic cages. Then, cerebroventricular and femoral catheters were inserted (as described above). Three days later, the vasopressor responses to i.v. or i.c.v. injections of 500 ng AII were determined. During the following 24 h, rats that received i.c.v. AII were maintained in individual metabolic cages, which allowed for a high degree of accuracy in the measurement of water intake by the inclusion of spill catches. At the end of the experiment, with rats under anaesthesia, both kidneys and the heart were excised, washed three times in saline, blotted and weighed.

Analytical procedures

Urinary volume (UV) was determined gravimetrically. Urinary sodium ($U_{Na}V$) and potassium (U_KV) were determined by flame photometry. Urinary creatinine and osmolality were measured with an automatic analyser (Hitachi 704, Kyoto, Japan). Kallikrein activity in urine was measured by using the synthetic substrate H-D-Val-Leu-Arg-*p*-nitroanilide (S2266, Kabi Diagnostica, Molndal, Sweden) in the presence of soybean trypsin inhibitor (Sigma Chemical Company, St. Louis, MI, U.S.A.) and expressed in nanokatals (1 nkat represents the enzyme activity able to cleave 1 nmol *p*-nitroaniline s^{-1} from substrate).

Statistical analysis

All data are expressed as mean ± s.e.mean. Multivariate repeated-measures ANOVA was performed to test for interaction between time and grouping factor. Univariate ANOVA then was used to test for differences among groups and over time. Differences within or between groups were determined using Student's paired or unpaired *t* test, respectively, with the Bonferroni multiple-comparison adjustment. Mathematical

and statistical analysis were performed with as STATVIEW II package (Brain Power, Los Angeles, CA, U.S.A.) on an Apple Macintosh IICX computer.

Results

Experiment 1. Effect of oral short-term administration of A-81988 on the basal blood pressure and on the pressor response to AII in adult rats

Rats given A-81988 in their drinking water for one week showed lower MBP values compared to controls (WKY rats: 96±1 versus 106±2 mmHg; SHR: 149±3 versus 175±4 mmHg, $P<0.05$ for both comparisons).

As shown in Figure 1a, b the pressor response to i.v. AII was significantly reduced in rats given A-81988 compared to controls. Figure 1c, d shows that the pressor response induced in SHR by 500 ng i.c.v. AII was greater than that observed in WKY rats. However, in both strains the response to i.c.v. AII was reduced in magnitude and duration by oral administration of A-81988.

Experiment 2. Effect of early oral administration of A-81988 on the development and heritability of hypertension in SHR

There was no significant difference between control and experimental groups of the 2nd G as far as the size of litters at birth were concerned (5 to 10 pups per litter).

As shown in Figure 2 (a, b), development of hypertension was attenuated by early oral administration of A-81988 in SHR (males: 136±5 versus 185±4 mmHg in controls; females: 136±6 versus 184±3 mmHg at 14 weeks, $P<0.01$ for both comparisons). In male SHR removed from therapy (Off-A-81988), attenuation of the hypertensive condition was still evident 7 weeks after discontinuation of the antagonist (173±4 versus 220±4 mmHg in age-matched never-treated controls, $P<0.05$). By contrast, the hypertensive phenotype was fully expressed in rats of the 3rd G. In particular, the trend of SBP to increase with ageing was similar in 3rd G female SHR and controls, whereas it was steeper in 3rd G male SHR so that a plateau was already reached at 11 weeks of age. Direct measurement of MBP confirmed the difference between A-81988-treated male rats, their offspring and controls at 14 weeks of age (101±7, 169±5 and 170±7 mmHg, respectively, $P<0.05$).

As shown in Figure 2c, d, no significant difference was observed between groups regarding HR. Body weight (Figure 2e, f) was lower in A-81988-treated rats compared to controls at 7 weeks of age and this difference persisted throughout the duration of the experimental period (247±4 versus 283±4 g in controls at 14 weeks, $P<0.01$). Body weight was not altered in the offspring of A-81988-treated rats.

As shown in Figure 3, A-81988-treated rats showed reduced pressor (17±2 versus 41±3 mmHg, $P<0.05$) and dipsogenic (29±6 versus 53±4 ml day^{-1}, $P<0.05$) responses to i.c.v. AII compared to those observed in controls. The pressor response to i.v. AII was also reduced (33±6 versus 45±4 mmHg in controls, $P<0.05$). By contrast, the effects of AII were not altered in Off-A-81988 SHR (i.c.v. AII: 36±2 mmHg and 53±2 ml day^{-1}; i.v. AII: 47±3 mmHg) as well as in their offspring (i.c.v. AII: 43±2 mmHg and 54±2 ml day^{-1}; i.v. AII: 45±3 mmHg).

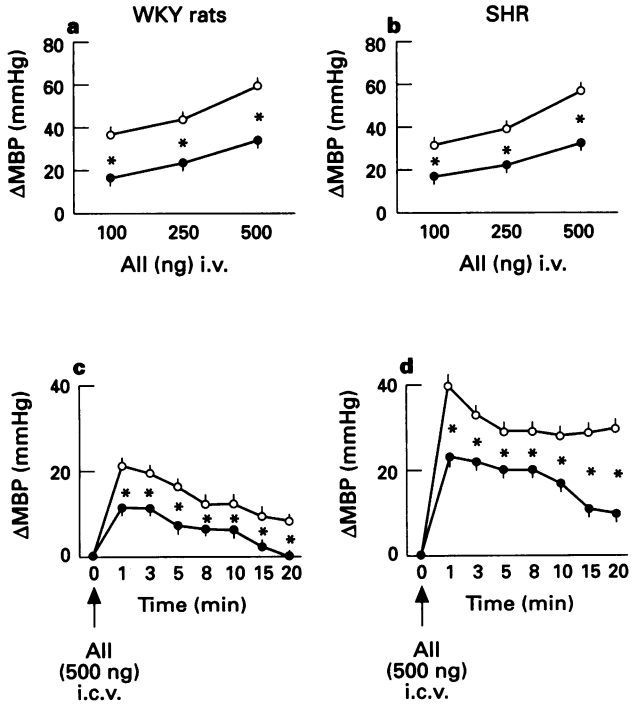

Figure 1 (a,b) Line graphs show changes in mean blood pressure (ΔMBP) induced by intravenous (i.v.) bolus injections of angiotensin II (AII) in Wistar Kyoto (WKY) rats and in spontaneously hypertensive rats (SHR) pretreated with oral A-81988 (●) or vehicle (○) for 1 week. Each group consisted of 6 rats. (c,d) Line graphs show changes in mean blood pressure (ΔMBP) induced by intracerebroventricular (i.c.v.) injection of 500 ng AII in WKY rats and SHR pretreated with oral A-81988 (●) or vehicle (○) for 1 week. Each group consisted of 6 rats. Data are mean±s.e.mean. *$P<0.05$ versus control group that received vehicle.

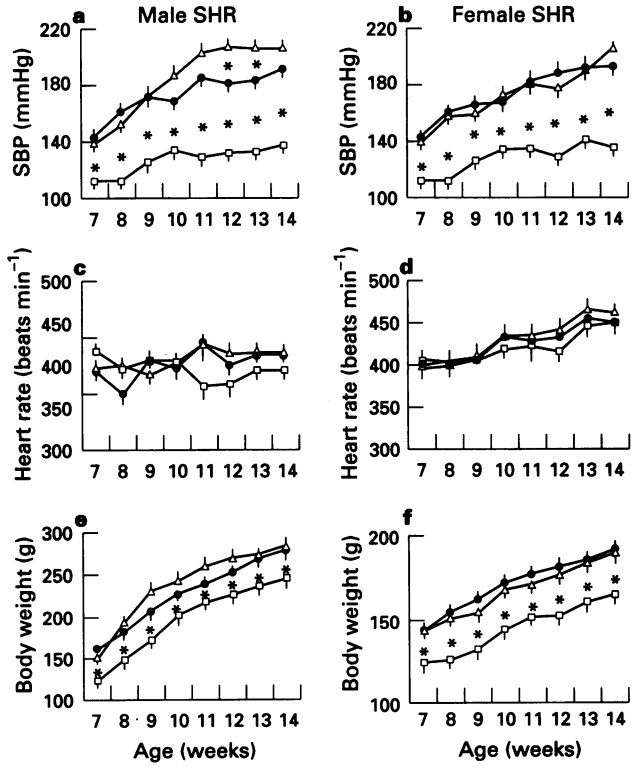

Figure 2 Line graphs show systolic blood pressure (SBP) heart rate and body weight values from 7 to 14 weeks of age in male and female spontaneously hypertensive rats (SHR). The following groups are shown: rats given early oral administration of A-81988 in tap water (□), their offspring (△) and controls (●). The last two groups drank tap water, instead of A-81988 solution. Each group consisted of 22 male and 22 female rats. Data are mean±s.e.mean. *$P<0.05$ versus control group that received vehicle.

No significant difference was observed among groups at 14 weeks of age regarding $U_{Na}V$, U_KV, urinary kallikrein, urinary creatinine and urinary osmolality (Table 1).

A-81988-treated SHR, but not their offspring, had lower heart/body weight ratios compared to controls at 14 weeks of age (3.21 ± 0.14, 3.82 ± 0.14 and 3.76 ± 0.07, respectively, $P < 0.01$). Heart/body weight ratio was also reduced in Off-A-81988 SHR compared to age-matched never-treated controls (3.44 ± 0.10 versus 4.06 ± 0.08 at 21 weeks of age, $P < 0.05$). Kidney/body weight ratios did not differ among groups (data not shown).

Discussion

A-81988 is a selective nonpeptidic AT_1-receptor antagonist, the specificity of which is suggested by the lack of affinity for adrenoceptors cholinoceptors, endothelin, vasopressin, bradykinin or PAF receptors (De *et al.*, 1992; Pollock *et al.*, 1993a; Lee *et al.*, 1994). Oral administration of A-81988, at doses ranging from 0.01 to 1 mg kg^{-1} per day, reportedly prevents hypertension caused by long-term inhibition of nitric oxide synthase (Pollock *et al.*, 1993b) or by severe reduction in renal mass (Pollock *et al.*, 1993a). Similar blood pressure lowering effects were observed by Lee *et al.* (1994) in 18-weeks old SHR, following the acute oral administration of the antagonist at 1 or 3 mg kg^{-1}. In the same model, oral administration of the antagonist for 4 weeks was able to normalize SBP and to prevent left ventricular hypertrophy. In the present study, we demonstrated that in adult SHR the antihypertensive effect of administration of A-81988 for 1 week is associated with inhibition of the vasopressor responses to i.c.v. or i.v. AII.

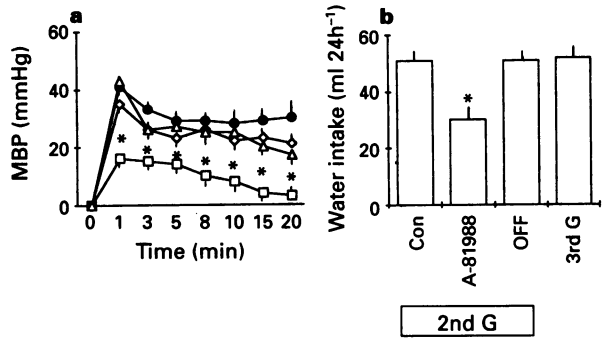

Figure 3 (a) Changes in mean blood pressure (ΔMBP) induced by i.c.v. bolus injection of 500 ng angiotensin II (AII) in spontaneously hypertensive rats (SHR) of the 2nd generation [controls (●), A-81988-treated (□), Off-A-81988 (◇), $n = 10$ in each group], and 3rd generation (△, $n = 6$). Data are mean ± s.e.mean. $^*P < 0.05$ versus control group, (b) Twenty-four hour water intake after intracerebroventricular bolus injection of 500 ng AII in spontaneously hypertensive rats (SHR) of the 2nd generation [A-81988-treated (□), controls (●), Off-A-81988 (◇), $n = 10$ in each group], and 3rd generation, offspring of Off-A-81988 rats (△, $n = 6$). Data are mean ± s.e.mean. $^*P < 0.05$ versus control group.

Previous studies showed that the magnitude of the antihypertensive action of ACE-inhibitors strongly depends on the duration of the treatment and on how early it is started. Indeed, these compounds are modestly effective in adult SHR, while the blood pressure lowering effect reportedly averages some 30 mmHg if treatment is started at weaning (Harrap *et al.*, 1990; Giudicelli *et al.*, 1993). Furthermore, administration of captopril since the foetal stage of life, completely prevents the development of hypertension and myocardial hypertrophy in SHR (Wu & Berecek, 1993). Our finding that early oral administration of an antagonist of AT_1-receptors was as effective as captopril confirms that the RAS plays an important role in the pathogenesis of arterial hypertension in this genetic model. Persistence of the antihypertensive effects after discontinuation of treatment indicates that early pharmacological inhibition of the RAS affects the adult cardiovascular phenotype, permanently. Not surprisingly, A-81988 prevents myocardial hypertrophy in SHR as does ACE-inhibition (Sen *et al.*, 1980). Indeed, AII is able to stimulate DNA turnover, and RNA and protein synthesis in cardiovascular tissues by activating various growth factors and oncogenes (Baker *et al.*, 1990). The possibility that remodelling of cardiovascular tissues contributes to the ability of A-81988 to prevent the development of hypertension, or, alternatively, is the consequence of blood pressure normalisation, remains to be elucidated.

Interestingly, the antihypertensive effect of A-81988 was associated with lower body weight values, an effect that reportedly occurs also in SHR given chronic treatment with ACE-inhibitors and that could reflect interference with sodium-fluid homeostasis (Harrap *et al.*, 1990). Indeed, reduction in total body fluid volume reportedly contributes to the antihypertensive effect that occurs during chronic blockade of the RAS (Harrap *et al.*, 1990).

Evidence for an enhanced activity of the RAS in the brain of SHR has been provided by a series of immunohistochemical and functional studies (Ganten *et al.*, 1983; Hermann *et al.*, 1984; Saavedra *et al.*, 1986). This alteration may play a role in the pathogenesis of genetic hypertension as suggested by the finding that early administration of ACE-inhibitors not only has antihypertensive effects in SHR but also inhibits their enhanced pressor and drinking responses to i.c.v. AI or AII (McDonald *et al.*, 1980; Unger *et al.*, 1981; Okuno *et al.*, 1983; Wu & Berecek, 1983). We found that the pressor response to i.c.v. AII was effectively inhibited by chronic oral administration of A-81988, and this inhibitory effect was greater than that exerted on the pressor response to i.v. AII (59 versus 27%). These findings favour the possibility that the antagonist can pass the blood-brain barrier and eventually exert its antihypertensive effect through a central mechanism. However, since A-81988 levels in the central nervous system were not determined in this study, a direct demonstration that the antihypertensive effect of the antagonist is mediated by a central, angiotensin-dependent mechanism is lacking. It is equally possible that normalization of blood pressure in the A-81988-treated rats alters the balance of factors regulating blood

Table 1 Effect of early oral administration of A-81988 on the urinary excretion of creatinine, sodium, potassuim, kallikrein and osmolality in 14 week old male SHR

	UCreat	$U_{Na}V$	U_KV	*UKall*	$U_{Osm}V$
Controls ($n = 22$)	11.1 ± 0.7	0.77 ± 0.11	1.11 ± 0.06	16.6 ± 1.6	11.24 ± 0.62
A-81988 ($n = 22$)	11.3 ± 0.6	0.71 ± 0.05	1.06 ± 0.05	13.4 ± 0.4	11.06 ± 0.48
3rd G ($n = 22$)	11.2 ± 0.5	0.60 ± 0.06	1.15 ± 0.05	16.6 ± 0.3	11.08 ± 0.42

Urine collections were obtained with rats in individual metabolic cages. No significant difference was observed among A-81988-treated rats, their offspring (3rd G), and controls as far as urinary creatinine (UCreat, μg day^{-1}), sodium ($U_{Na}V$, μmol day^{-1}), potassium (U_KV, μmol day^{-1}), and kallikrein (UKall, nkat day^{-1}) excretion and urinary osmolality ($U_{Osm}V$, mosmol day^{-1}) are concerned.

pressure, resulting in a depressed response to i.c.v. administration of AII. In preliminary experiments, we observed that acute i.c.v. administration of 10 μg A-81988 (a dose able to inhibit the pressor response to 500 ng i.c.v. AII by more than 80%) does not alter the blood pressure of SHR (Paolo Madeddu, unpublished observations 1994). Similarly, DePasquale *et al.* (1992) and Kawano *et al.* (1994) showed that neither acute nor chronic i.c.v. administration of DuP-753 lowers blood pressure of SHR, whereas the antagonist was effective by oral route. They concluded that, in this experimental model, DuP-753 decreases blood pressure by blockade of peripheral, not central, AT_1 receptors. However, a mild antihypertensive effect reportedly occurs in salt-sensitive SHR after the injection of DuP-753 into the anterior hypothalamic area of the brain (Yang *et al.*, 1992). Thus, brain AII may participate in the exacerbation of hypertension in conditions of sodium loading. On the other hand, failure of AII antagonists to affect basal blood pressure, when given i.c.v, might be attributable to their inability to reach receptors located in deep areas of the brain.

The antagonist A-81988 and captopril, though similar in many aspects (namely, the antihypertensive potency, the ability to alter the pressor and drinking responses to AII during treatment and the persistence of blood pressure effects after discontinuation), differ in the fact that responses to i.c.v. application of AI or AII were persistently decreased even after captopril withdrawal, whereas this effect was not seen in the Off-A-81988 SHR. Persistent alteration of the responses to i.c.v. AI after captopril withdrawal suggests that inhibition of central ACE, if it occurs in a critical phase, could lead to a permanent reduction in central content or activity of the enzyme and therefore to decreased local formation of AII. In addition, the reduced responses to i.c.v. AII in rats removed from captopril may be due to concomitant changes in central receptor number, affinity, and/or second messenger systems (Wu & Berecek, 1993). By contrast, antagonism of AT_1 receptors could reflexly increase renin release and plasma/tissue AII levels and stimulate angiotensin receptor expression (Lee *et al.*, 1994). These important differences should be taken into account when trying to explain why parental treatment with captopril attenuates the expression of the hypertensive genotype in the offspring never exposed to treatment (Wu & Berecek, 1993), whereas A-81988 does not. On the contrary, the rise in blood pressure was even accelerated in male SHR of the third generation (offspring of Off-A-81988 rats) possibly because they were exposed to elevated parental levels of renin and AII during foetal development. The findings that the drinking response to i.c.v. AI is blunted in the progeny of captopril-treated rats (Wu & Berecek, 1993) but not in the offspring of A-81988 SHR suggest that functional integrity of central angiotensinergic mechanism is essential for the maintenance of heritable hypertension.

One might speculate that failure of A-81988 to alter the phenotype of the following generation is due to activation of AT_2-receptors. However, this possibility appears unlikely since most of the cardiovascular effects of AII are mediated by AT_1-receptors.

Since, ACE inhibitors interfere with the metabolism of various vasoactive hormones such as bradykinin, enkephalins and substance P (Erdos, 1975), and since endogenous kinins could modulate the vasopressor action of AII (Madeddu *et al.*, 1994), mechanisms other than the RAS could be, at least in part, responsible for the effects of captopril on the blood pressure of the following generation. However, evidence for participation of endogenous kinins in the blood pressure effect of ACE-inhibitors in SHR is scant (Bao *et al.*, 1992). In addition, early pharmacological manipulation of the rat blood pressure phenotype with a receptor-antagonist of bradykinin does not alter the blood pressure levels of the following generation (Madeddu *et al.*, 1995).

Other possible, though only hypothetical, explanations for the difference between A-81988 and captopril include ability of the latter to produce psychobehavioural changes (nursing behaviour) and/or chemical modifications of parental DNA (methylation patterns), that could be a source of heritability (Lindpainter, 1994).

In conclusion, our results indicate that the RAS plays an important role in the development of hypertension in SHR. Furthermore, in contrast to results with captopril (Wu & Berecek, 1993), the protective effect of AII AT_1 receptors blockade does not attenuate the hypertensive phenotype of following generation.

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Additive effect of ADP and CGRP in modulation of the acetylcholine receptor channel in *Xenopus* embryonic myocytes

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1 We have previously shown that the activation of either protein kinase A (PKA) or protein kinase C (PKC) enhanced the responses of muscle membrane to acetylcholine (ACh) by increasing the mean open time of embryonic-type ACh channels in *Xenopus* cultured myocytes. In the present study, we further investigated the interaction between these two kinases in the modulation of ACh channels by using the receptor ligands, adenosine diphosphate (ADP) and calcitonin gene-related peptide (CGRP) which selectively activate PKC and PKA, respectively.

2 ADP concentration-dependently increased the mean open time of embryonic-type ACh channels and 0.3 mM ADP is sufficient to achieve the maximal potentiating effect. α, β-Methylene ATP and PMA (phorbol 12-myristate 13-acetate) but not adenosine, AMP, dibutyryl cyclic GMP have similar potentiating action.

3 Suramin (0.3 mM) pretreatment abolished the potentiating effect of ADP but left that of PMA unchanged.

4 CGRP increased the mean open time of embryonic-type ACh channels in a concentration-dependent manner and 1 μM CGRP produced the maximal effect.

5 The maximal effects of both ADP (0.3 mM) and CGRP (1 μM) in the prolongation of mean open time of ACh channels were additive.

6 These results suggest that the modulation of embryonic-type ACh channels by the endogenously released ligands via the activation of PKA and PKC is additive and possibly different sites of ACh channels may be involved in the potentiation effect of either PKC or PKA.

Keywords: ADP; CGRP; PKC; PKA; embryonic myocyte; ACh channel

Introduction

Protein phosphorylation, one of the principal mechanisms of regulating cellular metabolism, plays a major role in the regulation of synaptic function (Greengard, 1978). Neurotransmitter receptors are particularly appropriate targets for the modulation of synaptic transmission by protein phosphorylation, since they are crucial to the process of signal transduction across the postsynaptic membrane. The acetylcholine receptor (AChR) is the first receptor shown to be phosphorylated *in vitro*. The AChR in postsynaptic membranes isolated from the electric organs of *T. californa* and *E. electricus* was demonstrated to be phosphorylated by an endogenous protein kinase (Gordon *et al.*, 1977; Teichberg *et al.*, 1977). The major effect of phosphorylation of nicotinic AChRs appears to be the regulation of desensitization of the receptors to their agonists (Hemmings *et al.*, 1989). Intracellular recordings from rat soleus muscle treated with forskolin suggest that adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent phosphorylation of the AChR regulates its rate of desensitization (Middleton *et al.*, 1986; Albquerque *et al.*, 1986). In addition, treatment of rat primary myotube cultures with forskolin or cyclic AMP analogues increased the rate of desensitization, as analysed by intracellular recording and single-channel techniques (Middleton *et al.*, 1988; Mulle *et al.*, 1988). Treatment of chick myotubes with phorbol esters or diacylglycerol analogues also decreased the sensitivity of the AChR to acetylcholine and increased the rate of desensitization of the AChR, providing evidence that protein kinase C phosphorylation of the AChR also regulates its rate of desensitization (Eusebi *et al.*, 1985). The physiological role of desensitization or reduction of sensitivity of AChR remains unclear.

Recently, we have demonstrated that dibutyryl cyclic AMP, a membrane-permeable cyclic AMP analogue, significantly increased the opening frequency and the mean open time of the low-conductance ACh channels of cultured embryonic *Xenopus* myocytes (Fu, 1993). Calcitonin gene-related peptide (CGRP), a neuropeptide present at presynaptic motor nerve terminals, elevates cyclic AMP levels and stimulates the phosphorylation of ACh channels in myocytes (Laufer & Changeux, 1987; Miles *et al.*, 1989; Uchida *et al.*, 1990). We also showed that CGRP potentiates spontaneous synaptic currents and increases the mean open time of ACh channels in *Xenopus* embryonic myocytes (Lu *et al.*, 1993). The involvement of cyclic AMP-dependent protein kinase (PKA) in the action of CGRP was implicated, since intracellular loading of a PKA inhibitor into the myocyte prevented the CGRP effects. On the other hand, activation of protein kinase C (PKC) by phorbol esters also lengthens the open time of embryonic-type ACh channels in *Xenopus* cell cultures (Fu & Lin, 1993a). Furthermore, adenosine 5'-triphosphate (ATP), co-stored and released with ACh from presynaptic motor nerve terminals, has been reported to potentiate spontaneous transmitter release (Fu & Poo, 1991) and increase the mean open time of embryonic-type ACh channels through the binding of P_2-purinoceptor and the activation of PKC (Fu, 1994). ADP, a metabolite of ATP, is as effective as ATP in enhancing the ACh sensitivity of the myocytes (Fu, 1994). The aim of the present study was to explore further the interaction of both protein kinases in the modulation of ACh channels of embryonic cultured myocytes. We found that the activation of either PKA or PKC through the receptor ligands additively increase the mean open time of embryonic-type ACh channels. The present findings suggest that the concomitantly released neurotrophic factors may be synergistic in the physiological modulation of synaptic function during the early phase of synaptic development.

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Methods

Culture preparations

Cell culture of *Xenopus* myocytes were prepared as previously described (Bridgman *et al.*, 1984; Sanes & Poo, 1989). Briefly, the neural tube and the associated myotomal tissue of 1-day-old *Xenopus* embryos (stage 20–22; Nieuwkoop & Faber, 1967) were dissociated in Ca^{2+}- and Mg^{2+}-free Ringer supplemented with 0.15 mM EDTA. The cells were plated on clean glass coverslips and were used for experiments after incubation for 1-day at room temperature (20–22°C). The culture medium consisted of 50% (v/v) Ringer solution (composition, mM: NaCl 115, $CaCl_2$ 2, KCl 1.5, HEPES 10, pH 7.6), 49% L-15 Leibovitz medium (Sigma) and 1% foetal bovine serum (Gibco).

Electrophysiology

The cell-attached patch-clamp recording method was similar to that described previously by Hamill *et al.* (1981). Single ACh channel currents were measured with a patch-clamp amplifier (Axopatch-200A) in 1-day-old *Xenopus* myocyte culture at an applied pipette potential of +60 mV by using fire-polished and sylgard-coated glass electrodes (1–5 MΩ). The pipette was filled with Ringer solution containing low concentrations of ACh (1–5 nM). The current signals were filtered at 1 kHz and digitized by a digitizing unit (Neuro-Corder DR390) and stored on a videotape recorder for later playback. The data were digitized at 100 μs intervals and analyzed with PClamp programme (Axon Instruments). The amplitude and duration of individual events were measured and stored in the computer. Events corresponding to the opening of more than one channel were excluded from the open time analysis. Events with open time less than 1 ms were not analysed because of possible attenuation and distortion. Apparent open time histograms were fitted with a single exponential and the amplitude histograms were fitted with Gaussian distribution curves, using the least-squares method in both cases. All recordings were made at room temperature in culture medium. The drugs were bath-applied and the properties of single ACh channel were compared in different patches of the same myocyte before and after drug application. The results are expressed as the mean ± s.e.mean. Statistical significance was evaluated by Student's *t* test.

The following chemicals were used: acetylcholine (ACh), adenosine 5'-diphosphate (ADP), calcitonin gene-related peptide (CGRP), adenosine 5'-monophosphate (AMP), adenosine, phorbol 12-myristate 13-acetate (PMA), α, β-methylene ATP and dibutyryl cyclic GMP (db cyclic GMP) (Sigma) and suramin (FBA, Germany).

Results

Effect of ADP on the properties of single acetylcholine channel

Single channel recordings were made on isolated myocyte with micropipettes containing 1–5 nM ACh to examine the ACh channel properties before and after extracellular exposure of the myocyte to ADP. Figure 1a shows the typical patterns of single channel events observed before and after ADP (0.3 mM) exposure from different patches of the same myocyte when the myocyte was hyperpolarized +60 mV from rest. Two populations of ACh channels have been observed: a low-conductance channel with long open duration, which is the predominant population in 1-day-old cultures, and a high-conductance channel with short open duration. The current amplitudes were 4.58 ± 0.11 pA and 6.87 ± 0.09 pA ($n = 10$), respectively. We found that the amplitude of single channel currents for both types of ACh channels remained unchanged after ADP treatment (4.72 ± 0.19 pA and 6.97 ± 0.27 pA, $n = 10$ for low- and high-conductance ACh channels, respectively). However, the apparent mean channel open time of the low-conductance but not that of high-conductance ACh channels was increased after ADP treatment (Figure 1b and c). The ADP-induced increase in the mean open time of low-conductance ACh channels showed a steep dependence on the concentration of ADP between 0.1 and 0.3 mM (Figure 2) and 0.3 mM ADP was achieving the maximal effect. The mean open time of low-conductance ACh channels increased by 70% within 3 min after exposure to 0.3 mM ADP.

Potency of related nucleotides

Previous study has indicated that ATP enhanced the whole-cell response of the muscle membrane to ACh by acting through P_2-purinoceptors (Fu, 1994). We examined further the effects of various nucleotides on the open time of single ACh channel. α, β-Methylene ATP (0.3 mM), a slowly degraded derivative of ATP, increased the mean open time of low-conductance ACh channels (the mean open time before and after drug application was 4.23 ± 0.23 ms ($n = 30$) and 6.14 ± 0.51 ms ($n = 3$), respectively) (Table 1). ADP was as effective as α, β-methylene ATP in lengthening the open time of low-conductance ACh

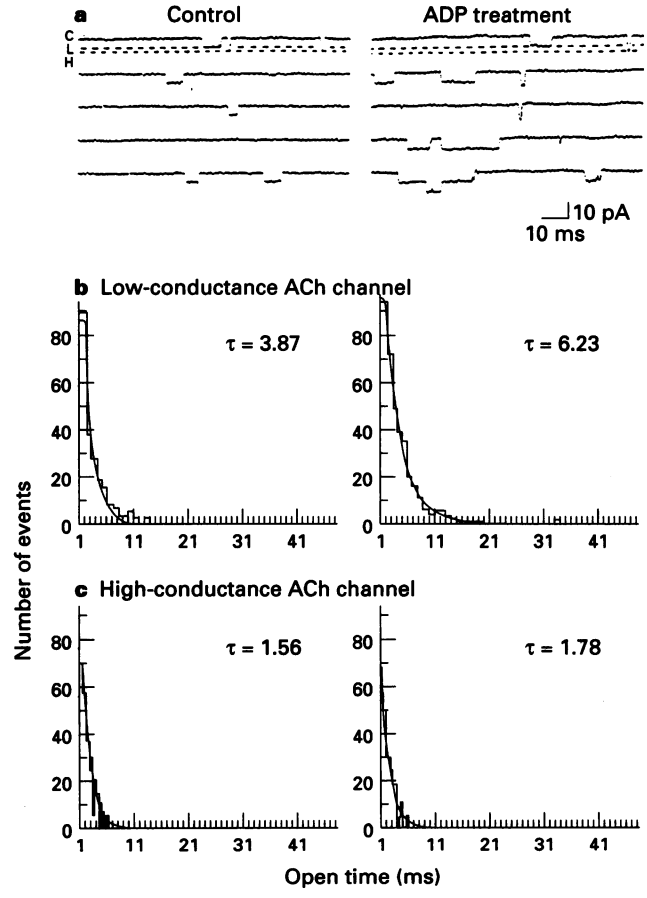

Figure 1 Effect of ADP on the properties of single acetylcholine (ACh) channel in *Xenopus* cultured myocyte. Recordings were made at +60 mV applied potential from cell-attached patches. The pipette was filled with Ringer containing 1 nM ACh. (a) Samples of recording of single ACh channel currents before (left panel) and after (right panel) bath application of 1 mM ADP were obtained from different patches of the same myocyte. - - -, The level of closed (C), low-conductance (L) and high-conductance (H) ACh channels; (b) and (c) showed the histogram of apparent open time of low- and high-conductance ACh channels before (left panels) and after (right panels) application of 1 mM ADP. Solid lines represent the best-fits with single exponential curves. The calculated mean open time is shown as τ.

channels. In contrast, adenosine monophosphate (AMP, 1 mM), adenosine (1 mM) or dibutyryl cyclic GMP (db cyclic GMP, 1 mM) were ineffective (the mean open time was 4.56 ± 0.44 ms, 4.40 ± 0.56 ms and 3.58 ± 0.58 ms, $n = 3$ each, respectively). PMA (phorbol 12-myristate 13-acetate, 1 μM), a protein kinase C activator was also tested for comparison, the mean channel open time after PMA application was 7.47 ± 0.66 ms ($n = 3$) (Table 1).

P_2-purinoceptor involved in the action of ADP

As shown above the potency of various nucleotides in increasing the mean open time of low-conductance ACh channels is consistent with the known order of potency for P_2-purinoceptor agonists (Dubyak & El-Moatassim, 1993). We examined further the effect of suramin, a P_2-purinoceptor antagonist, on the action of ADP. As shown in Figure 3, pretreatment with suramin (0.3 mM) inhibited the potentiating effect of ADP (1 mM). P_2-purinoceptor activation is thought to trigger a cascade of reactions involving transducer protein and phospholipase C, resulting in the activation of protein kinase C (PKC). For comparison, direct activation of PKC by the PKC activator, PMA (1 μM), showed a marked increase of the open time of low-conductance channels but suramin failed to abolish such a potentiation. These results suggest that the effect of ADP in lengthening the open time of low-conductance ACh channels is mediated through the activation of P_2-purinoceptors.

Interaction between ADP and CGRP in the potentiation of acetylcholine channel response

Our previous work indicated that calcitonin gene-related peptide (CGRP) enhanced the postsynaptic response at the developing neuromuscular junction by increasing the open time of embryonic-ACh channels through the activation of protein kinase A (PKA) (Lu *et al.*, 1993). As shown above, ADP prolonged the open time of embryonic-ACh channels through the activation of PKC. We examined further the interaction of these two protein kinases in the modulation of

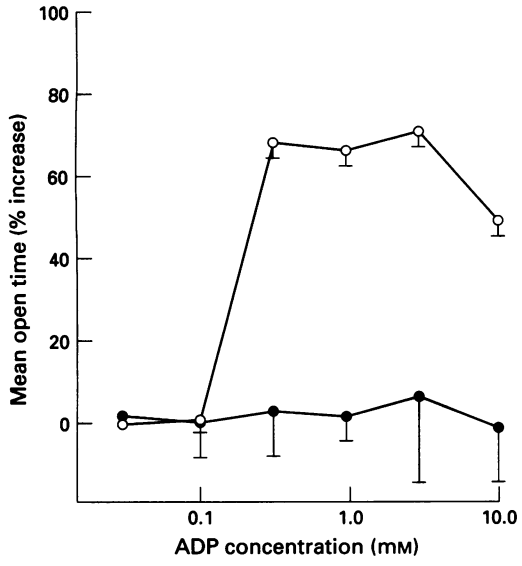

Figure 2 Concentration-dependent increase of the mean open time of single acetylcholine (ACh) channel by ADP in *Xenopus* cultured myocyte. Note that ADP concentration-dependently increased the mean open time of low-conductance (○) but not that of high-conductance (●) ACh channels. Points represent the percentage increase of channel mean open time in the presence and absence of ADP. Each point was determined for at least 4 separate recordings obtained from separated cultures. Data are presented as mean ± s.e.mean.

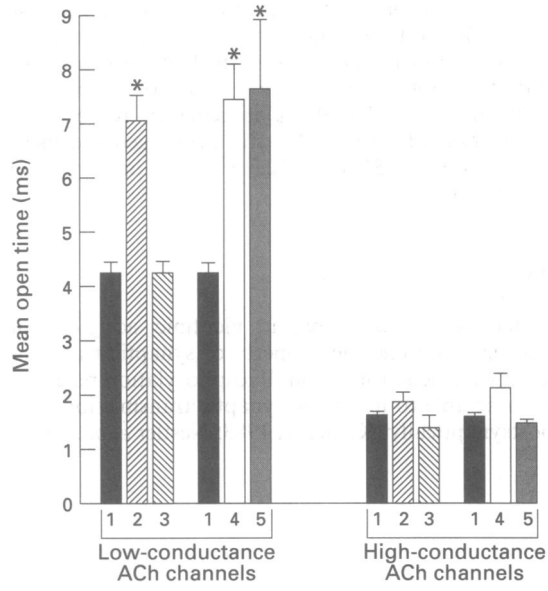

Figure 3 Involvement of P_2-purinoceptor in the potentiating effect of ADP on ACh channels. ADP and PMA (phorbol 12-myristate 13-acetate) were bath-applied at final concentrations of 1 mM and 1 μM, respectively. Suramin (0.3 mM) was given 10 min before the addition of ADP or PMA. Note that suramin pretreatment abolished the potentiating action of ADP but left that of PMA unchanged. *$P < 0.05$ as compared with control values. (1) Control; (2) ADP; (3) suramin pretreatment + ADP; (4) PMA; (5) suramin pretreatment + PMA.

Table 1 Comparison of the various nucleotides on the mean open time of ACh-activated single-channel in *Xenopus* cultured myocytes

Nucleotide	*Concentration*	n	*Mean open time (ms)* *Low-conductance*	*High-conductance*
Control	–	30	4.23 ± 0.23	1.86 ± 0.08
Adenosine	1.0 mM	3	4.40 ± 0.56	1.77 ± 1.08
AMP	1.0 mM	3	4.56 ± 0.44	2.03 ± 0.08
ADP	0.3 mM	12	7.11 ± 0.39*	1.99 ± 0.15
α,β-Methylene ATP	0.3 mM	3	6.14 ± 0.51*	1.80 ± 0.52
db cyclic GMP	1.0 mM	3	3.58 ± 0.58	1.76 ± 0.26
PMA	1.0 μM	3	7.47 ± 0.66*	2.47 ± 0.31

Data are presented as mean ± s.e.mean (n numbers of myocyte). ACh-activated single-channel was obtained from cell-attached patch recording on the surface of isolated day-1 *Xenopus* cultured myocytes and patch was hyperpolarized to +60 mV from rest. Pipettes were filled with Ringer containing 1–5 nM of ACh. *$P < 0.05$ as compared with control (Student's t test).
Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate; db cyclic GMP, dibutyryl cyclic GMP; PMA, phorbol 12-myristate 13-acetate.

ACh receptor channel. Figure 4 shows that CGRP concentration-dependently increased the mean open time of low-conductance ACh channels but did not affect that of high-conductance ACh channels. CGRP (1 μM) produced the maximal potentiating effect and the mean open time of low-conductance ACh channels increased by 65.5 ± 14.7% (n = 12). The concentration used in achieving maximal effect on ACh channels was 1 μM and 1 mM for CGRP and ADP, respectively. We thus compared the open time of ACh channels from different patches of the same myocyte before and after sequential bath application of ADP and CGRP. When the maximal effect of 1 mM ADP on low-conductance ACh channels was obtained (mean open time increased by 65 ± 26% of control, mean open time was 4.23 ± 0.23 ms (n = 30) and 6.97 ± 1.12 ms (n = 4) for control and ADP-treatment, respectively), further application of 1 μM CGRP still exerted a similar potentiation (mean open time increased by 148 ± 35% of control, mean open time was 10.49 ± 1.49 ms (n = 4) after CGRP plus ADP treatment), indicating that the effect of both ligands was additive (Figure 5a). When the order of drug treatment was reversed, i.e. CGRP was applied followed by ADP, the same additive effect of both agents on low-conductance ACh channels was observed (Figure 5b). In addition, sequential application of CGRP and PMA also exerted a similar additive effect on low-conductance ACh channels. The mean open time of low-conductance ACh channels before and after CGRP application were 4.48 ± 0.17 ms and 6.77 ± 0.49 ms (n = 3, 51.1 ± 10.9% increment), respectively. Further application of 1 μM PMA still prolonged the mean open time to 11.32 ± 0.54 ms (152.7 ± 36.5% increment as compared with control).

Discussion

The characteristics of postsynaptic nicotinic acetylcholine (ACh) channels determine the property of synaptic transmission at the neuromuscular junction. Phosphorylation reactions are known to be able to modulate synaptic transmission both pre- and postsynaptically (Kennedy, 1983; Nestler *et al.*, 1984).

The nicotinic ACh receptor (AChR) is a pentameric complex of four types of subunits, α, β, γ and δ, in the stoichiometry $\alpha_2\beta\gamma\delta$ (Changeux *et al.*, 1984; Galzi *et al.*, 1991). Phosphorylation of the muscle ACh receptor has been implicated in playing a role in the regulation of the receptor ion channel. Postsynaptic membranes isolated from *T. californica* contain at least three different protein kinases that phosphorylate the acetylcholine receptor (AChR) (Huganir & Miles, 1989). Cyclic AMP-dependent protein kinase (PKA) phosphorylates the γ and δ subunits of the AChR (Huganir & Greengard, 1983), protein kinase C (PKC) phosphorylates the α and δ subunits (Safran *et al.*, 1987), and tyrosine kinase phosphorylates the β, γ and δ subunits (Huganir *et al.*, 1984; Hopfield *et al.*, 1988; Qu *et al.*, 1990). Each of the protein kinases phosphorylate a single site on the relevant subunit(s) of the AChR and the locations of these sites have been proposed (Huganir *et al.*, 1984) or determined (Yee & Huganir, 1987; Safran *et al.*, 1987). Although data from many laboratories have suggested that the major functional effect of phosphorylation of the AChR is the regulation of its rate of desensitization, many

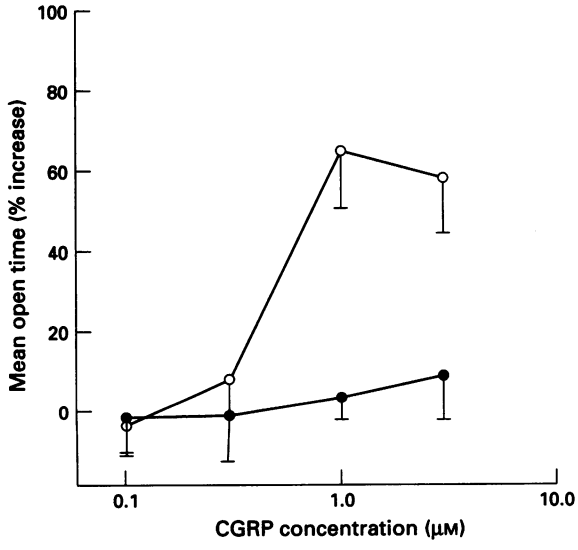

Figure 4 Concentration-dependent increase of the mean open time of ACh-activated single-channel by calcitonin gene-related peptide (CGRP) in *Xenopus* cultured myocyte. Points represent the percentage increase between bath application of CGRP and control in different patches of the same myocyte. Note that CGRP selectively prolonged the mean open time of low-conductance (○) channels but not that of high-conductance (●) ACh channels. Each point represents mean ± s.e.mean of three to six separate recordings from different cultures.

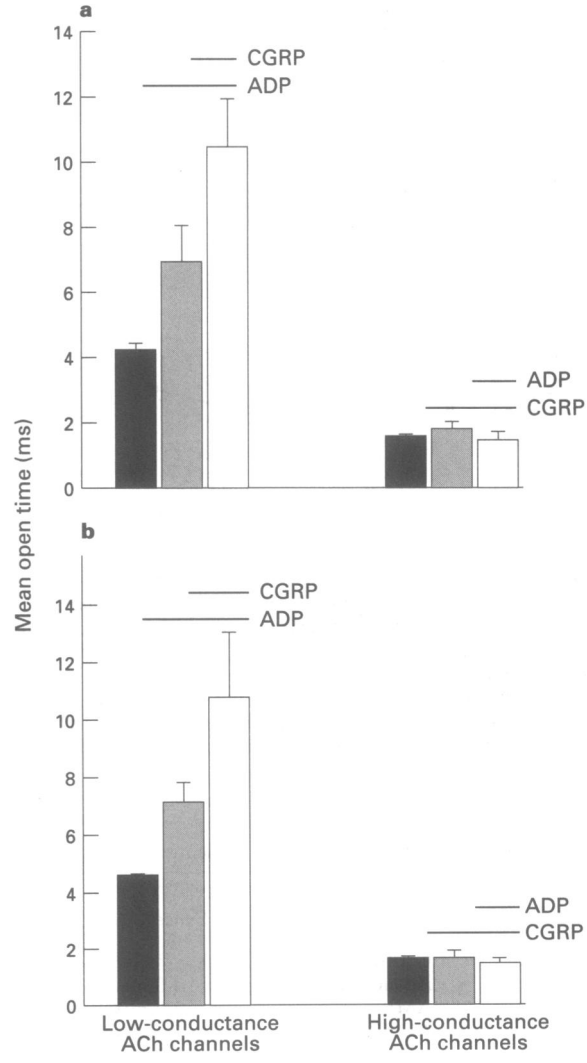

Figure 5 Additive effect of calcitonin gene-related peptide (CGRP) and ADP in lengthening the mean open time of single ACh channel. Different patches of the same myocyte were compared between the control (solid columns) and drug treatment. Note that when the maximal effect of ADP (1 mM) on the low-conductance ACh channels was obtained, further addition of CGRP (1 μM) still exerted an increase on channel open time (a) and *vice versa* (b). No effect on high conductance ACh channels was observed after the addition of both drugs.

recent studies have also shown that phosphorylation of the AChR may be involved in the regulation of subunit assembly in cultured chick muscle cells (Ross *et al.*, 1988), regulation of AChR redistribution (Wallace *et al.*, 1991) or increase of the mean open time of embryonic-type ACh channels in *Xenopus* cultured myocytes (Fu & Lin, 1993a; Lu *et al.*, 1993; Fu, 1994), indicating that phosphorylation may have effects on the physiological function of the AChR other than its effect on desensitization (Middleton *et al.*, 1986).

In previous studies, we have shown that either activation of PKA or PKC increases the mean channel open time of low-conductance ACh channels in embryonic *Xenopus* myocytes (Fu, 1993; Fu & Lin, 1993a). Since high concentrations of ACh will also cause the desensitization of ACh channels in the *Xenopus* cultured myocyte (Fu, 1993), we used very low concentrations of ACh (1–5 nM) in the patch pipette. Here we demonstrated that db cyclic GMP did not affect the properties of the single ACh channel, indicating that protein kinase G may not be involved in the modulation of ACh channels. CGRP and ATP, two principal neuromodulators present in the motor nerve terminals, were shown specifically to increase the open time of embryonic-type, low-conductance ACh channels (Lu *et al.*, 1993; Fu, 1994). Pharmacological experiments suggest that the CGRP actions were mediated by PKA, while ATP exerted its effect by binding to P_2-purinoceptors and activating PKC. ADP is as effective as ATP in the potentiation of ACh response (Fu, 1994). In the present study, we used ADP instead of ATP as the ligand for P_2-purinoceptor since ATP may have a depolarizing action on myocyte (Hume & Honig, 1986). ADP concentration-dependently increased the mean open time of low-conductance ACh channels in *Xenopus* cultured embryonic myocytes. The involvement of P_2-purinoceptor and PKC cascade in ADP effect is further supported by the inhibitory effect of suramin, a P_2-purinoceptor antagonist (Hoyle *et al.*, 1990). The effect of ADP and CGRP on the open time of ACh channels showed a steep dependence on concentration, suggesting strong cooperativity in one or more intracellular process triggered by these receptor ligands. The maximal effects of ADP and CGRP on the prolongation of the open time of low-conductance ACh channels are additive, implying that they may act through phosphorylating different sites of the ACh channels. This conclusion is further strengthened by the finding that additivity of ADP and CGRP on channel open time was also observed in the same membrane patch which contains the same population of single ACh channels (data not shown). Which subunits of ACh channel are phosphorylated by ADP and CGRP needs further investigation. The development, maintenance and modulation of synapses depends upon molecular interactions between the pre- and postsynaptic cells (Purves, 1986). The first messengers that are involved in the regulation of AChR have been extensively investigated (Smith *et al.*, 1987; Ross *et al.*, 1988; Miles *et al.*, 1989). The neuropeptide calcitonin gene-related peptide (CGRP) was an attractive candidate for a first messenger that regulates AChR phosphorylation (Uchida *et al.*, 1990), since it is a co-transmitter with ACh at the neuromuscular junction and is known to increase cyclic AMP concentrations in muscle (Laufer & Changeux, 1987) and potentiate synaptic response at the developing neuromuscular junction (Lu *et al.*, 1993). CGRP immunoreactivities were first detected at the embryonic stage 32 (40 h) in *larval Xenopus* (Peng *et al.*, 1989), which correlated with the culture stage we used (1-day-old culture plated with 1-day-old embryos). Although CGRP is not involved in the clustering of AChR (Peng *et al.*, 1989), CGRP may help synaptogenesis in other ways. ATP, the other trophic factor co-stored and co-released with ACh in the presynaptic motor nerve terminals (Dowdall *et al.*, 1974; Zimmerman, 1978), has been shown to have marked potentiating effects in spontaneous transmitter release (Fu & Poo, 1991) and postsynaptic ACh channel responses (Fu, 1994). The results of the present study suggest that the modulation of ACh channels in embryonic myocytes by activation of PKA and PKC is additive, indicating that the potentiating effects of concomitant-released neurotrophic factors on synaptogenesis may be synergistic.

Developmental changes of ACh receptor channels have been studied extensively in *Xenopus* muscle cells *in vitro* (Brehm *et al.*, 1984; Leonard *et al.*, 1984; Rohrbough & Kidokoro, 1990) as well as *in vivo* (Owens & Kullberg, 1989). Shortly after the initial insertion of ACh receptor channels, the majority of channels are an embryonic-type (low-conductance channels) which have a prolonged mean open time and lower conductance. The adult-type channels (high-conductance channels) are rare at the early stages and they have ~50% greater unitary conductance than embryonic-type channels and shorter mean open time. During development the relative population of the adult-type ACh channels increases. The modulatory effect of ATP and CGRP on low-conductance ACh channels also shows developmental change, the prolongation of channel open time by these two ligands declined or disappeared in older cultures (Fu & Lin, 1993b; Lu *et al.*, 1993). The effect of ATP and CGRP are thus restricted to the early phase of synaptogenesis. What is the functional significance of potentiating ACh responses by CGRP and ATP during the early phase of synaptogenesis? The immediate consequence of the potentiation is the enhancement of synaptic activity. At developing neuromuscular junctions, spontaneous synaptic activity appears immediately after nerve-muscle contacts (Xie & Poo, 1986). This activity plays a pivotal role in the maturation of synaptic connection, since maturation of synaptic specialization could be influenced by the presence of the electrical activity and the increase of intracellular Ca^{2+} concentration through the influx from ACh channels (Kidokoro & Saito, 1988; Lo & Poo, 1991). Lengthening the open time of embryonic-type ACh channels by the concomitantly released neurotrophic factors provides a means of elevating postsynaptic activity early in development. Our work thus provides evidence that two concomitantly released endogenous ligands like ATP and CGRP are capable of potentiating postsynaptic ACh channels and synaptic maturation synergistically at embryonic developing neuromuscular synapses via the activation of PKC and PKA, respectively.

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Contractile actions of thrombin receptor-derived polypeptides in human umbilical and placental vasculature: evidence for distinct receptor systems

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1 We studied the structure-activity profiles of four thrombin receptor-derived polypeptides (TRPs) (P5, SFLLR; P5-NH_2, SFLLR-NH_2; P7, SFLLRNP; P7-NH_2, SFLLRN) in contractile human placental artery (PA), umbilical artery (UA) and umbilical vein (UV) preparations and in a human platelet aggregation assay.

2 The contractile actions of the TRPs in the two arterial preparations were endothelium-independent, whereas in the UV tissue a contractile response was observed only in an endothelium-denuded preparation; no endothelium-mediated relaxation responses were observed in any of the vascular preparations.

3 In the three vascular preparations, the contractile responses required extracellular calcium and were attenuated by the tyrosine kinase inhibitor, genistein.

4 The relative contractile orders of potencies of the TRPs in the three vascular preparations were distinct from each other (PA: P7-NH_2 > P7 > P5-NH_2 > P5; UA: P7-NH_2 $\geqslant$ P5-NH_2 $\simeq$ P7 > > P5; UV: P5-NH_2 > > P7-NH_2 = P7 > > P5) and these were in turn distinct from the potency order observed in the platelet aggregation assay (P5-NH_2 $\geqslant$ P7-NH_2 > P7 > > P5).

5 Despite the markedly dissimilar TRP potency orders in the placental artery and umbilical vein preparations, the cDNA sequences for the thrombin receptor obtained by polymerase chain reaction cloning of cDNA from the two tissue sources were identical.

6 We conclude that the four tissues studied possess functionally distinct thrombin receptor systems that interact in a distinct way with agonist peptides. In view of the identity of the thrombin receptor cDNA in the two tissues displaying the most dissimilar structure-activity profiles, we suggest that in different tissues, differences in post-translational receptor processing or differences in receptor-effector coupling interactions may result in unique thrombin receptor systems that can display distinct structure-activity profiles.

Keywords: Thrombin receptor; umbilical artery; placental artery; umbilical vein; platelets

Introduction

It is now widely accepted that the ability of the serine protease, thrombin, to stimulate target cells involves the proteolytic activation of a specific G-protein-coupled thrombin receptor (Rasmussen *et al.*, 1991; Vu *et al.*, 1991; Coughlin *et al.*, 1992). The receptor's proteolytically-exposed amino-terminal sequence, beginning with serine-42 in the human receptor, is thought to act as an activating 'anchored' or 'tethered' ligand. Synthetic peptides based on the revealed N-terminal sequence of the thrombin receptor, containing up to 14 amino acids (amino acids designated by their single letter code) (i.e. S_{42}FLLRNPNDKYEPF or P14) are, on their own, capable of triggering the thrombin receptor, so as to mimic many of the diverse cellular actions of thrombin, ranging from the aggregation of platelets to the regulation of vascular contractility (Davey & Luscher, 1967; White *et al.*, 1980; 1984; DeMey *et al.*, 1982; Haver & Namm, 1984; Rapoport *et al.*, 1984; Walz *et al.*, 1985; 1986; DeBlois *et al.*, 1992; Muramatsu *et al.*, 1992). In structure-activity studies of the thrombin receptor-derived peptides (TRPs) done by us using vascular and gastric smooth muscle bioassay systems (Yang *et al.*, 1992; Hollenberg *et al.*, 1992; 1993) and by others using platelet aggregation assays (Chao *et al.*, 1992; Hui *et al.*, 1992; Sabo *et al.*, 1992; Vassallo *et al.*, 1992) it has become apparent that TRPs ranging in length from five (i.e. SFLLR or P5) to seven (i.e. SFLLRNP or P7) amino acids can exhibit distinct potencies in the different assay systems, equal to or even greater than the potency of the originally described TRP 14-mer, P14. In particular, we have observed that the orders of potencies of selected TRP agonists containing either a free or amidated carboxyl-terminal residue (e.g. P5, P5-NH_2, P7, P7-NH_2) can be used to distinguish between different thrombin receptor assay systems (Hollenberg *et al.*, 1993), so as to suggest the presence of pharmacologically distinct receptor subtypes in different tissues. The pharmacological approach using the TRPs has proved of considerable value for the delineation of functional thrombin receptors in intact vascular tissue (e.g. in rat aorta), wherein molecular probe assays (e.g. Northern blot analysis) have not proved sufficiently sensitive *in vivo* to detect receptor message in the vascular smooth muscle elements (Zhong *et al.*, 1992). Our work (Muramatsu *et al.*, 1992) and that of others (DeBlois *et al.*, 1992; Antonaccio *et al.*, 1993) indicates that TRPs, presumably acting via the thrombin receptor, can regulate contractility in vascular preparations from a number of species via two mechanisms: (1) the TRPs can cause an endothelial cell-dependent nitric oxide-mediated vasorelaxation (Muramatsu *et al.*, 1992), or (2) the TRPs can cause vascular contraction via a direct action on the smooth muscle elements by a mechanism that requires extracellular calcium (Muramatsu *et al.*, 1992; DeBlois *et al.*, 1992; Antonaccio *et al.*, 1993). In a study of the expression of the thrombin receptor in human arteries (Nelken *et al.*, 1992), *in situ* hybridization and immunohistochemical

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methods have localized the thrombin receptor in normal-appearing adult arteries almost exclusively in the endothelial layer.

Since the response profile for TRPs acting on human arterial samples has yet to be evaluated in any depth, the study described in this paper was designed with the following objectives in mind: First, we wished to use a readily accessible human vascular tissue source (placental and umbilical vessels) to evaluate the vasoactive properties of thrombin and the TRPs in human-derived tissues. Second, we wished to use a spectrum of TRP agonist probes to evaluate the structure-activity relationships for these peptides in the umbilical artery, umbilical vein and placental artery preparations; and we sought to compare the relative activities of the peptides in the three vascular preparations with their relative potencies in a platelet aggregation assay. We selected the four TRPs, SFLLR (P5), SFLLRNP (P7) and their carboxyamidated counterparts, SFLLR-NH_2 (P5-NH_2) and SFLLRNP-NH_2 (P7-NH_2) as receptor probes, since these four peptides have proved of value in previous work (Hollenberg *et al.*, 1993) to discriminate pharmacologically between distinct thrombin receptor systems in different tissues from the same species. Our study describes the evaluation of the activities of these polypeptides in the three placental vascular preparations, and in the platelet aggregation assay. Further, we describe the sequencing of cDNA for the thrombin receptor obtained from placental artery and umbilical vein RNA. The data point to the existence of distinct pharmacological receptor systems in the different placental vascular preparations that, nonetheless, contain the same receptor mRNA.

Methods

Preparation of tissue and contractile bioassay procedures

Full term placental tissue, with the attached umbilical cord, was obtained from normal vaginal deliveries, through the courtesy of the Maternity Care Centre of the Foothills Hospital, Calgary, Canada. Tissue was dissected immediately from the placenta and was transported to the laboratory in ice-cold Krebs-Henseleit buffer pH 7.4, of the following composition (mM): NaCl 115, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10. Placental artery segments (0.3 × 1 cm) were excised just at the placental surface where their structures arborize and penetrate the placental mass; umbilical cord sections (3 × 5 cm) were cut to facilitate the isolation of intact umbilical artery and umbilical vein samples for the preparation of helical strips. Vessels were trimmed free of Whartons jelly or other adhering material under microscopic visualization and helical strips (0.3 × 1 cm) were cut, routinely with the aid of a fine guide wire threaded through the vessel. In some experiments, the use of a guide wire was omitted to ensure the retention of an intact endothelium. In other experiments, tissue strips were rubbed firmly with damp filter paper to remove the endothelium, as documented previously by electron microscopic observation with umbilical artery preparations (Xie & Triggle, 1994). Tissues were mounted in a plastic organ bath (3 to 4 ml) under 2 g tension for equilibration at 37°C in the above described buffer, gassed with 95% O_2/5% CO_2 to maintain the pH at 7.4. Placental artery strips and umbilical artery strips were allowed to equilibrate 2–5 h until the baseline tension had stabilized; umbilical vein strips required 3–4 h for the stabilization of baseline tension. Tension was monitored isometrically with either Statham or Grass force-displacement transducers. The contractile integrity of each preparation was routinely assessed by challenging the tissue with a test concentration of 50 mM KCl, which caused a contractile response of 1.3 ± 0.1 g in the placental artery strip (mean ± s.e.mean for $n = 126$), of 1.0 ± 0.1 g in the umbilical artery strip (mean ± s.e.mean for $n = 120$) and a response of 1.7 ± 0.1 g in the umbilical vein preparation (mean ± s.e.mean for $n = 93$). The response of each tissue to 50 mM KCl was routinely used as a reference standard for normalizing the responses of different preparations to the TRPs, for which responsiveness was expressed as a percentage (% KCl) of the 50 mM KCl-mediated contraction. To assess the role of extracellular calcium, tissues were switched to a calcium-free Krebs-Henseleit buffer containing 0.1 mM EGTA, 10 min before the addition of agonist; after the addition of agonist, the buffer was replenished with calcium (2.5 mM) and the tissue was washed and re-equilibrated in calcium-containing buffer. In some experiments, a contractile response to 0.1 μM noradrenaline was also used to monitor tissue integrity. Reagents from stock solutions were added directly to the organ bath and concentrations were calculated accordingly. In order to avoid desensitization of the tissues to the TRPs, the following dosing intervals were used for the three vascular preparations: placental artery, 40–60 min; umbilical artery, 1–2 h; umbilical vein, 1–2 h. Because of desensitization of the umbilical vein preparation to the TRPs, concentration-effect curves were obtained by exposing each tissue only once to each TRP and expressing the response (% KCl) relative to the contraction caused by 50 mM KCl. This concentration of KCl was just at the top of its concentration-effect curve. Tissues were washed free from agonist at the plateau of the contractile response (about 3 to 5 min after adding agonist to the organ bath) and were washed 2–3 times further during the re-equilibration period between additional exposures to contractile agonists. Concentration-response curves for each TRP agonist in the three different vascular preparations were obtained with multiple tissue strips from over 120 tissue donors. In each experiment, using replicate tissue strips from an individual donor (4 to 7 strips per assay), the contractile responses to increasing concentrations of TRPs were normalized, as indicated above, as a percentage (% KCl) of the response elicited by 50 mM KCl. Data from 4–9 individual experiments for each concentration of TRP were pooled to construct the concentration-response curves.

Platelet aggregation assays

Platelet-rich plasma suspensions (300×10^6 ml^{-1}), anticoagulated with sodium citrate (1 ml of 0.105 M citrate per 9 ml of blood), were obtained by differential centrifugation of venipuncture samples obtained from healthy donors who denied taking either medications or alcohol for two weeks prior to the assays. Replicate platelet suspensions (0.2 ml) were constantly stirred (1,200 r.p.m.) at 37°C in a 4-channel aggregometer and reagents in a volume of 25 μl, were added to initiate the aggregation reaction. Turbidity was monitored constantly and both the rate and extent of aggregation were automatically recorded during the aggregation reaction. Measurements of aggregation were done minimally in triplicate for each TRP concentration; all experiments were done in the presence of 100 μM amastatin to minimize degradation of the TRPs. The response to a fixed concentration of P5-NH_2 (5 μM) was used as an internal standard to compare assays done with different platelet preparations. Concentration-effect curves for the four TRPs were constructed using the initial rate of aggregation as an index of platelet response. A semi-quantitative comparison of the platelet response to increasing concentrations of the four TRPs was also done using the initial microaggregation response (primary aggregation wave) as a monitor of platelet reactivity. This initial microaggregation response, detected by a transient decrease in turbidity was observed prior to the full platelet aggregation response, even in the absence of a full platelet aggregation reaction.

Peptides and other reagents

Thrombin receptor-derived peptides, based on the human receptor sequences, SFLLR (P5), SFLLR-NH_2 (P5-NH_2), SFLLRNP (P7) and SFLLRNP-NH_2 (P7-NH_2), were obtained from the Core Peptide Synthesis Laboratory at the Queens University, Department of Biochemistry, Kingston, ON, Ca-

nada, and through the courtesy of Dr. J. DiMaio, BioChem Therapeutic Inc., Laval, PQ, Canada. Stock peptide solutions, prepared in 50 mM sodium phosphate buffer, pH 7.4, were verified for peptide compositions and concentrations by quantitative amino acid analysis and by h.p.l.c. analysis. Lyophilized thrombin from human plasma, free from other clotting factors, possessing a specific activity of 3000 NIH units mg^{-1} (1 u ml^{-1} = 10 nM) was from Sigma, St. Louis, MO, U.S.A. (lot 21H9310, Cat. No. T-6759) as were the reagents, nifedipine, noradrenaline, indomethacin and amastatin. Genistein was from ICN biochemicals, Costa Mesa CA, U.S.A.

H.p.l.c. analysis of peptides

Liquid chromatographic analysis of aliquots ($\simeq$ 100 μl) of both stock peptide solutions and peptide samples (3 to 4 μg peptide) recovered from the organ bath during the course of a contractile bioassay was performed using a Vydac RPC 18 analytical column (0.5 x 30 cm) eluted with a gradient of acetonitrile (0 to 54% v/v) in 0.1% trifluoroacetic acid at a flow rate of 1 ml min^{-1}; absorbance was monitored at 214 nm.

Isolation and sequencing of receptor cDNA clones

Total tissue RNA was isolated from placental artery (PA) and umbilical vein (UV) tissue that was first rapidly dissected and cleaned, as for a bioassay procedure, and then quick-frozen. Four tissues (two PA and two UV samples) were processed for the preparation of RNA. In one instance, the PA and UV tissue came from the same donor; in other preparations, the PA and UV tissues were obtained from two separate donors. RNA was prepared with the use of the TRI-reagent (Molecular Research Centre, Cincinnati, OH, U.S.A.) and was reverse-transcribed (RT) with first strand cDNA synthesis kit and pd (N) 6 primer (Pharmacia) according to manufacturer's recommendations at 37°C for 60 min, followed by denaturing at 93°C for 5 min and flash-cooling to 4°C. The RT product was then diluted 3 x with water and 3 μl of this solution was used with sets of overlapping sequence-specific primers spanning the entire receptor cDNA coding sequence for polymerase chain reaction (PCR) amplification employing 1 unit of Ampli Taq polymerase in a 10 mM Tris HCl buffer, pH 8.3 (0.1 ml final vol), containing $MgCl_2$ (2.5 mM), KCl (50 mM), nuclease-free bovine albumin (5 μg) and 0.6 μM each of the four deoxynucleotide triphosphates.

The PCR primers used were: *Forward primers: (A)* 5' ATG GGG CCG CGG CGG CTG CT 3', targeted to amino acids M_1 to L_7 of the human receptor sequence. (B) 5' CCC GGT CAT TTC TTC TCA GGA A 3' targeted to amino acids P_{40} to N_{47} of the human receptor sequence, but using the rat receptor nucleotide sequence (131–152) for this domain. *Reverse primers:* (C) 5' CCT AAG TTA ACA - GCT TTT TGT ATA T 3' targeted from the stop codon to I_{419} of the human receptor sequence. (D) 5' AAT - CGG TGC CGG AGA AGT 3' targeted to amino acids P_{167}

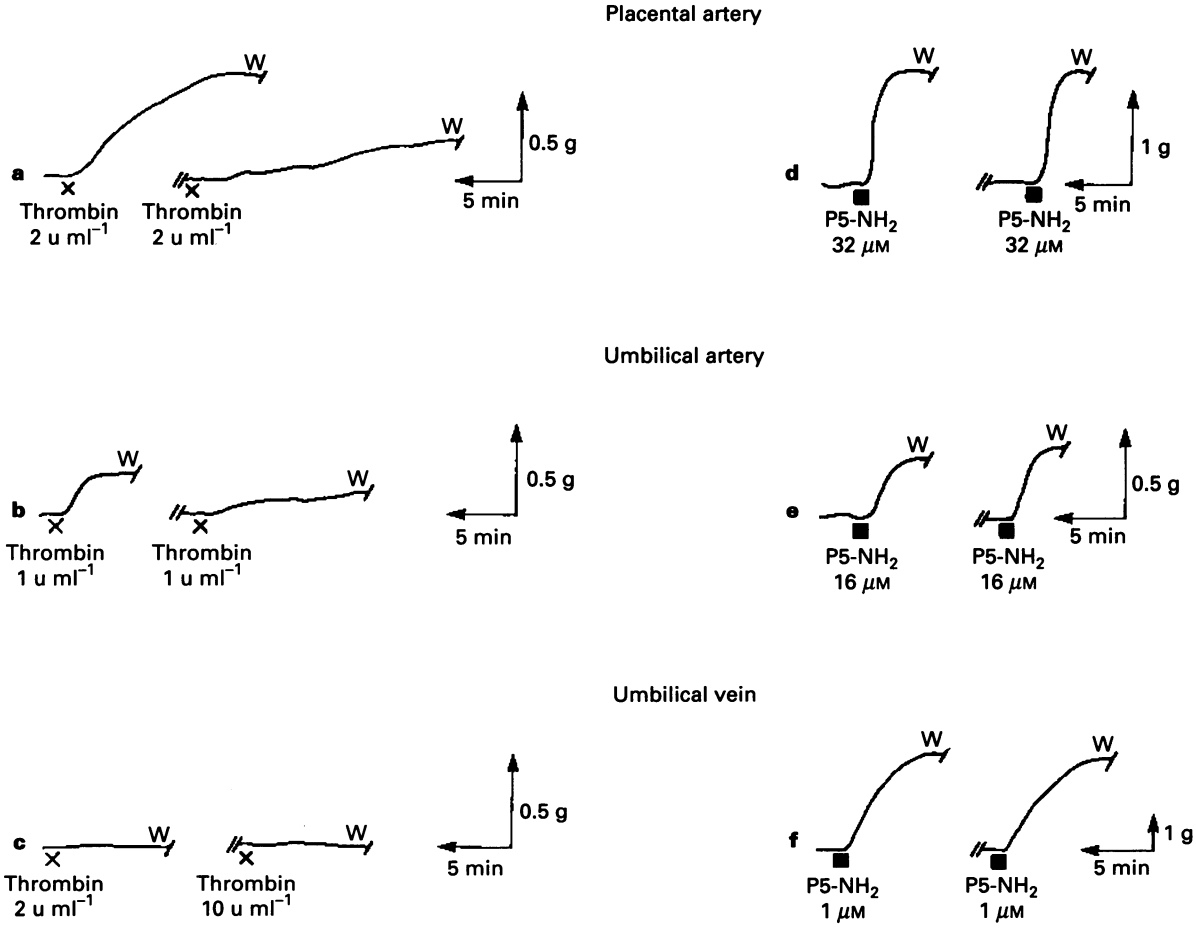

Figure 1 Responses of the placental artery, umbilical artery and umbilical vein to thrombin and P5-NH_2. The three tissue preparations were exposed either to thrombin (2 to 10 u ml^{-1}: left-hand panels a, b and c) or to P5-NH_2 (right-hand panels d, e and f) at 40 to 60 min intervals, followed by washing the tissue (W). Except for tracing (f) where the tissue was rubbed free of endothelium, all preparations possessed an intact endothelium. The scales for time and tension are shown to the right of each tracing. Each tracing (a to f), showing a continuous experiment for an individual tissue strip is representative of three or more independently conducted experiments.

to Y_{162} of human receptor sequence, but using the rat receptor nucleotides sequence (523–505) for this receptor domain. Primers (A) and (C) were used to obtain the complete coding region of the human receptor cDNA. Primers (B) and (C) were used to generate cDNA spanning the thrombin cleavage site up to the end of the coding region. Primers (B) and (D) were used as nested primers to confirm the identity of the PCR products. Amplification was allowed to proceed for 35 cycles, beginning with a 45 s denaturation period at 94°C followed by a 45 s reannealing time at 55°C and a primer extension period of 2 min at 72°C. The PCR products were purified by 1.5% agarose gel electrophoresis, and cloned in the pGEM-T vector (Promega) following transformation of *E. coli* strain DH5α cells. The insert was sequenced with M 13 universal, reverse sequencing primers and primers designed on the basis of human thrombin receptor sequence (Vu *et al.*, 1991), using the dideoxynucleotide sequencing method (Sanger *et al.*, 1977), employing a T7 DNA polymerase sequencing kit (Pharmacia).

Results

General responsiveness of the three vascular preparations to thrombin and P5-NH_2

Initially, we assessed the response characteristics of the placental artery (PA), umbilical artery (UA) and umbilical vein (UV) preparations to thrombin (1–10 u ml^{-1}) and to P5-NH_2, which was selected as a representative receptor-activating TRP. As illustrated in Figure 1 the intact PA and UA preparations exhibited contractile responses to both thrombin (tracings a and b) and P5-NH_2 (tracings d and e). However, as observed by us previously in other vascular preparations (Muramatsu *et al.*, 1992), once exposed to thrombin, the PA and UA tissues were desensitized to a second thrombin exposure (Figure 1a and 1b). Nonetheless, both the PA and UA tissues responded reproducibly to repeated exposures of P5-NH_2, provided the dose intervals were of 1 h or more (Figure 1, tracings d and e). In contrast with the PA and UA tissues, the umbilical vein tissue did not respond to thrombin (2–10 u ml^{-1}) either in intact preparations (Figure 1c) or in preparations rubbed free of endothelium (data not shown). Nonetheless, P5-NH_2 caused a robust reproducible contractile response of the endothelium-free UV preparation (Figure 1f), whereas the intact UV preparation was not responsive to P5-NH_2, even at concentrations as high as 100 μM (data not shown and see Figure 2). Although reproducible contractile responses of the endothelium-free UV preparation to P5-NH_2 were observed at relatively low concentrations ($\leqslant 2$ μM), provided the dose interval was greater than 1 h, higher concentrations ($\geqslant 5$ μM) caused some desensitization of the tissue. Therefore, concentration-effect curves were done by exposing UV preparations only once to a given concentration of the TRPs and expressing the response as a percentage (% KCl) of the tissue response to 50 mM KCl.

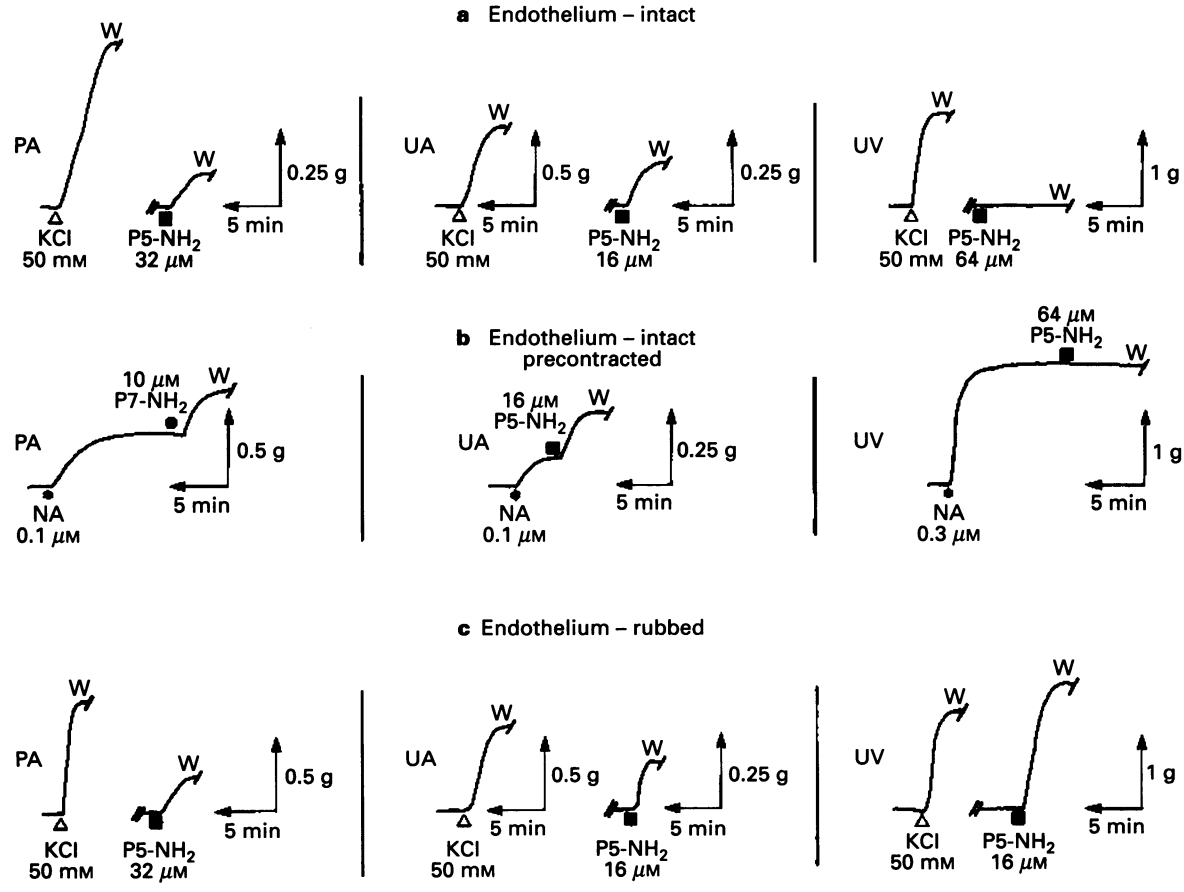

Figure 2 Evaluation of endothelial function. Individual tissue strips of placental artery (PA, left-hand panels), umbilical artery (UA, middle panels) or umbilical vein (UV, right-hand panels) were prepared either with the endothelium intact (top and middle tracings, a and b) or were rubbed free of endothelium (lower tracings, c). Tissue contractility was first monitored for each preparation with a test dose of either KCl or noradrenaline (NA); preparations were then exposed to P5-NH_2 either in the continuous presence of noradrenaline or after washing (W). The responses to P5-NH_2 relative to KCl can be compared in tissues with (top tracings, a) and without (bottom tracings, c) endothelium. The lack of a nitric oxide-mediated relaxation response is illustrated by the middle tracings (b). The tracings showing the responses of individual preparations are representative of three or more independently conducted experiments done with separate tissue strips.

Role of endothelium and endothelium-derived relaxing factors

In other arterial preparations (DeMey *et al.*, 1982; Rapoport *et al.*, 1984; Muramatsu *et al.*, 1992) thrombin or the TRPs can cause either an endothelium-dependent, nitric oxide synthase-mediated relaxation, or an endothelium-independent contraction. What was evident from the action of P5-NH_2 or P7-NH_2 in the PA and UA preparations, was that a contractile response was observed either in the presence or absence of an intact endothelium (Figure 2a and c, PA and UA tracings). Further, as opposed to our observations with rat aortic tissue (Muramatsu *et al.*, 1992), a contractile, rather than a relaxation response was detected in an endothelium-intact preparation that was precontracted with noradrenaline and then exposed to P5-NH_2 (Figure 2b, PA and UA tracings). A lack of a relaxation response to TRP in a noradrenaline-precontracted tissue was also observed for the UV preparation (Figure 2b, UV tracing). In view of the ability of the endothelium-intact UV preparation to release a relaxing factor presumed to be NO (Van de Voorde *et al.*, 1987; Chaudhuri *et al.*, 1991) we also examined the response of this preparation to P5-NH_2 in the presence of the NO-synthase inhibitor, L-NAME (0.1 μM); no contractile response was observed (not shown). Further, replenishing the organ bath with L-arginine, the precursor for NO synthesis, did not enable P5-NH_2 to cause a relaxation response in a UV preparation that was precontracted with 0.3 μM noradrenaline (e.g. see middle panel, Figure 2 and data not shown). Similarly, indomethacin (1 μM) was unable to unmask a contractile action of P5-NH_2 in the UV preparation, indicating that the production of prostanoids (e.g. prostaglandin) that might cause relaxation could not account for the inability of P5-NH_2 to cause a contractile response (also see below). As pointed out above, in the UV preparation, a TRP-induced contraction was only observed when the tissue was denuded of endothelium (Figure 2c, right-hand tracing). In all respects, the results using thrombin as an agonist (not shown) paralleled those obtained with the TRPs, except that in the endothelium-denuded UV preparation that contracted in response to P5-NH_2, no response was observed upon exposure to thrombin (up to 10 u ml^{-1}).

Role of extracellular calcium

In the absence of extracellular calcium, all three vascular contractile preparations failed to respond to P5-NH_2 (Figure 3). Nonetheless, upon replenishing extracellular calcium in the continued presence of the agonist, P5-NH_2, a robust contraction ensued (Figure 3, upper PA, middle UA and lower UV tracings). In keeping with these observations, pretreatment of the three tissues with the calcium channel antagonist, nifedipine (1 μM), attenuated the contractile actions of either P5-NH_2 or P7-NH_2 in the three preparations (response tracings not shown): in the PA and UA preparations, P5-NH_2-mediated contractions were inhibited by 50 ± 11% (mean ± s.e.mean for $n = 7$), in the presence of 1 μM nifedipine, whereas in the endothelium-free UV preparation, contractions caused by P7-NH_2 were virtually abolished by 1 μM nifedipine (not shown). The contractile response of all three tissues to 50 mM KCl was also blocked by 1 μM nifedipine (not shown).

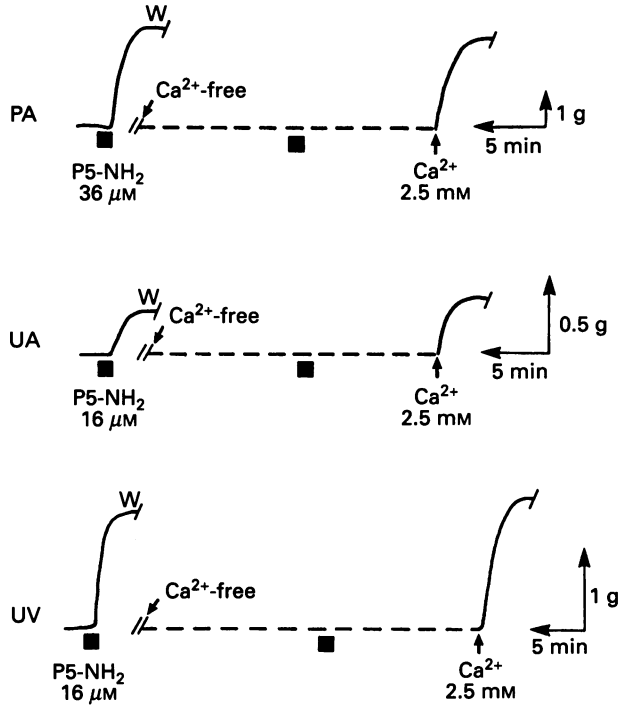

Figure 3 Role of extracellular calcium. Tissue strips were first assessed for responsiveness to P5-NH_2 (■), followed by washing (W) and transfer to a calcium-free buffer containing 0.1 mM EGTA. Tissues were again exposed to P5-NH_2 followed by reconstituting the buffer with 2.5 mM Ca^{2+} (arrow). The endothelium was removed from the umbilical vein (UV) preparation, but was intact in the placental artery (PA) and umbilical artery (UA) preparations.

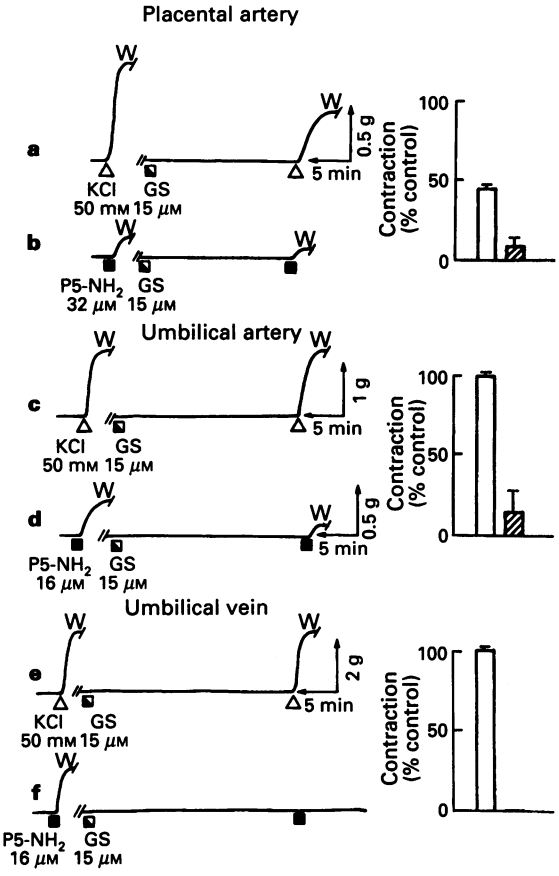

Figure 4 Inhibition of P5-NH_2-mediated contraction by genistein. The responsiveness of individual tissue strips (tracings a to f) was first assessed by exposure to either KCl (△, tracings a, c and e) or P5-NH_2 (■, tracings b, d and f) followed by washing (W). Genistein (□, 15 μM) was then added to the organ bath and the tissues were again challenged with either KCl (△) or P5-NH_2 (■). The histograms to the right show the responsiveness of the tissues to KCl (open columns) and P5-NH_2 (hatched columns) in the presence of genistein, as a percentage of the control response (mean ± s.e.mean for $n = 7$) observed in the absence of genistein.

Effects of inhibitors of cyclo-oxygenase and tyrosine kinase

In our previous work with gastric longitudinal smooth muscle preparations (Hollenberg *et al.*, 1992; 1993), we established that the contractile actions of P5-NH_2 and thrombin could be attenuated by either the cyclo-oxygenase inhibitor, indomethacin (1 μM), or by the tyrosine kinase inhibitor, genistein (15 μM). We were, therefore, interested to evaluate the effects of these enzyme inhibitors in the three placental vessel preparations. Although indomethacin (1 μM) caused a small ($35 \pm 6\%$, mean ± s.e.mean for $n = 7$) inhibition of the P5-NH_2-mediated contraction in the placental artery preparation, without affecting contractions caused by 50 mM KCl, this cyclo-oxygenase inhibitor had no effect on contractions caused by either P5-NH_2 or KCl in the UA or in the endothelium-denuded UV preparation (not shown). In contrast, genistein (GS, 15 μM) was able to attenuate P5-NH_2-mediated contractions in all three preparations, with essentially a complete and selective inhibition of P5-NH_2 action (compared with KCl-induced contractions) in the UV preparation (Figure 4). In the placental artery preparation, the inhibitory effect of genistein on P5-NH_2 action appeared to be partially non-specific, in that contractions caused by KCl were also inhibited, but to a lesser degree than those elicited by P5-NH_2 (Figure 4, top histogram). On the other hand, in the umbilical artery preparation the inhibition by genistein of P5-NH_2-induced contractions was selective (i.e. KCl action was unaffected: middle histogram, Figure 4), but not as complete as the inhibition by genistein in the umbilical vein preparation (lower histogram, Figure 4).

Concentration-effect curves

Concentration-effect curves were obtained for the contractile actions of P5, P5-NH_2, P7 and P7-NH_2 in the three vascular preparations (Figures 5 to 7) and for the platelet aggregation activity of the four polypeptides in a platelet-rich plasma preparation (Figure 8). Although there was some inter-tissue variability in responsiveness, as indicated by the error bars at each point in the several concentration-effect curves, it was possible to assign a relative order of potencies for the four polypeptides in each of the four bioassay systems (see below). Because of the intense desensitization caused by thrombin in the vascular preparations and because of the coagulation activity of thrombin in the platelet assay, it was not possible to compare directly the actions of the four TRPs with the action of thrombin (Figure 1 and data not shown). In the placental artery system, it was evident that the four TRPs did not all exhibit full intrinsic activity, since the maximal contractile forces, relative to the KCl response, differed somewhat (e.g. compare the maximal responses to P5-NH_2 with those of P7-NH_2 in Figure 5). This type of result, seen by us previously in a gastric contractile bioassay (Hollenberg *et al.*, 1992), was also evident to some extent in the umbilical artery preparation (Figure 6) but not in the umbilical vein preparation (Figure 7). Since the platelet response represents essentially an all-or-none phenomenon, it was perhaps not surprising that the intrinsic activity of all four polypeptides appeared to be equivalent (Figure 8).

In the two arterial preparations, P7-NH_2 was the most potent and P5 the least potent of the four polypeptides tested; however in these two preparations, the potency of P5-NH_2, relative to the other peptides, differed considerably (compare

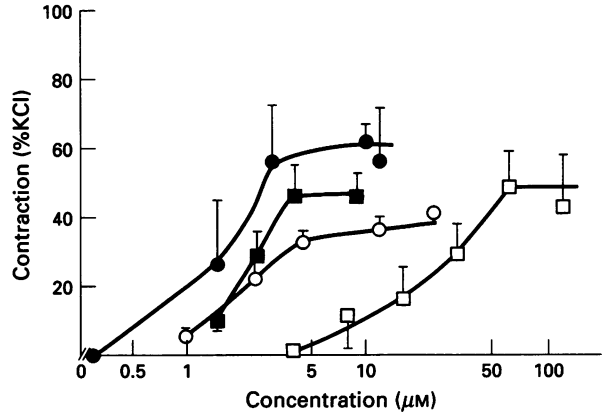

Figure 6 Concentration-response curves for the contractile actions of TRPs in the umbilical artery. The contractile responses of the umbilical artery preparations to different concentrations (μM) of the TRPs (x-axis) were expressed as a percentage (% KCl) of the tissue response to 50 mM KCl. Each data point represents the mean ± s.e.mean of 7 to 15 independent observations in different tissue preparations. The relative order of potencies in this system was P7-NH_2, (●) ≥ P5-NH_2, (■) ≃ P7, (○) > > P5 (□).

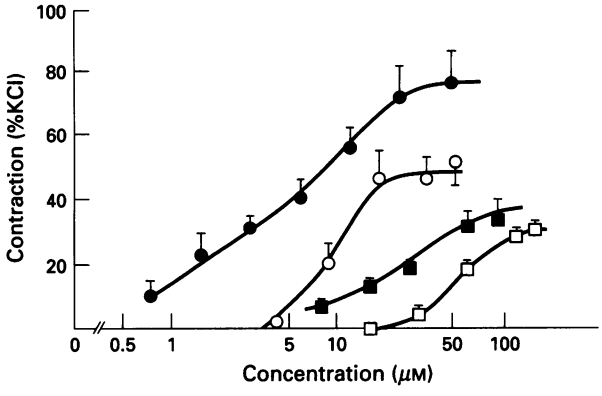

Figure 5 Concentration-response curves for the contractile actions of TRPs in the placental artery. The contractile responses of placental arterial tissues to increasing concentrations (μM) of the TRPs were expressed as a percentage (% KCl) of the tissue response to 50 mM KCl. Each data point represents the mean ± s.e.mean of 7 to 21 independent observations on different tissues. The relative order of potencies in this system was P7-NH_2, (●) > P7, (○) > P5-NH_2, (■) > P5 (□).

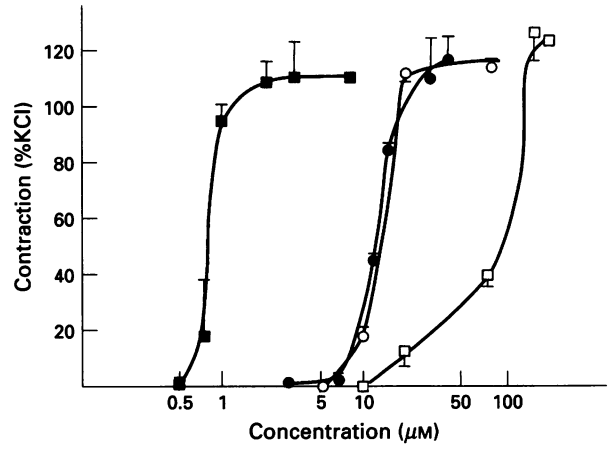

Figure 7 Concentration-response curves for the contractile actions of TRPs in the umbilical vein. The contractile responses of the endothelium-denuded umbilical vein preparation to different concentrations (μM) of the TRPs (x-axis) were expressed as a percentage (% KCl) of the response to 50 mM KCl. Each data point represents the mean ± s.e.mean of 7 to 12 independent observations in different tissues. The relative order of potencies in this system was P5-NH_2, (■) > > P7-NH_2, (●) = P7, (○) > > P5 (□).

Figures 5 and 6). In the PA preparation, the relative potency order was: P7-NH_2 > P7 > P5-NH_2 > P5, whereas in the UA preparation, the relative potency order was: P7-NH_2 ≥ P5-NH_2 ≃ P7 > > P5. In contrast, in the umbilical vein preparation, although P5 was, as in the other preparations, the least potent, P5-NH_2 was clearly the most potent of the four TRPs; both P7 and P7-NH_2 were equivalent, but lower in potency than P5-NH_2 (Figure 7). Thus, the relative order of potencies of the four TRPs in the UV preparation was: P5-NH_2 > > P7-NH_2 = P7 > > P5.

The platelet aggregation assays were done in the presence of amastatin (50–100 μM) to minimize peptide proteolysis by plasma amino peptidase (Coller *et al.*, 1992). As in the three vascular assays, in the platelet assay, P5 was the least potent of the four polypeptides (Figure 8). However, as in the UV system, but in contrast with the PA and UA assays, P5-NH_2 exhibited a comparatively high potency, equal to or even slightly greater than that of P7-NH_2 (Figure 8). The relative potency order shown in Figure 8, based on measures of the initial aggregation rate, was also observed when the data were expressed as a percentage of maximum aggregation (not shown). When platelet response was monitored semi-quantitatively, using the initial microaggregation shape change as an index of response, a reaction to P5-NH_2 (microaggregation at concentrations ≤1.25 μM) was routinely observed at a lower concentration than that of P7-NH_2 (microaggregation at concentrations ≥1.25 μM). Thus, the relative order of potencies of the four polypeptides in the platelet assay was P5-NH_2 ≥ P7-NH_2 > P7 > > P5. Quantitatively, it was possible to use the linear portions of the concentration-effect curves (Figures 5 to 8) to estimate the activities of P7-NH_2, P7 and P5-NH_2 relative to the activity of P5, as we have done previously (Hollenberg *et al.*, 1993), by calculating the relative effective concentration (EC) of a given agonist (R_{EC} = EC ÷ EC_{P5}) required to yield the same response as that caused by an equi-effective concentration of P5 (EC_{P5}) (Table 1). For instance, it can be seen that for P7, the R_{EC} values (0.12 to 0.14) relative to P5 were essentially the same in all three vascular preparations, whereas the R_{EC} value for P7 in the platelet assay differed considerably (0.06). In contrast with P7, the R_{EC} values for P7-NH_2 and P5-NH_2 differed markedly in all three vascular preparations. Thus, as summarized in Table 1, the relative biological activities of the four TRPs in the vascular and platelet assays could be seen to be distinct for each assay.

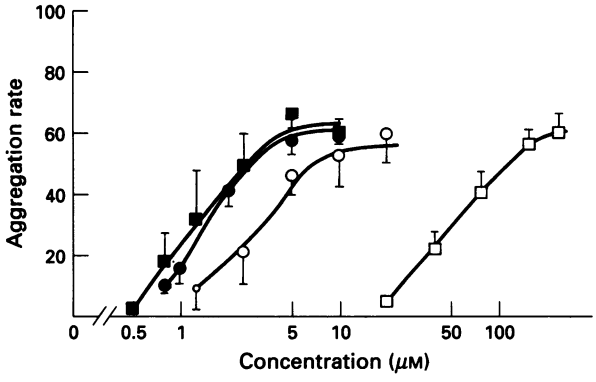

Figure 8 Concentration-response curves for TRP-induced platelet aggregation. The aggregation response of the human platelets to different concentrations (μM) of the TRPs (x-axis) were expressed as the initial rate of aggregation (% min^{-1}, y-axis). The experiments were done in the presence of amastatin (100 μM). Each data point represents the mean ± s.e.mean of 6 to 15 observations made in 3 independent experiments using different platelet samples. The relative order of potencies in this system was P5-NH_2, (■) ≥ P7-NH_2, (●) > P7, (○) > P5 (□).

H.p.l.c. analysis of peptides

As mentioned above, amastatin was added to the incubation medium for the platelet assay, in view of previous observations documenting the ability of this compound to block the degradation of TRPs by plasma aminopeptidase (Coller *et al.*, 1992). However, the protease inhibitor was routinely omitted from the vascular bioassay medium, since its presence at concentrations up to 50 μM was not found to affect the TRP-mediated contractile responses in the PA, UA and endothelium-denuded UV preparations. We had previously observed that, under the conditions of the vascular bioassay, no degradation of TRPs occurred for rat vascular and gastric smooth muscle preparations (Hollenberg *et al.*, 1993). Further, in the endothelium-containing UV preparation, the presence of amastatin (50 μM) failed to reveal a contractile action of P5-NH_2, indicating that endothelial clearance by proteolysis did not appear to be a factor related to the inability of this preparation to contract in response to P5-NH_2. We wished, nonetheless, to confirm with the three vascular preparations that the TRPs could be recovered intact from the organ bath at a time corresponding to the peak of contraction. As illustrated for P5-NH_2 in Figure 9, no degradation was observed during the course of an assay using placental artery or umbilical artery tissue; the same lack of degradation was observed using either

Table 1 Relative activities of the thrombin receptor derived peptide (TRPs) in human placental and umbilical vessels and platelets

	Relative activity (R_{EC})[a] value-relative to P5			
Peptide	*Placental artery*	*Umbilical artery*	*Umbilical vein*	*Platelets*
SFLLR (P5)	1	1	1	1
SFLLR-NH_2 (P5-NH_2)	0.33 ± .07	0.11 ± .04	0.01 ± .000	0.02 ± .001
SFLLRNP (P7)	0.12 ± .02	0.12 ± .02	0.14 ± .01	0.06 ± .001
SFLLRNP NH_2 (P7- NH_2)	0.02 ± .001	0.05 ± 0.001	0.12 ± .01	0.03 ± .001
Relative order of potencies	P7-NH_2 > 7 > P5-NH_2 > P5	P7-NH_2 ≥ P5-NH_2 ≈ P7 > > P5	$P5_2$-NH > > P7-NH_2 ≈ P7 > > P5	P5-NH_2 ≈ P7-NH_2 > P7 > > P5

[a] An activity ratio (R_{EC} = EC_{TRP} ÷ EC_{P5}) was calculated as outlined previously (Hollenberg *et al.*, 1993) and in the text as the ratio of the concentration of a given TRP agonist relative to the concentration of P5 required to cause the equivalent biological response. R_{EC} values were obtained at 3 or more levels of response along linear portions of the concentration-response curves. Values represent the averages ± s.e.mean.

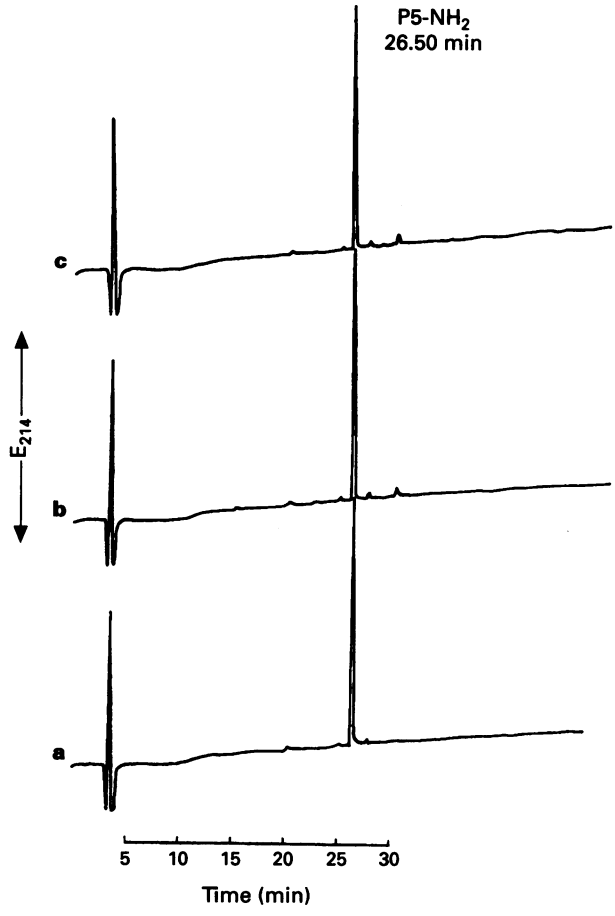

Figure 9 High performance liquid chromatography (h.p.l.c.) analysis of TRPs recovered from the bioassay organ bath. Each panel shows the h.p.l.c. profile for an individual sample of P5-NH_2, recovered from the organ bath in experiments using either placental artery (middle tracings, b) or umbilical artery (upper tracings, c) tissues; the analysis of control peptide solutions is shown in the lower tracing (a).

endothelium intact or endothelium-denuded umbilical vein preparations (not shown). We similarly confirmed that P5, P7 and P7-NH_2 were not degraded during the course of the three vascular bioassays (not shown). Thus, the difference in biological potencies that we observed could not be attributed differences in peptide degradation in the different vascular bioassay systems.

Receptor sequences obtained from PA and UV cDNA

Of the three vascular tissues studied, the umbilical vein and placental artery tissue yielded the most dissimilar structure-activity profiles for the TRPs. We thus selected these two tissues for the preparation of RNA in order to determine the thrombin receptor sequences in the tissues using the RT-PCR approach. The sets of overlapping PCR primers were targeted to the coding region of the thrombin receptor, beginning with the N-terminal methionine and ending at the carboxyterminal threonine (Vu *et al.*, 1991). Somewhat to our surprise, in view of the distinct structure-activity profiles for the TRPs in the PA and UV preparations, we found that the amino acid sequences corresponding to the cDNA sequences were identical in the two tissues and were essentially the same as those observed previously (Vu *et al.*, 1991) for the receptor cloned from a megakaryocyte cell line source. We did, however consistently find two differences in the vascular receptor sequences, compared with the originally published sequence (Vu *et al.*, 1991), with the following nucleotide changes: (1) base pairs 935–936 CG→GC, I_{237} unchanged, V_{238}→L_{238} and (2) base pairs 1315–1316, CG→GC, S_{364}→C_{364}. The base transversion at nucleotides 935–936 was entirely in agreement with previously published results, obtained with a human umbilical vein endothelial cell library (Bahou *et al.*, 1993). The base transversion at nucleotides 1315–1316 was confirmed in all of our experiments done to sequence independent receptor cDNA clones obtained from four independently prepared RNA samples (two from PA; two from UV) that were separately subjected to reverse transcription in four reactions done on two different occasions. The consistent finding of the two base transversions at residues 935–936 and 1315–1316 from 4 independently prepared RNA samples ruled out the possibility that these sequences arose as a result of PCR artifact.

Discussion

The main finding of our study was that all three placental vascular preparations possessed functional receptors for the thrombin receptor-derived peptides and that the relative potencies of P7, P7-NH_2, P5 and P5-NH_2 differed appreciably between the three contractile preparations; further, the relative orders of potencies of the four TRPs in the vascular preparations were all distinct from the one measured in the platelet aggregation assay (see Table 1). For all four bioassay preparations, a relatively consistent concentration-effect curve was observed for P5, the least potent of the four peptides, with responses occurring between 20 to 200 μM of peptide and an EC_{50} between 50 to 100 μM (Figures 5 to 8). Nonetheless, relative to P5, the activities of the other three polypeptides, as expressed by their R_{EC} values, differed substantially between the four bioassay systems, with P5-NH_2 showing the greatest variation, being least potent and closest to P5 in the placental artery assay ($R_{EC}=0.33$) and most potent relative to P5 ($R_{EC}=0.01$) in the umbilical vein preparation. Taken together, the distinct orders of agonist potencies for the same set of agonists in the four different assay systems point to distinct functional receptor subtypes, according to classical structure-activity criteria (Ahlquist, 1948).

In comparison with our previous work using rat and guinea-pig vascular and gastric bioassay systems (Hollenberg *et al.*, 1993), the distinct order of potencies for the four TRPs in the human PA and UV tissues were selected to determine the thrombin receptor cDNA sequences. In spite of the very distinct orders of potencies of the four TRP analogues in the PA and UV tissues, the cDNA sequences for the receptor obtained from PA and UV RNA were the same in both tissues; and these sequences were essentially the same as the one originally cloned from a human megakaryocyte-like cell line (Vu *et al.*, 1991) and from a human umbilical vein endothelial cell cDNA library (Bahou *et al.*, 1993). Of the two base transversions that we have detected in comparison with the originally published sequence (Vu *et al.*, 1991), one (base pairs 935–936, CG→GC) has been detected in a human umbilical vein endothelial cell cDNA library (Bahou *et al.*, 1993). The second transversion that we have detected (base 1315–1316, CG→GC) cannot be attributed to PCR artifact and may, as with the one detected by Bahou and coworkers (1993), represent genetic polymorphism of the thrombin receptor gene. Interestingly, the S_{364}→C_{364} mutation that we have detected would lead to a sequence homology with residues $S_{358}C_{359}C_{360}$ of the *Xenopus* thrombin receptor (Gerszten *et al.*, 1994). Such a substitution in transmembrane domain No. 7 of the human receptor might have a functional consequence.

How, one may ask, might tissues containing the same thrombin receptor mRNA possess functionally distinct receptor subtypes, as indicated by the different structure-activity profiles for the four TRPs? One possibility is that post-translational modification of the product of the same mRNA in different cell types might yield functionally different receptor subtypes. This situation would appear to be the case for the

murine bradykinin receptor, for which the transfection of the same cDNA into COS cells yielded two populations of receptors with distinct ligand binding properties (McIntyre *et al.*, 1993). To our knowledge, our work would represent the first description in intact tissues of a situation akin to the one described *in vitro* by McIntyre and coworkers (1993) for the murine bradykinin receptor, transfected into COS cells. A second possible explanation for the presence of pharmacologically distinct receptors resulting from the same mRNA can by hypothesized on theoretical grounds. Kenakin and coworkers have predicted that the coupling of an individual receptor to single or multiple transducer proteins might alter the relative potencies of agonists (Kenakin & Morgan, 1989). Very possibly, the types and content of G-proteins in the PA and UV preparations may differ sufficiently to alter the potency profiles of the TRPs in these tissues, according to the predictions of Kenakin & Morgan (1989). A third alternative to be considered is the possibility that a TRP such as P5-NH_2, in addition to activating the thrombin receptor, might also be capable of activating an entirely unrelated receptor that may be present in the vasculature, such as the recently cloned protease activated receptor, PAR-2 (Nystedt *et al.*, 1994). Further work will be required to evaluate these several possibilities in the PA and UV preparations. From a practical point of view, our data suggest that studies aimed at developing selective TRP agonists or antagonists for the thrombin receptor may require bioassay evaluations in a number of distinct intact tissues rather than solely in receptor transfection systems such as the frog oocyte (Vu *et al.*, 1991; Gerszten *et al.*, 1994).

The absence of an endothelium-mediated relaxation response to the TRPs in the placental vascular preparations merits comment in view of our own observations of a TRP-stimulated nitric oxide (NO)-mediated relaxation in endothelium-containing rat aortic tissue (Muramatsu *et al.*, 1992) and in view of the ability of thrombin to cause an NO-mediated relaxation in human mammary artery ring preparations (Yang *et al.*, 1994). In the endothelium-intact PA, UA and UV preparations (middle panel, Figure 2) a relaxation response in a precontracted tissue was not observed under conditions where we had readily observed a TRP-mediated relaxation in endothelium-intact rat aortic tissue (Muramatsu *et al.*, 1992; Hollenberg *et al.*, 1993). Further, the presence of L-NAME in the UV preparation at concentrations that would have blocked the synthesis of NO did not unmask a contractile response to P5-NH_2; a complete denudation of the endothelium was required to reveal a contractile response to P5-NH_2 in the UV preparation. Yet the presence of NO synthase has been observed by immunohistochemistry in UA and UV tissue (Buttery *et al.*, 1994). We suggest that although present in the endothelium of the PA, UA and UV tissues, the NO synthase is somehow refractory to activation by the TRPs, so as to yield too low a level of NO to cause a relaxant effect. Possibly, the comparatively high Po_2 to which the placental tissues are exposed in the organ bath may be a factor in the lack of a relaxant response of the preparations to the TRPs (Xie & Triggle, 1994).

In the UV tissue, the lack of a contractile response in endothelium-intact preparations to TRPs could not be attributed to the production of NO or a relaxant cyclo-oxygenase product and could not be explained by rapid degradation of the peptide by aminopeptidase, in contrast with the observations of Godin and coworkers (1994). We are thus unable at present to account for the unmasking of a contractile action of the TRPs in this tissue by removal of the endothelium. Possibly, in endothelium-intact preparations the TRPs cause the production of an as-yet-unidentified relaxing factor that completely offsets the contractile response. Alternatively, the intact endothelium may in some way present a physical barrier that limits access of the TRPs to the contractile elements in the UV tissue. Further work will be required to resolve this issue. Similarly, the inability of the UV preparation to respond to thrombin would suggest the presence of inhibitory factors that may affect the enzymatic activity of thrombin itself or that may in some way modulate receptor activity. Thus, the physiological interpretation of the response of the denuded UV preparation to the TRPs remains an open question.

The signal transduction pathway(s) whereby the TRPs regulate contractility in the PA, UA and UV preparations appear to be similar in some respects to those activated by TRPs in gastric and aorta-derived smooth muscle preparations (Muramatsu *et al.*, 1992; Hollenberg *et al.*, 1993; Antonaccio *et al.*, 1993). Like the contractile response of aortic tissue, the contractile response of the placental vessels to the TRPs all depended on the presence of extracellular calcium. Thus, the TRP-induced activation of phosphoinositide hydrolysis (Hung *et al.*, 1992) with the consequent inositol-tris-phosphate mediated elevation of intracellular calcium (Berridge, 1993), would not appear to be sufficient to generate a TRP-mediated contractile response. Also, like the contractile response caused by TRPs in a gastric tissue preparation, the contractile actions of the TRPs in the PA, UA and UV preparations were inhibited selectively (e.g. compared with KCl-induced contractions) by the tyrosine kinase inhibitor, genistein. In this regard, the TRP-mediated contraction of the UV preparation appeared to be selectively (compared with KCl) and completely inhibited by genistein, whereas the TRP-induced contractions of the UA and PA preparations were only partially blocked by genistein. In contrast with the sensitivity of the contractile actions of the TRPs in the gastric contractile assay to indomethacin (Hollenberg *et al.*, 1993), the contractile action of the TRPs in the placental vessels was essentially resistant to this cyclo-oxygenase inhibitor. Thus, further work appears warranted to explore the similarities and differences between the placental vessels and other contractile smooth muscle preparations, in terms of the signal transduction pathways activated by thrombin and the TRPs especially with a focus on a possible role for a tyrosine kinase pathway. In this respect, the potential ability of the functional receptor subtypes for the TRPs that we have observed in this study to couple to distinct signal transduction pathways in different tissues will be of considerable interest.

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Acute and chronic cardiac and regional haemodynamic effects of the novel bradycardic agent, S16257, in conscious rats

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1 We carried out experiments to assess the cardiac and regional haemodynamic effects of single or repeated injections of the novel bradycardic agent, S16257, (7,8-dimethoxy 3-{3-{[(1S)-(4,5-dimethoxybenzocyclobutan-1-yl)methyl]methylamino}propyl}1,3,4,5-tetrahydro-2*H*-benzapin 2-one), in conscious rats.

2 In the first experiment, male Long Evans rats were chronically instrumented for the measurement of cardiac or regional haemodynamics ($n=9$ in each group), and, on separate experimental days, were randomized to receive i.v. bolus injections of vehicle (5% dextrose) or S16257 at a dose of 1 mg kg^{-1}.

3 In animals instrumented for the measurement of cardiac haemodynamics ($n=9$), following injection of vehicle, there were no immediate changes, and 7-8 h later there were slight reductions in heart rate and mean arterial blood pressure only. Injection of S16257 caused an immediate, transient, pressor effect but thereafter there were reductions in heart rate, mean arterial blood pressure, cardiac index and total peripheral conductance, together with increases in stroke index and peak aortic flow. The integrated decreases in heart rate, mean arterial blood pressure, cardiac index and total peripheral conductance and increases in stroke index, peak aortic flow, dF/dt_{max} and central venous pressure following S16257 were all significantly greater than the changes after vehicle injection. After injection of S16257, the fall in heart rate and fall in cardiac index were not linearly related.

4 In animals instrumented for the measurement of regional haemodynamics ($n=9$). the bradycardic effect of i.v. S16257 was accompanied by reductions in renal, mesenteric and hindquarters blood flows and vascular conductances that were greater than the changes seen following injection of vehicle, but only for the first 1 h. Considering animals instrumented for the measurement of cardiac and regional haemodynamics together, the bradycardic effect of S16257 was greater the higher the resting heart rate.

5 In the second experiment, animals chronically instrumented for the measurement of cardiac or regional haemodynamics ($n=9$ in each group) were given s.c. injections of S16257 (1 mg kg^{-1}) on four consecutive days. The general patterns of change in cardiac and regional haemodynamics following s.c. injection of S16257 were as described above for i.v. injection, although the rates of onset of effects were slower. The bradycardic effect of S16257 was less on the first, than on the subsequent, three days.

6 Overall, these results indicate that the bradycardic action of S16257 is not associated with any signs of negative inotropic action. Only the initial depressor effect of i.v. S16257 is associated with reductions in renal, mesenteric and hindquarters flow and vascular conductance significantly greater than those seen after vehicle injection. With repeated s.c. injection of S16257, there are no signs of desensitization to its bradycardic actions, nor impairment of regional perfusion. If these results extrapolate to the clinical setting, it seems likely that S16257 will have beneficial bradycardic effects, with no concurrent undesirable actions on other aspects of cardiovascular function.

Keywords: Bradycardic agent; S16257; cardiac haemodynamics; regional haemodynamics

Introduction

The development of specific bradycardic agents (Kobinger & Lillie, 1984) offers a new therapeutic strategy in patients with ischaemic heart disease or congestive heart failure, since these drugs can reduce heart rate without impairing cardiac function (e.g., Guth *et al.*, 1987; Krumpl *et al.*, 1988; Van Bogaert *et al.*, 1990; Johnston *et al.*, 1991; Chen & Slinker, 1992; Gout *et al.*, 1992; Furukawa *et al.*, 1992; Breall *et al.*, 1993; Bosmith *et al.*, 1993; Marshall *et al.*, 1993; Wynsen *et al.*, 1994; Rouse & Johnson, 1994; Rouse *et al.*, 1994). One of the first specific bradycardic agents to be developed, namely zatebradine (UL-FS 49; (7,8-dimethoxy 3-{3-{[2-(3,4-dimethoxyphenyl)-ethyl]methylamino}-propyl}1,3,4,5-tetrahydro-2*H*-benzazepin 2-one dihydrochloride) (Kobinger & Lillie, 1984), has structural similarities to verapamil, but causes bradycardia at much lower doses than are needed to reduce contractility of electrically driven atria, or to relax precontracted aorta strips (Kobinger & Lillie, 1984).

Zatebradine has been shown to block the pacemaker current (I_f) in sheep cardiac Purkinje fibres (Van Bogaert *et al.*, 1990), and recently, a new specific bradycardic agent, S16257, (7,8-dimethoxy 3-{3-{[(1S)-(4,5-dimethoxy-benzocyclobutan-1-yl)methyl]methylamino}propyl} 1,3,4,5-tetrahydro-2*H*-benzazepin 2-one), related to zatebradine, was shown to be equipotent with the latter in slowing the diastolic depolarization of rabbit sino-atrial node cells (Thollon *et al.*, 1994). In spite of the profound bradycardic effects these agents can have, little attention has been paid to their integrated cardiac and regional haemodynamic effects *in vivo*, or to the reproducibility of their cardiovascular actions. Although recent studies by Rouse & Johnson (1994) and Rouse *et al.*, (1994) showed that acute central haemodynamic effects of the bradycardic agent, ZD 7288, (which is structurally dissimilar to zatebradine and S16257) were secondary to its bradycardic action in anaesthetized and conscious dogs, there was no assessment of regional haemodynamics, nor of the reproducibility of the effects seen. Therefore, the objectives of the present work were to

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Table 1 Pretreatment resting cardiovascular variable in two groups of Long Evans rats (n = 9 in each): one group was randomized to receive i.v. bolus injections of vehicle or S16257 on different days, the other was given s.c. injections of S16257 on four consecutive days

	Intravenous		*Subcutaneous*			
	Pre-Vehicle	*Pre-S16257*	*Pre-S16257 Day 1*	*Pre-S16257 Day 2*	*Pre-S16257 Day 3*	*Pre-S16257 Day 4*
Heart rate (beats min^{-1})	358±7	347±8	373±7	372±6	361±6	358±7
Mean blood pressure (mmHg)	101±2	101±2	101±2	98±2	98±2	100±2
Cardiac index (ml min^{-1} 100 g^{-1})	23.2±1.4	23.3±1.1	24.7±1.2	27.2±1.3*	28.5±1.3*	27.9±0.9*
Stroke index (μl min^{-1} 100 g^{-1})	65.3±4.5	67.4±3.8	66.5±3.6	73.6±3.9*	79.1±3.4*	78.2±2.6*
Peak aortic flow (ml min^{-1} 100 g^{-1})	97.2±4.8	96.4±4.8	103.3±4.6	109.4±5.4*	114.8±5.4*	113.9±4.5*
dF/dt_{max} (1 min^{-2} 100 g^{-1})	401±24	398±23	436±19	465±20*	487±23*	484±18*
Total peripheral conductance (μl min^{-1} $mmHg^{-1}$ 100 g^{-1})	230±15	229±9	246±12	278±16*	292±14*	279±8*
Central venous pressure (cmH_2O)	4.3±0.4	4.4±0.2	5.6±0.3	5.0±0.2	4.6±0.2	5.0±0.2

Values are mean±s.e.mean; *$P<0.05$ versus Day 1 for the s.c.group.

assess the cardiac and regional haemodynamic effects of S16257 following a single i.v. injection, and also following repeated s.c. injection (to simulate oral ingestion, but without disturbing the animals).

Methods

All experiments were carried out on male, Long Evans rats (346–450 g) bred in the Biomedical Services Unit (Queen's Medical Centre, Nottingham).

Cardiac haemodynamics

About 8 days before experiments were run, each animal had an electromagnetic flow probe (Skalar, Delft) implanted around the ascending aorta via a transthoracic approach, under sodium methohexitone anaesthesia (40–60 mg kg^{-1}, i.p., supplemented as required) (Gardiner *et al.*, 1990b). Following surgery, animals were given ampicillin (Penbritin, Beecham, 7 mg kg^{-1}, i.m.) and returned to individual home cages with free access to tap water and food (Biosure, GLP grade, 41B (M)). At least 6 days later animals were briefly anaesthetized (sodium methohexitone 40 mg kg^{-1}, i.p.). One group of animals (n=9) had an intra-arterial catheter implanted in the distal abdominal aorta (via the ventral caudal artery), and 3 catheters implanted in the right jugular vein, 2 for administration of S16257 or vehicle, and 1 fashioned and positioned for recording central venous pressure (Gardiner *et al.*, 1990b). The other group (n=9) had an intra-arterial and an intravenous catheter implanted for recording arterial and central venous pressure, respectively, and a s.c. catheter implanted for administering S16257. Animals were allowed to recover for at least 24 h before experiments were begun.

Cardiac haemodynamic data (mean thoracic aortic flow, peak thoracic aortic flow, maximum rate of rise of aortic flow (dF/dt_{max}), instantaneous heart rate, mean arterial pressure, central venous pressure, stroke volume and total peripheral conductance) were digitised by a custom-built microprocessor and stored on disc for off-line analysis (Gardiner *et al.*, 1990b).

All variables, except heart rate, mean arterial and central venous pressures were factored by body weight (i.e., cardiac index = mean thoracic aortic flow 100 g^{-1}; stroke index = stroke volume 100 g^{-1}).

Regional haemodynamics

Animals had miniaturized pulsed Doppler flow probes (Haywood *et al.*, 1981) sutured around the left renal and superior mesenteric arteries, and the distal abdominal aorta (below the level of the ileocaecal artery) to monitor blood flow to the hindquarters (Gardiner *et al.*, 1990b). All surgery was carried out under sodium methohexitone anaesthesia (40–60 mg kg^{-1}, i.p., supplemented as required). Following surgery, animals were given ampicillin (7 mg kg^{-1}, i.m. Penbritin, Beecham) and returned to individual home cages with free access to tap water and food. At least 7 days later, animals were briefly anaesthetized (sodium methohexitone 40 mg kg^{-1}, i.p.). One

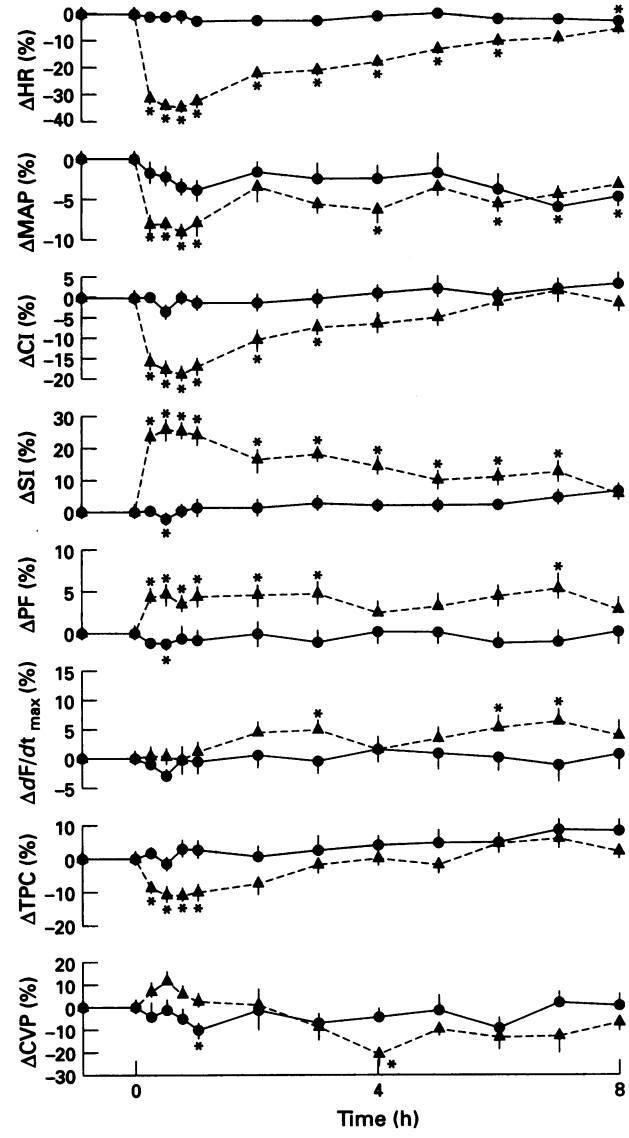

Figure 1 Cardiovascular changes over 8 h following i.v. injection of vehicle (●) or S16257 at 1 mg kg^{-1} (▲) in the same Long Evans (n=9) on 2 experimental days. Values are mean±s.e.mean; *$P<0.05$ versus pre-injection baseline. HR = heart rate; MAP = mean arterial blood pressure; CI = cardiac index; SI = stroke index; PF = peak aortic flow; dF/dt_{max} = maximum rate of rise of aortic flow; TPC = total peripheral conductance; CVP = central venous pressure.

Table 2 Integrated (AOC, $AUC_{0\text{-}1h}$) cardiovascular responses to i.v. vehicle or S16257 in the same conscious Long Evans rats (n = 9)

	Vehicle	*S16257* (1 mg kg^{-1})
Heart rate (AOC; % h)	−3±1	−33±2*
Mean blood pressure (AOC; % h)	−3±1	−8±1*
Cardiac index (AOC; % h)	−3±1	−18±1*
Stroke index (AOC, AUC; % h)	−2±1	+21±2*
Peak aortic flow (AOC, AUC; % h)	−2±1	+4±1*
dF/dt_{max} (AOC, AUC; % h)	−3±1	+2±2*
Total peripheral conductance (AUC, AOC; % h)	+4±1	−10±1*
Central venous pressure (AOC, AUC; % h)	−9±3	+9±2*

Values are mean±s.e.mean. *$P<0.05$ versus vehicle.

Table 3 Pretreatment resting cardiovascular variables in two groups of Long Evans rats ($n=9$ in each): one group was randomized to receive i.v. bolus injections of vehicle or S16257 on different days, the other was given s.c. injections of S16257 on four consecutive days

	Intravenous		*Subcutaneous*			
	Pre-Vehicle	*Pre-S16257*	*Pre-S16257 Day 1*	*Pre-S16257 Day 2*	*Pre-S16257 Day 3*	*Pre-S16257 Day 4*
Heart rate (beats min^{-1})	309±6	314±10	329±8	324±8	321±8	318±9
Mean blood pressure (mmHg)	100±2	97±2	104±2	107±1	105±1	104±2
Renal Doppler shift (kHz)	5.2±0.3	5.1±0.3	6.2±0.5	6.6±0.4	6.7±0.4	6.3±0.4
Mesenteric Doppler shift (kHz)	7.1±0.7	7.5±0.6	6.9±0.5	6.4±0.5	7.2±0.8	7.3±0.7
Hindquarters Doppler shift (kHz)	3.6±0.3	3.7±0.4	4.6±0.3	4.5±0.3	4.6±0.4	4.6±0.4
Renal conductance ([kHz $mmHg^{-1}$]10^3)	52±3	53±3	60±4	61±4	64±5	61±4
Mesenteric conductance ([kHz $mmHg^{-1}$]10^3)	71±6	78±7	66±5	60±6	69±9	71±8
Hindquarters conductance ([kHz $mmHg^{-1}$]10^3)	36±3	38±4	44±3	42±3	44±4	44±4

Values are mean±s.e.mean.

group ($n=9$) of animals had an intra-arterial catheter implanted in the distal abdominal aorta (via the ventral caudal artery) for blood pressure and heart rate recording and 2 catheters implanted in the right jugular vein for S16257 or vehicle administration. The other group of animals ($n=9$) had an intra-arterial and a s.c. catheter (for S16257 administration) implanted. Animals were allowed to recover for at least 24 h before experiments were begun.

In animals instrumented for measurement of regional haemodynamics, continuous recordings (on a Gould ES 1000 system) were made of mean and phasic arterial blood pressure, instantaneous heart rate and mean and phasic Doppler shift signals from renal, mesenteric and hindquarters probes. The latter were monitored to ensure the signals were of an acceptable quality (signal: noise>20:1). Vascular conductance changes were calculated from mean Doppler shift signals and mean arterial blood pressure (Gardiner *et al.*, 1990b).

Responses to i.v. injection of vehicle or S16257

Pilot experiments Pilot experiments were carried out in 2 animals instrumented for measurement of blood pressure and heart rate only. On 5 consecutive days S16257 was given i.v. at doses of 0.1, 0.3, 1.0, 3.0 and 10 mg kg^{-1}. These doses reduced heart rate by a mean of 7, 7, 31, 55 and 56%, respectively. Hence, a dose of 1 mg kg^{-1} S16257 was chosen for the full studies, since this was about the ED_{50} for its bradycardic effect.

Full experiments Animals received vehicle or S16257 (1 mg kg^{-1}, i.v.), on separate experimental days, in random order. Each experimental run began at 07 h 00 min, and i.v. injection was given in a bolus of 100 μl. The times at which measurements were made (0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7 and 8 h) were determined from pilot experiments.

Responses to s.c. injection of S16257

Pilot experiments Pilot experiments were carried out in 2 animals instrumented for measurement of blood pressure and heart rate only. These animals received s.c. injections of S16257 (1 mg kg^{-1}) at 07 h 00 min on 4 consecutive days, and mean arterial blood pressure and heart rate were measured every 1 h for 8 h. This experiment demonstrated that the maximum bradycardic effect of S16257 given s.c. occurred between 2–3 h after injection, as opposed to about 1 h when the same dose was given i.v., but the magnitude was the same as that seen after i.v. dosing.

Full experiments Animals received S16257 at 1 mg kg^{-1} s.c. on 4 consecutive experimental days. Each experimental run began at 07 h 00 min, and s.c. injection was given in a bolus of 100 μl. The times at which measurements were made (1, 2, 3, 4, 5, 6, 7 and 8 h) were determined from the pilot experiments.

Data analysis

All calculations (mean±s.e.mean, % changes, areas over or under curves) were done using a Pascal Turbo programme (that also performed the within-run and between-run analyses using Friedman's test (Theodorsson-Norheim, 1987)); unpaired comparisons were by Mann-Whitney U tests; a P value<0.05 was taken as significant. Correlations were assessed by the Spearman ranks test.

Drugs

S16257 was supplied by Institut de Recherches Internationales Servier (France). S16257 was dissolved in sterile isotonic (5%) dextrose.

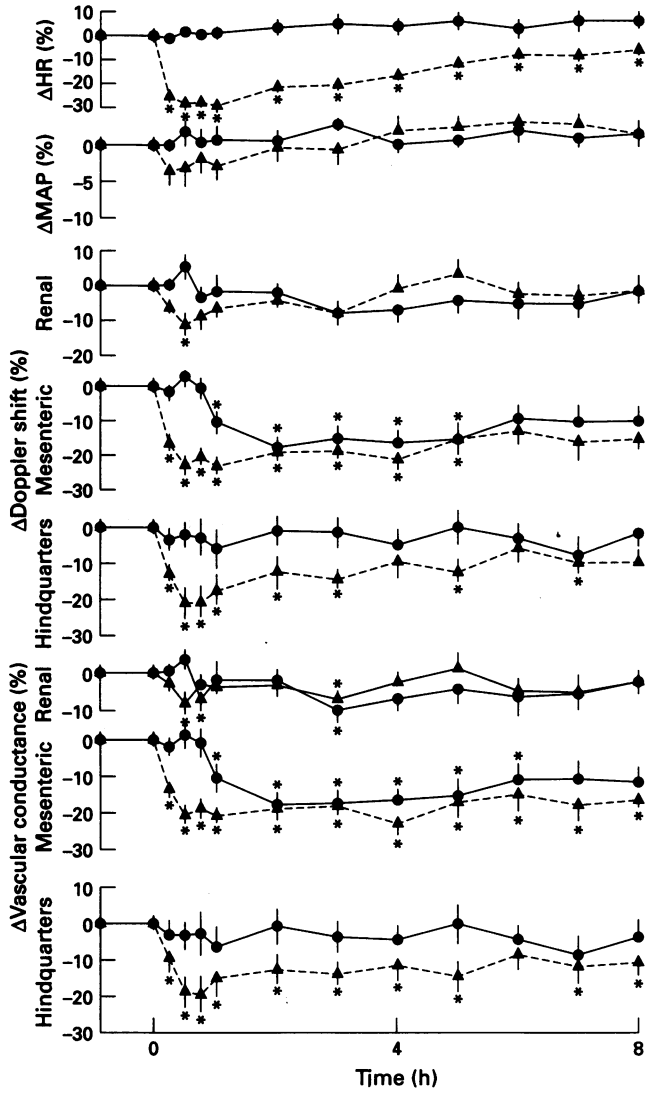

Figure 2 Cardiovascular changes over 8 h following i.v. injection of vehicle (●) or S16257 at 1 mg kg^{-1} (▲) in the same Long Evans rats (n=9) on 2 experimental days. Values are mean ± s.e.mean; *P<0.05 versus pre-injection baseline. HR = heart rate; MAP = mean arterial blood pressure.

Results

Responses to i.v. injection of vehicle or S16257

Cardiac haemodynamics Resting values for cardiovascular variables in animals instrumented for the measurement of cardiac haemodynamics are shown in Table 1.

Cardiovascular changes following injection of vehicle There were no acute changes following injection of vehicle (Figure 1). However, 7 and 8 h after injection of vehicle there were slight reductions in mean arterial blood pressure and heart rate, but no change in other variables (Figure 1).

Cardiovascular changes following injection of S16257 at 1 mg kg^{-1} Injection of S16257 caused an immediate, but slight and transient pressor effect, as also seen in the animals instrumented for regional haemodynamics; thereafter, there was a modest fall in mean arterial blood pressure, but a marked bradycardia (Figure 1). The latter was accompanied by a significant reduction in cardiac index, although stroke index showed a sustained increase (Figure 1); there was a modest rise in peak aortic flow, and dF/dt_{max} was maintained or increased slightly (Figure 1). The higher the resting heart rate, the greater was the bradycardic effect of S16257, but the fall in heart rate and the fall in cardiac index were not linearly related (data not shown). Over the first 1 h following injection of S16257 there was a slight reduction in total peripheral conductance and a tendency for central venous pressure to rise (Figure 1). Subsequently, central venous pressure fell slightly (Figure 1).

Comparison of integrated responses to vehicle and S16257 The temporal drift in some of the variables in the vehicle-injected group tended to obscure the immediate differences between the effects of treatment with vehicle and treatment with S16257 (Figure 1) if integrated responses were considered over the whole 8 h post-injection period. Therefore, the analysis of integrated responses was confined to the 1 h following injection, when the effects of S16257 were most marked (Figure 1). Relative to the effects of vehicle, S16257 caused significantly greater reductions in heart rate, mean arterial blood pressure, cardiac index, and total peripheral conductance, together with significantly greater increases in stroke index, peak aortic flow, dF/dt_{max} and central venous pressure (Table 2).

Regional haemodynamics Resting values for cardiovascular variables in animals instrumented for the measurement of regional haemodynamics are shown in Table 3.

Cardiovascular changes following injection of vehicle There were no acute changes following injection of vehicle (Figure 2), but between 1–5 h later, there were significant reductions in mesenteric flow and conductance (Figure 2). This is a phenomenon we have described previously (Gardiner *et al.*, 1990a; 1994); it is likely it represents a waning of the hyperaemic vasodilatation in the gut induced by food ingestion during the dark cycle (rats are nocturnal feeders). There was also a slight fall in renal vascular conductance (see Gardiner *et al.*, 1990a; 1994), but no significant changes in mean arterial blood pressure, heart rate, or hindquarters haemodynamics (Figure 2).

Cardiovascular changes following injection of S16257 at 1 mg kg^{-1} There was a slight, transient pressor response immediately following injection of S16257, but thereafter there

Table 4 Integrated (AOC, $AUC_{0\text{-}1h}$) cardiovascular responses to i.v. vehicle or S16257 in the same conscious Long Evans rats (n = 9)

	Vehicle	*S16257* (1 mg kg^{-1})
Heart rate (AOC; %h)	−2±1	−28±2*
Mean blood pressure (AUC, AOC; %h)	+2±2	−5±1*
Renal flow (AOC; %h)	−3±1	−10±1*
Mesenteric flow (AOC; %h)	+5±2	−20±2*
Hindquarters flow (AOC; %h)	−8±2	−19±3*
Renal conductance (AOC; %h)	−3±1	−7±2*
Mesenteric conductance (AOC; %h)	−6±2	−17±2*
Hindquarters conductance (AOC; %h)	−8±2	−17±3*

Values are mean ± s.e.mean. *P<0.05 versus vehicle.

were no significant changes in mean arterial blood pressure (Figure 2). However, there was a substantial and sustained bradycardia (Figure 2). There were slight reductions in renal flow and vascular conductance, and larger reductions in mesenteric and hindquarters flow and vascular conductance (Figure 2).

Comparison of integrated responses to vehicle and S16257 As with animals instrumented for the measurement of cardiac haemodynamics (see above), the temporal drift in cardiovascular variables following injection of vehicle (Figure 2) meant that consideration of integrated responses over the full 8 h recording period obscured the clear, early differences between the effects of vehicle and S16257. However, analysis of the changes over the 1 h following injection showed significantly greater reductions in heart rate, mean arterial blood pressure, and renal, mesenteric and hindquarters blood flows and vascular conductances after S16257, than after vehicle (Table 4).

Responses to s.c. injection of S16257

Cardiac haemodynamics Resting values for cardiovascular variables in animals instrumented for the measurement of cardiac haemodynamics are shown in Table 1. Cardiac index, stroke index, peak aortic flow, dF/dt_{max} and total peripheral conductance were slightly lower on Day 1 than on subsequent days.

Subcutaneous injection of S16257 caused no immediate effects, in contrast to the animals given S16257 by i.v. injection (see above).

Table 5 Cardiovascular changes following s.c. injection of S16257 in the same conscious, Long Evans rats ($n = 9$) on 4 consecutive days

		1 h	*2 h*	*3 h*	*4 h*	*5 h*	*6 h*	*7 h*	*8 h*	*Integrated response* (% h)
Δ Heart rate (%)	Day 1	−19±3*	−27±2*	−26±2*	−18±2*	−14±2*	−11±2*	−9±2*	−7±2*	−128±12
	Day 2	−22±2*	−32±2*	−30±1*	−27±1*	−21±1*	−18±2*	−14±2*	−13±2*	−170±7†
	Day 3	−16±2*	−32±2*	−30±2*	−28±1*	−20±2*	−16±1*	−16±2*	−16±1*	−164±5†
	Day 4	−12±2*	−29±1*	−31±2*	−29±2*	−22±1*	−20±1*	−18±2*	−13±2*	−166±8†
Δ Mean blood pressure (%)	Day 1	−2±2	−5±2*	−8±2*	−5±2*	−3±2	−3±2	−4±2	−3±2	−36±11
	Day 2	−3±2	−4±1*	−8±1*	−4±1*	−3±2	−6±2	−4±1	−3±1	−34±7
	Day 3	−2±1	−3±2	−5±1*	−6±2*	−4±2	−2±1	−5±1*	−4±1*	−31±7
	Day 4	−2±2	−3±1	−4±2	−6±3	−7±2*	−5±2	−6±1*	−5±1*	−38±8
Δ Cardiac index (%)	Day 1	−5±2*	−6±2*	−3±4	1±3	4±3	1±4	4±4	6±2*	−28±8
	Day 2	−12±2*	−15±2*	−13±1*	−9±1*	−6±2*	−5±3	1±2	−3±2	−64±9†
	Day 3	−8±2*	−15±2*	−13±2*	−14±2*	−8±1*	−7±2*	−6±2*	−6±2*	−75±10†
	Day 4	−4±3	−12±3*	−14±3*	−10±3*	−6±3	−6±3	−6±3	3±3	−70±13†
Δ Stroke index (%)	Day 1	19±5*	30±5*	31±5*	25±5*	21±4*	14±5*	15±3*	14±3*	162±29
	Day 2	15±3*	25±3*	25±3*	24±2*	18±3*	16±4*	18±3*	12±4*	147±17
	Day 3	9±2*	25±3*	24±2*	20±3*	15±2*	11±2*	11±2*	11±2*	123±9
	Day 4	9±3*	24±4*	25±4*	26±3*	20±3*	17±3*	14±2*	11±3*	141±19

Values are mean±s.e.mean; *$P<0.05$ for change; †$P<0.05$ versus Day 1 response (Friedman's test).

Table 6 Cardiovascular changes following s.c. injection of S16257 in the same conscious, Long Evans rats ($n = 9$), on 4 consecutive days

		1 h	*2 h*	*3 h*	*4 h*	*5 h*	*6 h*	*7 h*	*8 h*	*Integrated response* (% h)
Δ Peak flow (%)	Day 1	3±2	7±2*	9±2*	9±3*	9±3*	6±3*	7±3*	6±2*	55±17
	Day 2	1±2	4±2*	6±1*	7±1*	6±1*	7±1*	8±1*	5±1	44±8
	Day 3	1±1	5±2	7±2*	6±2*	6±1*	4±1*	4±1*	2±1	37±7
	Day 4	3±2	5±2	6±2*	8±2*	6±2*	5±1*	4±2*	3±2	42±10
Δ dF/dt_{max} (%)	Day 1	9±1	5±2	7±3*	10±3*	10±3*	6±4	8±4*	6±3	55±20
	Day 2	−2±2	1±2	3±2	6±2*	6±1*	6±2*	9±1*	4±2	39±9
	Day 3	0±2	2±3	7±3	3±3	8±3*	6±2*	6±2*	2±2	42±12
	Day 4	3±3	3±4	5±3	6±3	7±3	5±2*	4±3	4±3	46±16
Δ TPC (%)	Day 1	−3±2	0±3	6±5	7±4	8±5	5±5	9±5	10±4	−19±8
	Day 2	−8±3*	−11±3*	−5±1*	−6±2*	−3±4	2±4	5±2	1±3	−42±11
	Day 3	−6±2*	−12±3*	−9±2*	−8±4*	−4±2	−5±2	−1±3	−1±3	−54±11
	Day 4	−1±4	−10±4*	−10±4	−3±5	1±4	0±5	0±4	2±4	−49±12
Δ CVP (%)	Day 1	−1±4	−6±5	−12±5*	−13±5*	−16±5*	−13±5*	−16±3*	−12±4*	−90±27
	Day 2	5±2*	4±3	−3±5	2±5	−1±6	2±4	−2±5	2±6	48±17†
	Day 3	10±4	11±6	8±6	9±6	12±4*	11±8	10±7	−1±2	102±24†
	Day 4	10±5	12±4	14±7	7±5	6±4	2±4	3±3	−3±7	74±21†

Values are mean±s.e.mean; *$P<0.05$ for change; †$P<0.05$ versus Day 1 response (Friedman's test). TPC = total peripheral conductance; CVP = central venous pressure.

On the first day, injection of S16257 caused a bradycardia that was maximal 2–3 h after injection (Table 5). Although slower in onset than the bradycardic response to i.v. injection of S16257, the nadirs in heart rate following both routes of administration were similar. There was a slight, but significant, bradycardia even 8 h after s.c. injection of S16257 (Table 5), but by 24 h after injection resting heart rate was not different from the pre-injection value.

On the three subsequent days, injection of S16257 evoked bradycardias that were not significantly different from each other, but were significantly greater than that seen on the first experimental day (Table 5).

On the first day, S16257 caused only a slight fall in cardiac index, but on the subsequent three days, the integrated falls in cardiac index were similar to each other and significantly greater than on day 1 (Table 5). Generally, the falls in heart rate and cardiac index on each experimental day were not linearly related (data not shown).

The patterns of change in the other variables were similar on all four experimental days (Tables 5 and 6).

Regional haemodynamics Resting values for cardiovascular variables in animals instrumented for the measurement of regional haemodynamics are shown in Table 3. Subcutaneous injection of S16257 caused no immediate effects, in contrast to the animal given i.v. injections (see above). The patterns of change in heart rate on the four days following S16257 were similar to those described above, i.e., the integrated response was significantly less on the first day than on the subsequent days (Tables 7 and 8). All other variables, with the exception of

Table 7 Cardiovascular changes following s.c. injection of S16257 in the same conscious, Long Evans rats ($n = 9$) on 4 consecutive days

		1 h	*2 h*	*3 h*	*4 h*	*5 h*	*6 h*	*7 h*	*8 h*	*Integrated response* (% h)
Δ Heart rate (%)	Day 1	−22±2*	−22±3*	−22±1*	−18±2*	−14±2*	−11±2*	−10±3*	−9±2*	−124±11
	Day 2	−25±2*	−25±1*	−27±1*	−21±2*	−17±2*	−17±2*	−13±2*	−11±2*	−150±10†
	Day 3	−20±3*	−29±2*	−27±2*	−26±1*	−20±1*	−18±2*	−16±2*	−14±2*	−164±8†
	Day 4	−18±2*	−29±3*	−30±2*	−28±2*	−21±2*	−19±2*	−18±2*	−14±3*	−168±13†
Δ Mean blood pressure (%)	Day 1	−2±1	−3±1*	−2±1	−2±1	0±2	−1±1	−1±1	1±1	−16±6
	Day 2	−4±1*	−6±1*	−5±1*	−6±1*	−4±1*	−4±1*	−3±2	−4±1*	−35±8
	Day 3	−3±1*	−5±1*	−4±1*	−5±1*	−6±1*	−2±1	−5±1*	−3±1*	−32±7
	Day 4	−3±1*	−5±2*	−8±1*	−6±1*	−5±1*	−4±2*	−5±2*	−3±2	−39±8
Δ Renal flow (%)	Day 1	−1±2	−7±5	−4±3	−4±3	−5±3	−3±3	−6±5	−4±3	−45±13
	Day 2	−6±3	−13±4	−6±5	−6±2	−3±4	−3±5	−9±4	−5±3	−59±16
	Day 3	−4±4	−9±4	−9±4	−9±4	−7±4	−4±5	−10±3	−6±3	−65±20
	Day 4	−1±4	−9±3	−8±5	−11±2	−4±3	−6±3	−5±2	−7±3	−55±11
Δ Mesenteric flow (%)	Day 1	−11±4	−19±1*	−20±2*	−17±2*	−14±3*	−13±3*	−11±5*	−11±5*	−115±16
	Day 2	−14±3*	−20±4*	−21±3*	−15±4*	−14±5*	−19±4*	−16±4*	−16±4*	−128±23
	Day 3	−11±2*	−21±4*	−21±4*	−19±4*	−17±4*	−15±4*	−10±6*	−11±5*	−124±20
	Day 4	−11±2*	−23±4*	−21±4*	−22±4*	−18±3*	−14±4*	−16±3*	-10±4*	−129±15
Δ Hindquarters flow (%)	Day 1	−13±4*	−12±5*	−14±4*	−13±5*	−7±3	−8±4	−12±7	−10±6	−90±26
	Day 2	−13±4*	−13±4*	−22±9*	−14±4*	−9±4*	−12±5*	−4±6	−8±5	−99±27
	Day 3	−5±4	−15±4*	−15±4*	−15±4*	−14±3*	−12±4*	−12±4*	−10±4*	−97±18
	Day 4	−9±5	−19±5*	−17±4*	−15±4*	−10±5*	−11±5	−5±6	−7±6	−101±25

Values are mean ± s.e.mean; *$P < 0.05$ for change; †$P < 0.05$ versus Day 1 (Friedman's test).

Table 8 Cardiovascular changes following s.c. injection of S16257 in the same conscious, Long Evans rats ($n = 9$) on 4 consecutive days

		1 h	*2 h*	*3 h*	*4 h*	*5 h*	*6 h*	*7 h*	*8 h*	*Integrated response* (% h)
Δ Renal conductance (%)	Day 1	1±2	−4±4	−1±3	−1±3	5±3	−2±4	−6±4	−5±3	−38±13
	Day 2	−2±4	−8±4	−1±5	−1±3	2±4	1±5	−6±4	−1±3	−38±12
	Day 3	−1±4	−4±4	−5±3	−4±3	−1±4	−2±5	−5±4	−3±3	−45±16
	Day 4	2±4	−4±2	0±5	−5±4	2±4	−2±4	0±2	−3±4	−32±11
Δ Mesenteric conductance (%)	Day 1	−9±4	−17±1*	−18±2*	−15±2*	−14±4*	−12±3*	−11±4	−12±5	−106±14
	Day 2	−10±4	−16±3*	−16±4*	−10±4*	−10±4*	−16±4*	−13±4*	−13±4*	−102±21
	Day 3	−8±2	−17±4*	−17±3*	−14±5*	−12±4*	−13±4*	−5±6	−8±6	−100±19
	Day 4	−8±3	−18±4*	−14±4*	−16±5*	−13±3*	−10±5	−11±5	−7±4	−99±17
Δ Hindquarters conductance (%)	Day 1	−11±5	−9±5	−12±4	−11±5	−7±4	−6±5	−11±7	−11±6	−84±27
	Day 2	−9±4	−8±5	−18±10	−8±5	−5±4	−8±6	0±6	−4±6	−78±26
	Day 3	−1±5	−10±5	−11±4*	−10±4*	−9±3	−10±4*	−8±5	−7±4	−73±18
	Day 4	−7±5	−14±5	−10±4	−10±4	−5±5	−7±6	1±7	−3±8	−78±22

Values are mean ± s.e.mean; *$P < 0.05$ for change (Friedman's test).

mesenteric flow and conductance, showed only slight and variable changes (Tables 7 and 8). The pattern of change in mesenteric haemodynamics was similar to that seen after vehicle (see above).

Discussion

Although the electrophysiological and other cardiac effects of specific bradycardic agents have been delineated (see Introduction), the acute cardiovascular consequences of administering these drugs *in vivo* are less well known, and somewhat contradictory. For example, Kobinger & Lillie (1984) found that zatebradine (0.3 mg kg^{-1}, i.v., 1 min before) caused marked bradycardia and a slight reduction in diastolic blood pressure (about 10 mmHg) in chloralose-anaesthetized cats. However, in conscious dogs, zatebradine (1 mg kg^{-1}, i.v.) caused bradycardia with no change in blood pressure. The magnitude of the bradycardia was greater the higher the resting heart rate. Subsequently, Krumpl *et al.* (1988) found that zatebradine (0.5 mg kg^{-1} infused over 5 min) in conscious dogs caused bradycardia but no significant change in cardiac output or stroke volume. However, Johnston *et al.* (1991) reported that a single dose of zatebradine (0.3 mg kg^{-1} in 3 ml given i.v. over 5 min) in anaesthetized, closed-chest dog, caused bradycardia and a reduction in cardiac output, accompanied by a rise in total peripheral resistance, but no change in stroke volume or mean arterial blood pressure 30 min later. Marshall *et al.* (1993) observed that oral dosing (0.1 – 10 mg kg^{-1}) of dogs with another specific bradycardic agent, ZD 7288, unrelated to zatebradine, had no effect on resting heart rate, whereas in conscious rats there was a dose-dependent (1 – 100 mg kg^{-1}) bradycardia, but no change in systemic arterial blood pressure averaged over the 6 h following dosing. Subsequently, Rouse & Johnson (1994) and Rouse *et al.* (1994) produced evidence that the acute haemodynamic effects of ZD 7288 (0.02 – 1 mg kg^{-1}, i.v.) were secondary to the bradycardia. In contrast, Adachi (1994) found that i.v. infusion (30 – 300 μg kg^{-1} min^{-1}) of the structurally unrelated bradycardic agent, E4080, caused dose-dependent reductions in mean aortic blood pressure in conscious dogs, but with no change in heart rate. Adachi (1994) suggested that E4080 was exerting an anti-tachycardic action under these conditions, since the occurrence of reflex sympathetic activation was apparent from dose-dependent increases in plasma noradrenaline.

Against this background we considered it useful to establish the *in vivo* cardiac and regional haemodynamic responses to the specific bradycardic agent, S16257 (Thollon *et al.*, 1994), following single i.v. injection to quantify its acute effects, and following repeated s.c. injection to establish its chronic effects. Although, in a clinical setting, S16257 would be given by mouth, repeated administration by gavage to conscious, chronically-instrumented rats would have made undisturbed haemodynamic measurements impossible. Therefore, in order to stimulate the slower drug absorption following oral, compared to i.v., administration, the chronic dosing of S16257 was achieved through a catheter implanted s.c.

Responses to single i.v. injection of S16257

The present experiments showed that, relative to vehicle, i.v. injection of S16257 caused marked reduction in heart rate. As observed by Kobinger & Lillie (1984) for zatebradine, the higher the resting heart rate, the greater was the bradycardic effect of S16257. This is consistent with S16257 acting directly on sino-atrial node cells to inhibit the pacemaker current (I_f), and hence fix the rate of diastolic depolarization (Thollon *et al.*, 1994) irrespective of the level of sympathetic and vagal tone. Although cardiac index fell with S16257, the percentage reduction was half that seen in heart rate, due to a substantial increase in stroke index. The early fall in mean arterial blood pressure was less than would have been expected from the fall in cardiac index because vascular conductance was reduced, particularly in the mesenteric and hindquarters vascular beds, and less so in the kidney. It is likely these early regional vasoconstrictor responses were due to reflex and neurohumoral activation subsequent to arterial baroreceptor unloading, as a result of the fall in cardiac index (Charlton & Baertschi, 1982), but with the substantial increase in stroke index and in diastolic interval, it is not immediately obvious how the pattern of baroreceptor discharge would have changed. Such reflex mechanisms would usually involve activation of the sympathoadrenal and renin-angiotensin systems, and release of vasopressin (Gardiner & Bennett, 1985; Schadt & Ludbrook, 1991; see Adachi, 1994), and hence it would be of interest to know how therapeutic agents that interfere with these mechanisms influence the responses to S16257.

Although total peripheral conductance fell, and this, alone, might have reduced venous return, S16257 caused a rise in central venous pressure. While it is feasible this was due to active venoconstriction (involving the mechanisms mentioned above), it is probable that the prolongation of diastole caused by S16257 contributed importantly to the rise in central venous pressure. The latter effect would normally be accompanied by activation of cardiopulmonary receptors, the influence of which might oppose those elicited by unloading of arterial baroreceptors (see Thorén, 1979, for review). Hence, the degree of peripheral vasoconstriction following i.v. S16257 probably reflected this interaction. Since S16257 is related to zatebradine, which is structurally similar to verapamil, and can cause vasorelaxation at high doses (Kobinger & Lillie, 1984), any direct vascular effect of S16257 would have been expected to cause dilatation. Such an action would have opposed any reflex vasoconstriction, and hence should have enhanced the fall in blood pressure resulting from the fall in cardiac index. The finding that the hypotensive effect of S16257 was slight indicates that any putative vasodilator action it was exerting was minimal. These observations also argue against S16257 exerting any marked effect on central nervous mechanisms at the dose used, although we cannot exclude such an action.

In association with the effects of S16257 discussed above, it was clear that the compound did not reduce peak aortic flow or dF/dt_{max}. Considering that afterload increased (i.e. total peripheral conductance fell), then this is good evidence for the absence of a negative inotropic effect of S16257 (De Wildt & Sangster, 1983).

Responses to repeated s.c. injections of S16257

Although after S16257 was given by s.c injection, the rate of onset of its bradycardic effect was slower, the maximal effect and its duration were similar to those after i.v. injection. More notably, repeated administration of S16257 on four consecutive experimental days produced no signs of desensitization to its bradycardic effects. Indeed, the integrated response on the first day was less than on the subsequent three days, although the overall pattern of response was remarkably similar. It is feasible that pharmacokinetic factors accounted for the differences between the response to S16257 on the first day, compared to subsequent days.

The somewhat lesser bradycardic effect of S16257 on the first experimental day was accompanied by a smaller fall in cardiac index and total peripheral conductance, consistent with the latter being secondary to the fall in cardiac index (see above). As after i.v. injection, however, the marked bradycardic action of s.c. S16257 was associated with a lesser reduction in cardiac index, due to the substantial increase in stroke index. This effect, which was very reproducible over the four experimental days, was accompanied by slight increases in peak aortic flow and dF/dt_{max}, indicating that S16257 was devoid of negative inotropic effects, even with repeated administration. The fact that s.c. S16257 had only slight, variable hypotensive effects on any of the experimental days is consistent with the absorption of S16257 being sufficiently slow to allow the reflex reduction in total peripheral conductance more effectively to oppose the fall in cardiac index.

In conclusion, it is clear that S16257 can have marked and reproducible, bradycardic effects without any signs of negative inotropic action, or detrimental effects on regional haemodynamics, even with chronic exposure over four days. If these results extrapolate to the clinical setting, it seems likely that S16257 will have beneficial bradycardic effects, with no concurrent undesirable actions on other aspects of cardiovascular function.

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Effect of swimming on vascular reactivity to phenylephrine and KCl in male rats

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1 The present study aimed to examine whether there is any change in vascular responsiveness to phenylephrine and KCl during exercise, and whether the vascular endothelium plays a role in these changes.

2 Adult male rats were subjected to a swimming schedule every day for 5-6 weeks. Studies were performed *in vitro* on thoracic aortae.

3 Maximum contractile response to phenylephrine of endothelium-intact thoracic aortic rings (passive tension 1.0 g) obtained from swimming rats (1.2 ± 0.2 g, $n=8$) was lower than that of sedentary control rats (2.1 ± 0.2 g, $n=8$). When the endothelium was removed, however, the dose-response curves of both groups of rats were shifted to the left with an increase in maximum responses and they were no longer significantly different (max. tension, swimming rats: 3.2 ± 0.3 g, $n=6$, control rats: 3.4 ± 0.4 g, $n=5$).

4 Indomethacin did not significantly alter the dose-response curves. A similar effect to that obtained by removal of the endothelium was observed when methylene blue and indomethacin were both added.

5 Passive tension in the range of 2.5-3.0 g, caused a significant increase in active tension developed to phenylephrine (1 μM for endothelium-intact and 0.1 μM for endothelium-denuded) of thoracic aortic rings of both swimming and sedentary control rats compared to their corresponding groups when using passive tension of 1.0-1.5 g.

6 The reduction in responses to phenylephrine of endothelium-intact thoracic aortic rings of swimming rats persisted with the use of a passive tension of 3.0 g. The presence of 300 μM N^G-nitro-L-arginine (L-NOARG) caused a significant leftward shift of the curve with an increase in maximum responses when a passive tension of either 1.0 or 3.0 g was applied to the rings. However, for the rings with a passive tension of 1.0 g, L-NOARG caused a smaller increase in maximal contractile responses to phenylephrine of the rings of sedentary controls than those of swimming rats.

7 There was no difference in the dose-response curves to depolarizing concentrations of KCl (20, 40, 80 and 120 mM) of endothelium-intact thoracic aortic rings from swimming and sedentary control rats. When the endothelium was removed, however, the dose-response curves of both groups of animals were shifted to the left with an increase in maximum responses. Moreover, the responses to KCl of endothelium-denuded thoracic aortic rings of swimming rats were greater than those of sedentary control rats.

8 These results suggest that there were changes in vascular responsiveness to phenylephrine and KCl during exercise. The fall in sensitivity to phenylephrine with no change in KCl responses, and the increase in maximum responses to phenylephrine in the presence of L-NOARG in endothelium-intact aortae (passive tension 1.0 g) from swimming rats, were due to an increase in spontaneous release and upregulation of phenylephrine-stimulated release of EDRF/NO, and may not be a consequence of an increase in prostaglandins or a decrease in the production of endothelial constrictors by vascular endothelium. EDRF/NO may play an important role in modulating local vasodilatation.

Keywords: EDRF; endothelium; exercise; KCl; phenylephrine; swimming

Introduction

It has long been known that exercise training causes beneficial changes in the cardiovascular system, one effect being to lower resting blood pressure, both in laboratory animals (Lutgemeier *et al.*, 1987; Noma *et al.*, 1987; Overton *et al.*, 1988) and in man (Meredith *et al.*, 1991; Seals & Reiling, 1991). A decrease in resting peripheral resistance in spontaneously hypertensive rats (SHR) (Tipton *et al.*, 1979) and an increase in iliac and coronary blood flow in rats (Buttrick *et al.*, 1986; Yancey & Overton, 1993) have also been found. In conscious dogs, acute treadmill exercise causes dilatation of the circumflex coronary artery, decreased coronary vascular resistance and increase in coronary blood flow (Berdeaux *et al.*, 1991; Wang *et al.*, 1993). An increase in human leg vasodilator capacity was also found in the elderly following exercise by jogging (Martin III *et al.*, 1990). However, the mechanism responsible for these changes is not yet understood.

In *in vivo* studies, Harri (1979) reported that long term swimming of the rat caused a decrease in sensitivity of blood pressure responses to phenylephrine, and increased vasodilator response to isoprenaline, a β-adrenoceptor agonist. Similar results were also reported by Pavlik *et al.* (1976) and Wiegman *et al.* (1981), using noradrenaline injected via the jugular vein. In the *in vitro* preparations, however, conflicting results have been obtained. Edwards *et al.* (1985) found no changes in vascular responsiveness to noradrenaline of thoracic aortic strips between exercise trained and sedentary control rats, while Delp *et al.* (1993) found diminished sensitivity to noradrenaline with no changes in maximum responses of endothelium-intact aortic rings of exercise training rats. Ohkubo *et al.* (1992) demonstrated that exercise training by swimming altered the vascular fatty acid composition by increasing the potential for prostacyclin production which may be involved in the increase in vasodilator capacity in exercise-trained rats.

Since 1980, it has become apparent that endothelium derived relaxing factor (EDRF) recently defined as nitric oxide (NO) (Palmer *et al.*, 1987), generated by vascular endothelium,

plays an important role in vasodilatation in many circumstances. It is released from the endothelial cells under both basal conditions and during stimulation with acetylcholine (Griffith *et al.*, 1984; Rubanyi *et al.*, 1985; Busse *et al.*, 1993). Physical stimuli, such as elevation shear stress due to increased blood flow, can also induce EDRF release and promote vasodilatation (Gerova *et al.*, 1983; Smiesko *et al.*, 1985). Thus, it is possible that the hyperaemia associated with strenuous exercise, such as swimming, may cause an increase in spontaneous release of EDRF/NO from the endothelial cell, which may contribute to a dilatation of the vascular smooth muscle, in order to achieve enough blood flow to the skeletal muscle during exercise (Amstrong & Laughlin, 1984; Sinoway *et al.*, 1987). Therefore, the present study aimed to examine (1) whether there are any changes in vascular responsiveness to the α_1-adrenoceptor agonist, phenylephrine and to KCl during exercise, and (2) whether the vascular endothelium plays a role in these changes. Studies were performed *in vitro* on thoracic aortae obtained from adult male rats, which were subjected to a swimming schedule every day for 5–6 weeks. Dose-responses curves to phenylephrine, and depolarizing concentration of KCl were studied in rings of endothelium-intact or denuded thoracic aortae. The effects of passive tension, indomethacin, methylene blue, and N^G-nitro-L-arginine (L-NOARG) on the dose-response curves to phenylephrine were also investigated.

Methods

Adult male Wistar rats, initial weight 350–420 g, were used in the study. The animals were housed at 25°C on a 10 h dark and 14 h light cycle. All rats were allowed free access to food and drinking water. Animal weights were recorded on the first and the last day of swimming schedule.

Exercise was performed by swimming in a round fibre tank (100 cm in diameter and 70 cm height) containing tap water approximately 45 cm deep and maintained at room temperature (28–29°C). A maximum of only ten rats was allowed to swim each time. The swimming schedule followed the protocol of Ohkubo *et al.* (1992). Briefly, the swimming time on the 1st day was 10 min, and then increased in 10 min steps everyday. At 100 min per day, swimming was carried out in two routines of 50 min each. The twice-daily swimming was further increased 50 min each, in steps of 10 min each day until the maximum swimming time of 90 min was achieved. Rats were then allowed to swim at this maximum swimming time every day for another two weeks. Animals were used for the study of vascular reactivity changes during the following week.

Blood pressure and heart rate measurement

Five animals of each group were selected randomly after completion of the swimming schedule for measurement of blood pressure and heart rate. Animals were anaesthetized with Nembutal (60 mg kg^{-1}, i.p.). A tracheal tube was inserted into the trachea. A polyethylene catheter was cannulated through the right common carotid artery and connected to a pressure transducer and polygraph for monitoring blood pressure and heart rate. The data for basal mean arterial blood pressure and heart rate were collected at 20 min after equilibration.

Organ bath studies

Animals were killed by cervical dislocation, and the descending thoracic aorta was removed, rinsed with oxygenated Krebs solution (37°C), and placed in a Petri dish containing the same solution. All fat and connective tissue were removed. Two adjacent rings were cut, each approximately 7 mm long. From one, the endothelial layer was removed by mechanical disruption, by the method modified from Furchgott & Zawadzki (1980) as previously described (Jansakul *et al.*, 1989).

Each ring was placed in a 20 ml organ bath by use of two vertically placed stainless steel stirrup hooks passed through its lumen, the lower being fixed, and the other connected to an isometric force transducer (Grass FT03C). Connections from both were recorded continuously on a Grass polygraph (7DWU). Each organ bath contained Krebs-Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ $7H_2O$ 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2 EDTA 0.024 and ascorbic acid 0.09 at 37°C, continuously bubbled with 95% O_2 and 5% CO_2. Prior to addition of drugs, rings were equilibrated for 60 min under a resting tension of 1.0 g, and the Krebs solution replaced every 10 min.

After equilibration, the presence of a functional endothelium was tested as follows. Each aortic ring was preconstricted with 3×10^{-7} M phenylephrine (PE) for 5–8 min (by which time the response had plateaued), and dilator responses to 10^{-6} M acetylcholine recorded. Vasodilatation (80–90%) to acetylcholine occurred with endothelium-intact, but no dilatation was observed for endothelium-denuded rings. After this test, 45 min was allowed (with washing every 10 min) for re-equilibration prior to determination of dose-response relationship to phenylephrine or KCl.

Effects of swimming, indomethacin and methylene blue on vasoconstrictor responses to phenylephrine

After testing for the presence of functional endothelium as above, tissues were incubated for 45 min. A cumulative dose-response curve to phenylephrine was obtained. Following multiple washing to remove phenylephrine, the tissues were then incubated for a further 45 min in the presence of indomethacin (10^{-6} M). The second dose-response curve to phenylephrine was then obtained. Using the same procedure, the third dose-response curve to phenylephrine was obtained in the presence of both indomethacin (10^{-6} M) and methylene blue (10^{-5} M).

Passive-active tension relationship

After testing for the presence of functional endothelium as above, tissues were incubated for 30 min. The aortic rings were stretched to increase their passive tension by 0.5 g for each stage from 1.0 to 3.0 g. At each stage, the tissues were further equilibrated for another 10 min, the tissues were then exposed to phenylephrine (10^{-6} M for endothelium-intact and 10^{-7} M for endothelium-denuded rings) for 5–8 min, at which time the maximum responses were reached. This was followed by multiple washing to remove the drug and re-equilibration for 20–30 min; the rings were then adjusted to the next passive tension and the above procedure repeated.

Effects of swimming, passive tension and L-NOARG on vasoconstrictor response to phenylephrine

Only rings with intact-endothelium were studied. Passive tension of 1 g was applied to one of the aortic rings. After testing for the presence of a functional endothelium, a test for complete blockade of EDRF release by a specific nitric oxide synthase inhibitor, L-NOARG, was made as follows. The tissues were incubated with 3×10^{-4} M L-NOARG for 30 min and were then exposed to 3×10^{-6} M phenylephrine in the presence of L-NOARG for 5 min and dilatation in response to 3×10^{-6} M acetylcholine recorded. In the present study, no dilatation to acetylcholine was found. After multiple washing to remove the drugs, the aortic rings were re-equilibrated for 30 min in the presence of 3×10^{-4} M L-NOARG. A cumulative dose-response curve to phenylephrine was obtained.

Another aortic ring with intact endothelium obtained from the same animal as above, was equilibrated under a resting tension of 3.0 g. After testing for the presence of a functional endothelium as described above, the tissues were re-equilibrated for 45 min. A cumulative dose-response curve to

phenylephrine was obtained. This was followed by multiple washing to remove the drug and the tissues were re-equilibrated for 20 min, at which time the tissue tension had returned to normal (3.0 g). The tissues were then incubated with 3×10^{-4} M L-NOARG for 30 min. The second dose-response curve to phenylephrine was obtained in the presence of L-NOARG.

Effects of swimming on vasoconstrictor responses to KCl

Discrete responses to KCl were obtained with depolarizing concentrations of KCl (20, 40, 80, 120 mM). In these experiments, amounts of KCl (mM) were substituted for NaCl in the Krebs-Henseleit solution so that the tonicity of the solution remained the same.

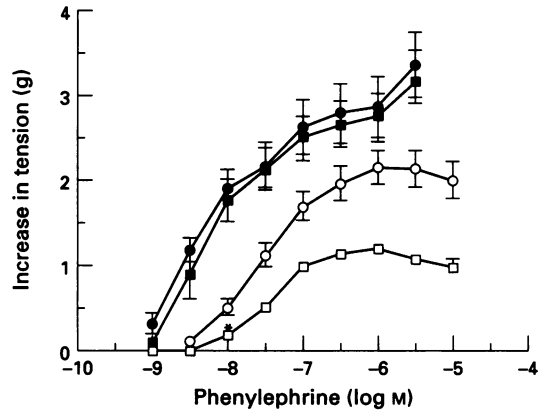

Figure 1 Constrictor responses to phenylephrine of endothelium-intact and denuded thoracic aortic rings obtained from swimming and sedentary control rats. Each point represents the mean ± s.e.mean from 5–8 experiments. (□) Swimming, endothelium intact; (■) swimming, endothelium-denuded; (○) sedentary control, endothelium intact; (●) sedentary control, endothelium-denuded rings. *Significantly different from the means obtained from endothelium-intact aortic rings of sedentary control rats, $P < 0.05$.

Drugs

All drug solutions were prepared daily and kept on ice until used. (–)-Phenylephrine HCl (Sigma, U.S.A.) was dissolved in a solution containing NaCl 9.0 g l^{-1}, NaH_2PO_4 0.19 g l^{-1} and ascorbic acid 0.03 g l^{-1}. Acetylcholine chloride (Sigma, U.S.A.), methylene blue (Sigma, U.S.A.) and N^G-nitro-L-arginine, (Sigma, U.S.A.) were dissolved in distilled water. Indomethacin was dissolved in 0.1% sodium carbonate (Na_2CO_3) solution.

Statistical analysis

Absolute tension developed was measured throughout so that comparisons could be made of both sensitivity and the maximal responsiveness of endothelium-intact and denuded thoracic aortic rings. The drug concentration which produced 50% of the maximal response for the drug (EC_{50}) was derived from regression analysis over the linear portion of the dose-response curve (Diem & Leutner, 1970). Other data are expressed as mean ± s.e.mean of 5–15 experiments ($n = 5-15$), and tests of significance made with ANOVA and Fisher PLSD or Student's unpaired t test. In all cases, a P value of 0.05 or less was considered statistically significant.

Results

The weights of the swimming and sedentary control rats at the beginning of the studies were not different (swimming, 369.9 ± 9.6 g, $n = 15$; sedentary control, 361.6 ± 3.5 g, $n = 15$). Swimming rats lost weight during the studies (324.9 ± 6.9 g, $n = 15$, $P > 0.05$), while sedentary control rats gained weight (381.6 ± 4.2 g, $n = 15$, $P > 0.05$). There were no differences in

Table 1 EC_{50} and maximum contractile responses to phenylephrine (Phe) and KCl of thoracic aortic rings obtained from swimming and sedentary control rats using passive tension of either 1.0 or 3.0 g.

	EC_{50} (95% c.l.) (nM)		*Maximum responses (± s.e.mean)* (Increase in tension, g)	
Endothelium	Present	Removed	Present	Removed
Passive tension 1.0 g				
Swimming				
Phe	35.0(21.0–59.0)	1.8(.01–33.0)	1.2 ± 0.1	3.2 ± 0.3*
Phe + Indo	45.0(35.0–56.0)	0.9(0.002–45.0)	0.9 ± 0.2	3.1 ± 0.4*
Phe + Indo + MB	12.1(8.3–17.8)[a]		3.4 ± 0.3[a]	
Phe + L-NOARG	4.4(3.1–6.2)[a]		4.7 ± 0.5[a]	
KCl	32.1(28.0–36.9)	17.7(11.1–27.6)*	1.7 ± 0.1	2.8 ± 0.2*
Sedentary control				
Phe	25.0(9.5–65.0)	4.0(1.3–13.0)	2.1 ± 0.2	3.4 ± 0.4*
Phe + Indo	20.0(6.4–63.0)	5.4(1.8–16.0)	2.5 ± 0.2	3.5 ± 0.4*
Phe + Indo + MB	17.7(11.5–27.2)		3.4 ± 0.2[a]	
Phe + L-NOARG	4.2(3.0–6.0)[a]		3.6 ± 0.6[a]	
KCl	36.5(31.8–42.0)	18.7(11.8–29.7)*	1.6 ± 0.2	2.1 ± 0.2*
Passive tension 3 g				
Swimming				
Phe	22.0(11.0–45.0)		1.6 ± 0.3	
Phe + L-NOARG	4.5(2.7–7.7)[a]		5.3 ± 0.8[a]	
Sedentary control				
Phe	9.7(3.1–30.0)		2.5 ± 0.3	
Phe + L-NOARG	1.6(1.0–2.5)[a]		5.0 ± 0.3[a]	

Values were obtained in the presence or after removal of endothelium, in the presence of indomethacin (Indo, 1 μM), indomethacin (1 μM) plus methylene blue (MB, 10 μM), and N^G-nitro-L-arginine (L-NOARG, 300 μM). *Significantly different from results obtained in the presence of endothelium of their corresponding group ($P < 0.05$). [a]Values were significantly different from the values obtained with phenylephrine alone within each activity group, $P < 0.05$.

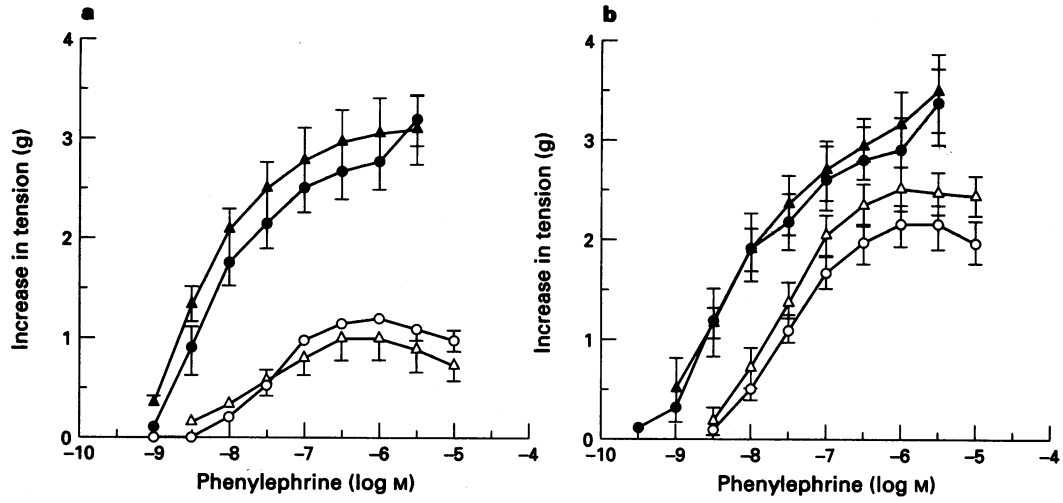

Figure 2 Effects of indomethacin (1 μM) on constrictor responses to phenylephrine of endothelium-intact and denuded thoracic aortic rings of (a) swimming rats and (b) sedentary control rats. Each point represents the mean ± s.e.mean from 5–8 experiments: (○) endothelium intact; (●) endothelium-denuded; (△) endothelium intact, in the presence of indomethacin; (▲) endothelium-denuded, in the presence of indomethacin.

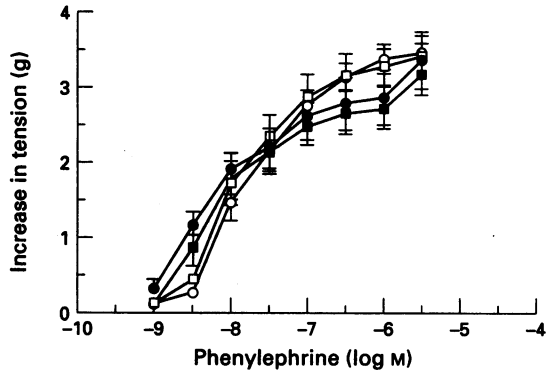

Figure 3 Effects of indomethacin (1 μM) and methylene blue (10 μM) on constrictor responses to phenylephrine of endothelium-intact thoracic aortic rings obtained from swimming and sedentary control rats. Each point represents the mean ± s.e.mean from 6–7 experiments: (■) swimming, endothelium-denuded; (●) sedentary control, endothelium-denuded; (□) swimming, endothelium intact in the presence of indomethacin and methylene blue; (○) sedentary control, endothelium intact in the presence of indomethacin and methylene blue.

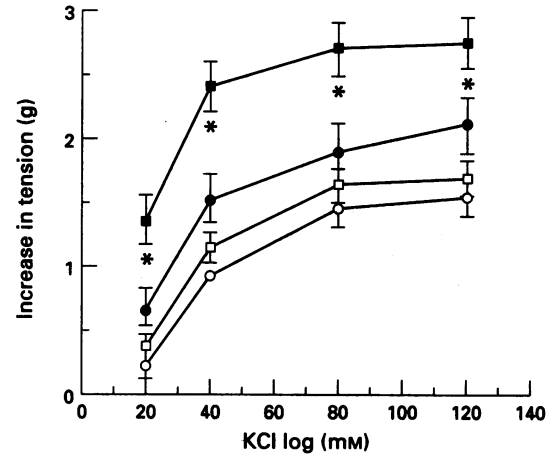

Figure 4 Constrictor responses to KCl of endothelium-intact or denuded thoracic aortic rings obtained from swimming or sedentary control rats. Each point represents the mean ± s.e.mean from 6–8 experiments: (□) swimming, endothelium intact; (■) swimming, endothelium-denuded; (○) sedentary control, endothelium intact; (●) sedentary control, endothelium-denuded. *Significantly different from the mean obtained from endothelium-denuded aortic rings of sedentary control rats, $P < 0.05$.

mean arterial blood pressure between these two groups (swimming, 167.9 ± 12 mmHg; sedentary control, 186.0 ± 16.0 mmHg, $n = 5$). Swimming animals, however, had a significantly decreased heart rate compared to sedentary controls (swimming, 371 ± 6.4 beats min^{-1}, $n = 5$; sedentary control, 404 ± 5.1 beats min^{-1}, $n = 5$, $P > 0.05$). The weight of thoracic aortic rings (7 mm long) obtained from swimming and sedentary control rats were not significantly different (swimming, 9.2 ± 0.5 mg, $n = 15$; sedentary control, 8.4 ± 0.7 mg, $n = 15$).

When using passive tension of 1.0 g, both phenylephrine and KCl produced concentration-related constriction of the rat thoracic aortic rings. Figure 1 shows vasoconstrictor responses to phenylephrine of endothelium-intact and -denuded thoracic aortic rings obtained from swimming and sedentary control rats. The responses to phenylephrine of endothelium-intact thoracic aortic rings obtained from swimming rats were lower than those of sedentary control rats at almost all concentrations ($10^{-8} - 3 \times 10^{-6}$ M) studied, with a decrease in maximum contractile responses (max.tension, swimming: 1.2 ± 0.1 g, $n = 8$, control: 2.1 ± 0.2 g, $n = 8$, $P > 0.05$). When the endothelium was removed, however, the dose-response curves of both groups of rats were shifted to the left with an increase in maximum responses which were no longer significantly different between swimming and control animals (max.tension, swimming rats: 3.2 ± 0.3 g, $n = 6$, control rats: 3.4 ± 0.4 g, $n = 5$). However, the EC_{50} values of phenylephrine in responses of the aortic rings from swimming or sedentary control rats either with or without endothelium were not significantly different (Table 1).

The effects of indomethacin on the vasoresponsiveness to phenylephrine are shown in Figure 2. Indomethacin (10^{-6} M) did not alter the dose-response curves to phenylephrine of the endothelium-intact or -denuded thoracic aortic rings obtained from either swimming (a) or sedentary control (b) rats. However, when methylene blue (10^{-5} M) was also added to the preparation of endothelium-intact thoracic aortic rings of both swimming and sedentary control rats, the dose-response curves to phenylephrine were shifted to the left with increase in

maximal responses and were no longer different from those obtained from endothelium-denuded rings of both groups of animals (Figure 3 and Table 1).

Vasoconstrictor responses to depolarizing concentrations of KCl (20–120 mM) of endothelium-intact and denuded thoracic aortic rings obtained from swimming and sedentary control rats are shown in Figure 4. There was no difference in dose-response curves to KCl of endothelium-intact thoracic aortic rings obtained from swimming and sedentary control rats. When the endothelium was removed, however, the vasoconstrictor responses to KCl of both groups of animals were significantly shifted to the left with an increase in maximum contractile responses. In addition, the contractile responses to KCl of endothelium-denuded thoracic aortic rings of swimming rats were greater than those of sedentary controls (Figure 4 and Table 1).

With differences in passive tension applied to the aortae, the active tension developed by thoracic aortic rings of both swimming and sedentary control rats to a maximum concentration of phenylephrine (10^{-6} M) were significantly different. The passive tension in the range of 1.0–2.0 g did not cause a significant difference in active tension developed compared to their corresponding groups. When the passive tension was increased up to 2.5–3.0 g, however, it did significantly increase the active tension developed compared to its corresponding group under a passive tension of 1.0–2.0 g. Nevertheless, in the case of endothelium-intact aortic rings obtained from swimming rats, the responses to phenylephrine were significantly lower than those of sedentary controls for all levels of passive tension applied. On the other hand, after removal of endothelium of the rings obtained from both swimming and sedentary control rats, the responses to phenylephrine were not different between these two groups at all levels of passive tension studied (Figure 5).

Figure 6 shows the effects of L-NOARG and passive tension (1.0 g and 3.0 g) on constrictor responses to phenylephrine of endothelium-intact aortic rings obtained from swimming and sedentary control rats. When the aortic rings were set up with a passive tension of 3.0 g, the responses to phenylephrine were lower in the rings of swimming rats than in those of sedentary controls at all concentrations studied. The presence of L-NOARG, caused a significant leftward shift of the dose-response curves and the EC_{50} values were reduced about 5 fold, with an increase in maximum responses, which were no longer significantly different. For the aortic rings with a passive tension of 1.0 g, although the response to phenylephrine of swimming rats was lower than those of sedentary control and L-NOARG caused a significant shift of the curves to the left of both swimming and sedentary control rats, the rings of swimming rats had higher maximum responses to phenylephrine than did those of sedentary control rats (Figure 6 and Table 1).

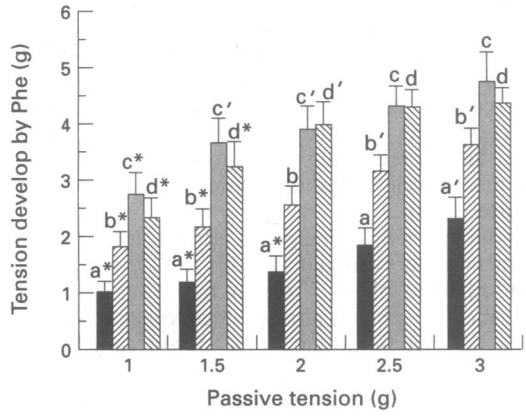

Figure 5 Effects of passive tension on constrictor responses to phenylephrine (1 μM for endothelium-intact and 0.1 μM for endothelium-denuded rings) of endothelium-intact and denuded thoracic aortic rings obtained from swimming or sedentary control rats. Each point represents the mean ± s.e.mean from 5 experiments: (■) swimming, endothelium intact; (▩) swimming, endothelium denuded; (▨) sedentary control, endothelium intact; (▨) sedentary control, endothelium denuded rings. a* Significantly different from a; b* significantly different from b; c* significantly different from c; d* significantly different from d ($P < 0.05$). Values of (a) were significantly different from those of (b) and (c) at all levels of passive tension; values of (b) were significantly different from those of (d) at all levels of passive tension ($P < 0.05$).

Discussion

The present study demonstrates that there is a decrease in reactivity to phenylephrine, an α-adrenoceptor agonist, but not to KCl, of endothelium-intact thoracic aortic rings obtained from swimming rats compared to those from sedentary control rats. The decrease in reactivity is defined as a lower maximum

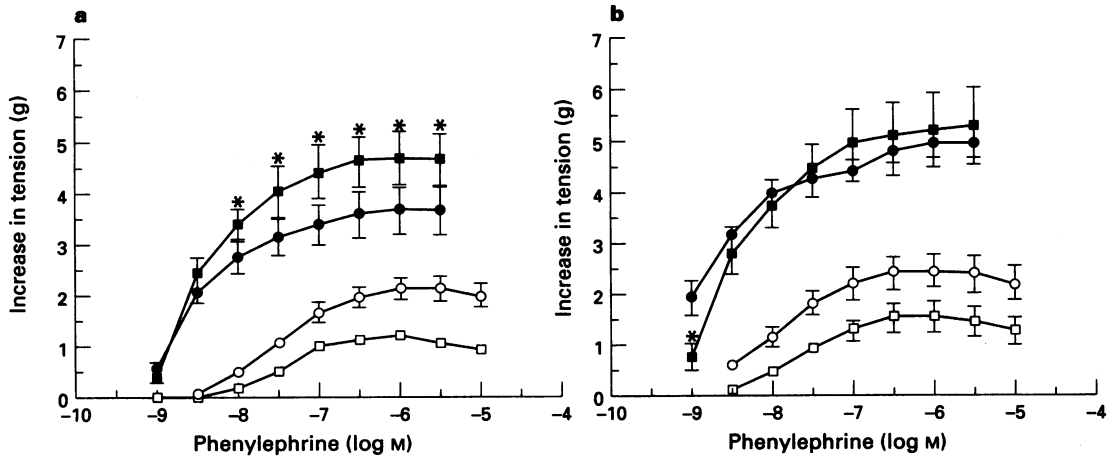

Figure 6 Effects of N^G-nitro-L-arginine (L-NOARG) on constrictor responses to phenylephrine of endothelium-intact thoracic aortic rings obtained from swimming and sedentary control rats under passive tension either of 1.0 (a) or 3.0 g (b); (□) swimming; (■) swimming in the presence of L-NOARG; (○) sedentary control; (●) sedentary control in the presence of L-NOARG. *Significantly different from sedentary control in the presence of L-NOARG, $P < 0.05$.

contractile response to phenylephrine of swimming rats than that of sedentary controls. A similar finding using noradrenaline and KCl was reported in exercise training of Yucatan miniature swine (Oltman *et al.*, 1992). In the dog, however, Rogers *et al.* (1991) demonstrated that contractile responses of the endothelium-intact epicardial coronary arteries (rings) to noradrenaline and phenylephrine were not different between exercised and sedentary control dogs. In exercise trained rats (both running and swimming) Edwards *et al.* (1985) reported no changes in contractile responses to noradrenaline of endothelium-intact thoracic helical strips between those obtained from exercise trained and sedentary control rats. Delp *et al.* (1993), using abdominal aortic rings of young adult male normotensive Sprague Dawley rats, found no difference in dose-response curves to phenylephrine between exercise training and sedentary controls, but did find a significant decrease in vascular sensitivity to noradrenaline and KCl with no difference in maximum contraction between these two groups. These inconsistent results are probably due to differences in animal species and strain and/or differences in vascular tissues and the type of tissue used. Whereas the present study used thoracic aortic rings of Wistar rats, Edwards *et al.* (1985) used helical strips of aortae. It is well known that the manipulation of aortae while cutting into helical strips destroys some of the vascular endothelium (Furchgott, 1993). Delp *et al.* (1993) however, used abdominal aortic rings from the area distal to the renal arteries and proximal to the bifurcation of the iliac arteries of Sprague Dawley rats. It is possible that different sections along the dorsal aorta have differences in vascular receptors and/or reactivity. The blunting of contractile responses to phenylephrine, but not to KCl in the present study, suggests that there are some differences during exercise between responses of the thoracic aorta mediated via the α-adrenoceptor and voltage-operated channel. KCl does not stimulate endothelial NO production whereas phenylephrine does. Thus, a possible reason for this could be an up-regulation of phenylephrine-mediated NO responses in tissue that otherwise had greater smooth muscle contractile responses as a result of exercise.

Watanabe *et al.* (1991) demonstrated that there was an increase in plasma vasodilator prostaglandin E_2 after acute exercise by swimming in rats. In addition, in the *ex vivo* model studied, Ohkubo *et al.* (1992) found an increase in vascular tissue production of prostacyclin, another vasodilator substance, after exercise training in the rat. Thus, it is possible that the decrease in sensitivity to phenylephrine of endothelium-intact thoracic aortic rings of swimming rats compared to those of sedentary control may be attenuated by increasing production of vasodilator prostaglandins from vascular tissue. In order to investigate this possibility, responses to phenylephrine were obtained in the presence of indomethacin, a cyclo-oxygenase inhibitor. Indomethacin did not alter the dose-response curves to phenylephrine in either swimming or control groups. Therefore, the possibility that changes in vascular reactivity to phenylephrine during exercise training are unlikely to be due to increased production of vasodilator prostaglandins.

The decrease in vascular responsiveness to phenylephrine of endothelium-intact thoracic aortic rings of swimming rats in the present studies are unlikely to be due to changes in tissue size or smooth muscle mass, since there was no significant difference in tissue weight of endothelium-intact aortic rings between swimming and sedentary control rats. In addition, when using different passive tensions for the thoracic aortic rings (when it would be expected that at the higher passive tensions applied, the tissues which had a bigger mass would develop the higher active tensions), the smaller responses to phenylephrine of swimming rats, compared to those of sedentary control rats, persisted, and this difference was abolished after removal of endothelium. Delp *et al.* (1993) found no differences of the wall thickness and the effects of stretch on passive tension developed of thoracic aortic rings obtained from treadmill running exercise and sedentary control rats. In their experiments, the maximum stretch of the thoracic aortic rings was about 150% above the initial unstretched outer diameter, and the passive tension developed was about 2.7 g, and this passive tension was used for their pharmacological responsiveness studies. They found no difference in tension developed to phenylephrine of the intact endothelium aortae between those obtained from treadmill running exercise and from sedentary control rats. Thus, it is possible that the passive tension of 1.0 g, which was used in most of the present study, may not be appropriate for the physiological adaptation to swimming. Therefore, in the present study dose-response curves to phenylephrine with a passive tension of 3.0 g were a similar range to those of Delp's group (1993). Results show that the response to phenylephrine of the aortic rings with endothelium-intact from swimming rats were still lower than those of sedentary control rats as was found with a passive tension of 1.0 g and these differences disappeared in the presence of L-NOARG. This finding confirms that the lower responsiveness to phenylephrine of aortic rings of swimming rats is not due to an increase in smooth muscle mass during exercise.

The possible mechanisms for the decrease in responsiveness to phenylephrine of endothelium-intact aortic rings of swimming rats in the present study may (1) involve an alteration in adrenoceptor number at the vascular smooth muscle or (2) be modulated by the vascular endothelium. Recently it has been documented that the vascular endothelium plays an important role in controlling vascular responsiveness by producing a variety of factors mediating relaxation (EDRF/NO), vascular smooth muscle cell hyperpolarization, and vasoconstriction (Vanhoutte, 1993).

To examine whether changes in adrenoceptor-mediated responsiveness at the vascular smooth muscle and/or the vascular endothelium plays a role in the lowering of vascular responsiveness to phenylephrine of endothelium-intact thoracic rings of exercise trained rats, dose-response curves to phenylephrine were also observed in rings of endothelium-denuded thoracic aortae. Also, the dose-response curves to phenylephrine of endothelium-denuded rings were obtained in the presence of indomethacin, a cyclo-oxygenase inhibitor, in order to confirm that prostaglandins did not play a part in these changes. Removal of the vascular endothelium caused a significant shift of the curves to the left with an increase in maximum responses to phenylephrine of both exercise-trained and sedentary control rats. Moreover, the two curves obtained were no longer different, and indomethacin did not alter dose-response curves to phenylephrine of any groups studied. This suggests that vascular endothelium plays an important role in reducing vasoconstrictor responses to phenylephrine of endothelium-intact thoracic aortic rings of exercise trained rats, but not by altering the α-adrenoceptor mediated response in the vascular smooth muscle during exercise training. These results are analogous to those reported by Oltman *et al.* (1992), who found a selective alteration at the vascular smooth muscle for noradrenaline and adenosine receptor-mediated responses in exercise training miniature swine, defined by a decrease in maximum contraction to phenylephrine and reduced vasodilatation to adenosine of proximal coronary artery of the exercise trained animals, whether in the presence or absence of vascular endothelium. The reason for this may be the difference in animal species and/or type of vascular tissues used. In young adult Sprague Dawley rats, however, Delp *et al.* (1993) reported that exercise training by treadmill running, diminished the vascular sensitivity (no changes in maximum contractile response) to noradrenaline but not to phenylephrine of endothelium-intact abdominal aortic rings and the reduced sensitivity was abolished by removal of the endothelium. In addition, removal of the vascular endothelium did not alter the maximum contractile responses to noradrenaline of either exercise-trained rats or sedentary controls. This led them to suggest that there is an endothelium-dependent mechanism involving α_2-adrenoceptor enhancement of the abdominal aortic rings during exercise training in the rat. The anomaly between the present study and that of Delp *et al.* may be due to differences of type of exercise, animal strain and/or section along the dorsal aorta.

However, removal of the vascular endothelial layer may remove both vasodilator and vasoconstrictor substances generated by the endothelium. Thus, the role of the endothelium in attenuation of the vasoconstrictor response to phenylephrine during exercise training in the present study may involve (1) an increase in spontaneous and/or stimulated production of EDRF/NO and then attenuation of the vasoconstriction to phenylephrine and/or (2) a decrease in the production of vasoconstrictor substances e.g. endothelin (Yanagisawa *et al.*, 1988) and then diminishing the facilitation of the phenylephrine-induced vascular response. In order to examine this possibility, the thoracic aortic rings with endothelium were used and incubated with indomethacin, a cyclo-oxygenase inhibitor, and methylene blue, a presumed inhibitor of the effect of EDRF by abolishing cyclic GMP formation elicited by the EDRF (Gruetter *et al.*, 1981). The dose-response curves to phenylephrine were performed in the presence of these inhibitors, allowing the normal generation of endothelin and other substances by the vascular endothelium. The dose-response curves to phenylephrine of the endothelium-intact tissues in the presence of indomethacin and methylene blue were shifted to the left with an increase in maximum responses to the same extent as those obtained from endothelium-denuded thoracic aortic rings of both exercise training and sedentary control rats. Furthermore, L-NOARG, a specific nitric oxide synthase inhibitor, which is able to block both basal and agonist-stimulated production of endothelium nitric oxide (Frew *et al.*, 1993), also shifted the dose-response curves of phenylephrine to the left with increase in maximum responses in both groups of rats. Beside this, in the case of the aortic rings which had a passive tension of 1.0 g, the curves to phenylephrine of those obtained from swimming rats were higher in maximum contractile responses than those of sedentary controls, but the differences were not found when the tissues had a passive tension of 3.0 g. These findings suggest that the blunting of maximum contractile response to phenylephrine of endothelium-intact rings is due to an attenuation by the increase in released EDRF/NO from vascular endothelium, and not to a decrease in the production of vasoconstrictors and/or changes in α-adrenoceptor mediated responses at the vascular smooth muscle. This possibility was also supported by Sessa *et al.* (1994) who have shown an enhanced production of EDRF/NO and nitric oxide synthase gene expression at the endothelial cell in chronically exercised dogs.

In the present study no change was found in dose-response curves to KCl of endothelium-intact thoracic aortic rings between exercised and sedentary control rats. This may be due to changes in vascular smooth muscle for voltage-operated channel-mediated responses during exercise training. The present study also tested this hypothesis, by studying the dose-response curves to KCl of endothelium-denuded rings. KCl caused an increase in both sensitivity and maximum contractile response of endothelium-denuded aortic rings of both swimming and sedentary controls. Moreover, the responses to KCl of swimming rats were higher than those of sedentary controls to all doses of KCl. This suggests that there is an increase in both sensitivity and reactivity to KCl of vascular smooth muscle during exercise in rats. Therefore, even though there is an increase in spontaneous release of EDRF/NO from vascular endothelium, the dose-response curves to KCl were restored to the same level as those of sedentary controls.

In conclusion, the present studies suggest that strenuous and long-term exercise by swimming in adult male rats causes an increase in both spontaneous and stimulated release of EDRF/NO from the vascular endothelium to attenuate the vasoconstrictor response to phenylephrine and KCl.

Some parts of this work have been presented at the International Congress of Pharmacology, 24–29 July 1994, Montreal, Canada.

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Protein kinase C in rat brain cortex and hippocampus: effect of repeated administration of fluoxetine and desipramine

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1 Recent evidence indicates that changes in the activity of cyclic AMP-dependent protein kinase may be involved in neuroadaptive mechanisms after chronic treatment with antidepressants. The aim of this study was to investigate the effect of repeated administration of fluoxetine (FL) and desipramine (DMI) on the distribution and activity of protein kinase C (PKC) in subcellular fractions of rat cortex (Cx) and hippocampus (Hc) under basal conditions and in response to a single *in vivo* administration of 5-$HT_{2A/2C}$ agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI).

2 Rats were treated for 21 days with FL (5 mg kg^{-1} day^{-1}, i.p.) or DMI (10 mg kg^{-1} day^{-1}, i.p.). DOI was injected to groups of rats receiving repeated doses of antidepressants or to control rats 1 h before *ex vivo* PKC assay. Distribution of PKC was determined by [^{3}H]-phorbol-12,13-dibutyrate ([^{3}H]-PDBu) binding and PKC activity by the Amersham enzyme assay system.

3 Autoradiography of tissue sections revealed decreased [^{3}H]-PDBu binding in CA_1 region of hippocampus (by 18%) and paraventricular thalamic nucleus (by 28%) of rats after repeated administration of FL.

4 *In vitro* exposure of brain sections to 50 μM FL resulted in significant decreases (by 23-32%) of [^{3}H]-PDBu binding in six out of seven regions examined; exposure to 100 μM FL reduced [^{3}H]-PDBu binding (by 36-52%) in all regions. In contrast, exposure of brain sections to 100 μM DMI failed to alter specific [^{3}H]-PDBu binding in brain sections.

5 The activity of PKC in subcellular fractions of Cx and Hc was significantly (by 40-50%) decreased in rats given repeated doses of FL or DMI. A single administration of either drug was without effect.

6 A single *in vivo* administration of DOI to control rats resulted in reduced PKC activity (by 30-40%) in the particulate fraction of both Cx and Hc. This response to DOI was similar in DMI-treated rats but was not seen in rats given repeated doses of FL. A single administration of DOI to animals given repeated doses of FL resulted in PKC activities higher than those seen in rats treated with FL alone.

7 The results indicate that repeated administration of FL and DMI produced similar changes in basal PKC activity but differentially affected the PKC response to the 5-$HT_{2A/2C}$ receptor agonist, DOI. The effect on basal PKC activity may result from a post-receptor action of antidepressants; the alteration of PKC response to DOI after fluoxetine could be due to receptor-mediated desensitization of the signalling system.

Keywords: Protein kinase C; fluoxetine; desipramine; rat brain; 5-$HT_{2A/2C}$ receptor; [^{3}H]-PDBu binding

Introduction

Fluoxetine is a selective 5-hydroxytryptamine(5-HT) reuptake inhibitor widely used as an effective antidepressant drug which, in contrast to tricyclic antidepressants, lacks significant affinity to various neurotransmitter receptors and does not have significant sedative, anticholinoceptor and/or cardiovascular effects (Beasley *et al.*, 1992). Most antidepressant drugs ultimately affect the 5-hydroxytryptaminergic transmission by their important effects on several processes in 5-hydroxytryptaminergic neurones, including neuronal activity, synthesis, enzymatic degradation, release and reuptake of 5-HT. Chronic treatment with some antidepressants induces homologous desensitization of 5-HT-stimulated phosphoinositide hydrolysis as well as simultaneous downregulation of 5-HT_{2A} sites (Peroutka & Snyder, 1980; Kendall & Nahorski, 1985; Conn & Sanders-Bush, 1987; Sanders-Bush *et al.*, 1989). However, the new selective 5-HT uptake inhibitors such as fluoxetine, fluvoxamine and citalopram have not been shown to alter consistently the number of 5-HT_{2A} receptors (Sanders-Bush *et al.*, 1989). Our earlier autoradiographic study (Hrdina & Vu, 1993) has shown that repeated treatment of rats with fluoxetine increases the density of 5-HT_{2A} receptors in several brain regions. Changes in 5-HT_{2A} receptor density has not always been correlated with changes in phosphoinositide turnover after chronic antidepressant treatment. For example, sertraline, a potent 5-HT uptake inhibitor, causes no down-regulation of 5-HT_{2A} receptors and yet produces a decrease in [^{3}H]-inositol phosphate formation upon stimulation with 5-HT (Sanders-Bush *et al.*, 1989).

Since receptor changes represent only the first step in the action of antidepressants, recent studies have been focused on neuronal signal transduction processes beyond the receptor level as potential targets for the action of antidepressants. Recagni *et al.* (1992) has shown that chronic treatment with desipramine and fluoxetine alters the adenosine 3':5'-cyclic monophosphate (cyclic AMP) dependent phosphorylation system in rat brain associated with the microtuble fraction and suggested that this system could represent an intracellular target involved in the biochemical mechanism of action of antidepressant drugs. Protein kinase C (PKC) is another pivotal enzyme in phosphorylation of cellular proteins and its activity has been associated with regulation of 5-HT receptor-triggered signals (e.g. 5-$HT_{2A/2C}$), neurotransmitter release and neuronal plasticity (Nishizuka, 1988; Wang & Friedman, 1990). Recently, imipramine was reported to prevent *in vitro* the inhibitory effect of the PKC activator, phorbol ester, on noradrenaline (NA)-induced accumulation of inositide phos-

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phates in brain slices (Nalepa & Vetulani, 1991). The role of PKC in the chronic effects of antidepressant drugs has not yet been studied. The aim of the present experiments was to investigate the effect of repeated administration of antidepressants on PKC localization and activity, and on PKC responses to 5-HT receptor stimulation by a 5-$HT_{2A/2C}$ receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) (Appel *et al.*, 1990) in subcellular fractions of rat brain tissue.

Methods

Treatments

Male Sprague-Dawley rats, weighing 150 to 175 g, were injected i.p. daily for 21 days with fluoxetine (5 mg kg^{-1}), desipramine (10 mg kg^{-1}) or the equivalent volume of 0.9% saline as a vehicle. Animals were killed 48 h later in the case of fluoxetine or 24 h later in the case of desipramine. The time periods between the last daily dose of drugs and death were chosen to minimize residual drug interference with the assays, and were based on the difference in plasma half-lives between the two drugs. Other groups of animals received a single dose of fluoxetine or desipramine and were used for PKC activity determination 48 h or 24 h later. In experiments in which the PKC responses to 5-$HT_{2A/2C}$ receptor stimulation *in vivo* were studied, the 5-$HT_{2A/2C}$ agonist, DOI was injected i.p. in a dose of 10 mg kg^{-1} 1 h before the *ex vivo* PKC assay. Control rats were treated with corresponding volumes (2.5 ml kg^{-1}) of saline solution.

[^{3}H]-phorbol-12,13-dibutyrate ([^{3}H]-PDBu) binding

[^{3}H]-PDBu binding in homogenates was determined as described by Horsburgh *et al.* (1991). Rats were killed by decapitation, their brains dissected out and quickly removed. Cortical or hippocampal tissue was homogenized in 10 volumes of buffer (0.32 M sucrose, 5 mM benzamidine, 2 mM dithiothreitol, 3 mM EGTA, 0.5 mM $MgSO_4$, 0.5 mM $ZnSO_4$, 0.1 mM phenylmethylsulphonylfluoride, 0.1 mg ml^{-1} leupeptin, 0.05 mg ml^{-1} pepstatin and 0.1 mg ml^{-1} aprotinin) for 10 s in a Polytron (setting 6). Homogenates were then centrifuged at 10,000 *g* and 4°C for 8 min to precipitate nuclei and cytoskeleton. The resulting supernatant was then centrifuged at 100,000 *g* at 4°C for 1 h. The resulting pellet, resuspended in the original volume of buffer, and supernatant constituted the particulate and soluble fractions of the tissue, respectively. Assay tubes (final volume of 1 ml) contained the incubation buffer (50 mM Tris-HCl, 10 mM $(CH_3COO)_2Mg.4H_2O$, 1.4 mM $CaCl_2$, 0.4 mM EGTA, 50 mM KCl, 4 mg ml^{-1} bovine serum albumin (BSA) and 100 μl ml^{-1} phosphatidylserine, pH 7.5) without or with 2 μM cold PDBu (for determination of total and non-specific binding, respectively) and 2.5 nM [^{3}H]-PDBu. Incubation (in triplicate) was started by the addition of 20 μl homogenate, continued at 4°C for 2 h, and was terminated by the addition of 5 ml ice-cold buffer (20 mM Tris-HCl, 1 mM $CaCl_2$ and 10 mM $(CH_3COO)_2Mg.H_2O$, pH 7.5), followed by subsequent filtration through Whatman GF/B filter discs (dipped in ice-cold 20 mM Tris-HCl buffer containing 0.1% polyethyleneimine) and 4 additional washes. The filters were dried and the retained radioactivity was determined by liquid scintillation spectrometry. The specific binding of [^{3}H]-PDBu accounted for 80–95% of total binding.

[^{3}H]-PDBu binding in brain sections was determined by quantitative autoradiography as described by Worley *et al.* (1986). Coronal brain sections (15 μm thick) were prepared as described earlier (Hrdina *et al.*, 1990) and were incubated for 1 h at 23°C in a buffer (50 mM Tris-HCl, 1 mM $CaCl_2$ and 100 mM NaCl; pH 7.7) containing 2.5 nM [^{3}H]-PDBu. To determine the non-specific binding, adjacent sections were incubated in the presence of 1 μM cold PDBu. Specific binding in sections accounted for 80–90% of total binding. For autoradiography, the sections were dried, apposed to tritium-sensitive Hyperfilm (Amersham, Des Plaines, IL, U.S.A.) and kept at 4°C for 4 days. Every tenth section was stained with cresyl violet to facilitate the identification of brain structures by the atlas of Paxinos & Watson (1986). Autoradiograms were quantified by use of a Microcomputer Imaging Device (Hrdina *et al.*, 1990).

Protein kinase C activity

PKC activity in subcellular tissue fractions was measured with the Amersham enzyme assay system. A PKC-specific target peptide and all necessary co-factors were provided in the kit. Soluble and particulate fractions of cortical or hippocampal tissues were prepared as described above for [^{3}H]-PDBu binding. Soluble fractions were subsequently diluted in 8 volumes and particulate fractions in 4 volumes of homogenate buffer. Assay tubes (with final incubation volumes of 75 μl) contained 25 μl of component mixture (3 mM $Ca(C_2H_3O_2)_2$, 2 mol% L-α-phosphatidyl-L-serine, 6 μg ml^{-1} phorbol 12-myristate 13-acetate, 225 μM peptide and 7.5 mM dithiothreitol in 50 mM Tris-HCl containing 0.05 volumes sodium azide, pH 7.5) and 25 μl homogenate (blank tubes representing non-specific phosphorylation contained 25 μl homogenate buffer instead). The reaction was initiated by the addition of 25 μl of Mg-ATP buffer (10 μCi ml^{-1} [^{32}P]-ATP, 150 μM ATP and 45 mM $(CH_3COO)_2Mg.4H_2O$ in 50 mM Tris-HCl containing 0.05 volumes sodium azide, pH 7.5) to each tube. Incubation proceeded for 15 min at room temperature (25°C) and was terminated by the addition of 100 μl 'stop' reagent (dilute acidic reaction-quenching reagent) to each tube. An aliquot of solution from each tube (125 μl) was blotted onto individual peptide-binding papers which were then placed in a 5% acetic acid bath (10 ml/paper) for 10 min at room temperature. This solution was then decanted and replaced with fresh 5% acetic acid for a second 10 min wash. Papers were dried and the retained radioactivity determined by liquid scintillation spectrometry. The number of pmol phosphate transferred per minute by PKC to the PKC-specific peptide substrate was calculated. The inter-assay coefficient of variation was 19% ($n=14$) for PKC determination in soluble fraction from cortex of control animals. The design of experiments allowed for parallel measurements of samples from control and treated animals.

Protein content

In order to avoid interference of reagents present in the homogenate buffer with the protein assay, a modified version of the Lowry protein assay (1951) was used. Aliquots of homogenate were made up to a volume of 950 μl with distilled water and 50 μl of absolute trichloroacetic acid was added to each tube to precipitate proteins. The tubes were then centrifuged for 5 min. The resulting supernatants were aspirated and discarded, and the resulting pellets were resuspended in 200 μl of 0.1 M NaOH. The method of Lowry *et al.* (1951) was subsequently used to determine the protein content of each tube.

Drugs used

Labelled phorbol-12,13-dibutyrate ([^{3}H]-PDBu; specific activity, 18.6 Ci $mmol^{-1}$) and [^{32}P]-ATP tetra(triethylammonium) salt (specific activity, 3000 Ci $mmol^{-1}$) were purchased from New England Nuclear (Boston, MA, U.S.A.). PDBu was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), (±)-DOI hydrochloride from Research Biochemicals Inc. (Natick, MA, U.S.A.) and the protein kinase C enzyme assay system from Amersham (Des Plaines, IL, U.S.A.). Fluoxetine hydrochloride was generously donated by Eli Lilly & Co. (Indianapolis, IN, U.S.A.) and desipramine hydrochloride by Ciba-Geigy Canada Ltd. Other chemicals used were of purest grade available.

Statistics

Data are expressed as mean ± s.e.mean. Two-way ANOVA with *post hoc* Neuman-Keuls test for group differences and Student's *t* test (two-tailed) were used to determine the statistical difference between the various means.

Results

PKC activity

The mean activity of PKC in the soluble and particulate fractions from cerebral cortex and hippocampus of control rats (Table 1) and the relative distribution of activity between the two fractions observed in our study are similar to those reported by Wieloch *et al.* (1991). Two-way ANOVA with acute DOI and repeated administration of antidepressants as the two factors showed a highly significant effect of antidepressants on PKC activity in both fractions of cortex and hippocampus ($F=14.3$ and $F=11.7$, $P<0.0001$, in soluble and particulate fractions, respectively of cortex; $F=7.15$, $P<0.003$ and $F=12.0$, $P<0.0001$ for the respective fractions from hippocampus). There was also a highly significant interaction between single DOI and repeated antidepressant administration on PKC activity in both cortical fractions ($F=18.8$ and $F=11.8$, $P<0.0001$ for soluble and particulate fraction, respectively) as well as in the hippocampus ($F=7.15$, $P<0.003$ and $F=27.3$, $P<0.0001$ for the respective fractions). Repeated administration of fluoxetine produced significant decreases in basal PKC activity in both soluble and particulate fraction of the cerebral cortex and hippocampus (Figures 1 and 2). In comparison, repeated desipramine administration resulted in significant reduction of PKC activity in both fractions from cortex but only in particulate fraction from the hippocampus. Single administration of either fluoxetine or desipramine had no significant effect on PKC activity in subcellular fractions from cortex or hippocampus (Table 1). A single injection of the 5-$HT_{2A/2C}$ agonist, DOI (10 mg kg^{-1}, i.p.) resulted in a significant reduction of cytosolic PKC activity in cortex (by 40%) but not in hippocampus. Unexpectedly, the PKC activity in particulate fractions from both cortex and hippocampus was found to be significantly decreased (by about 60%) 1 h after DOI injection (Figures 1 and 2). The PKC response to DOI challenge was similar, even more pronounced, in rats given repeated doses of desipramine and given DOI 24 h after the last injection of the antidepressant (Figures 1 and 2). However, in animals treated with repeated doses of fluoxetine, a single injection of DOI did not produce the suppression of PKC activity in soluble fraction of cortex seen with DOI alone, and the reduction of PKC activity in particulate fraction of both cortex and hippocampus was reversed (Figures 1 and 2). In fact, PKC activity in response to DOI was higher in these animals than in those receiving only fluoxetine.

Table 1 Effect of a single administration of fluoxetine and desipramine (DMI) on basal protein kinase C (PKC) activity in rat cerebral cortex and hippocampus

Region / *treatment* / *Cerebral cortex*	*PKC activity* (nmol min^{-1} mg^{-1} protein) *Soluble*	*Particulate*
Control	3.05 ± 0.17	1.06 ± 0.05
Fluoxetine	2.70 ± 0.11	0.71 ± 0.12
DMI	3.00 ± 0.49	0.92 ± 0.18
Hippocampus		
Control	3.73 ± 0.17	1.11 ± 0.10
Fluoxetine	4.05 ± 0.09	0.96 ± 0.06
DMI	3.32 ± 0.21	0.85 ± 0.01

The values are the means ± s.e.mean from four animals in each group. Fluoxetine (5 mg kg^{-1}, i.p.) and desipramine (10 mg kg^{-1}, i.p.) were given in a single dose and *ex vivo* PKC assay (in duplicate) was done 1 h later. No statistically significant differences between the groups were found by ANOVA.

[^{3}H]-PDBu binding

Repeated administration of fluoxetine failed to produce significant changes in [^{3}H]-PDBu binding in subcellular fractions from rat cerebral cortex homogenate (Table 2). The

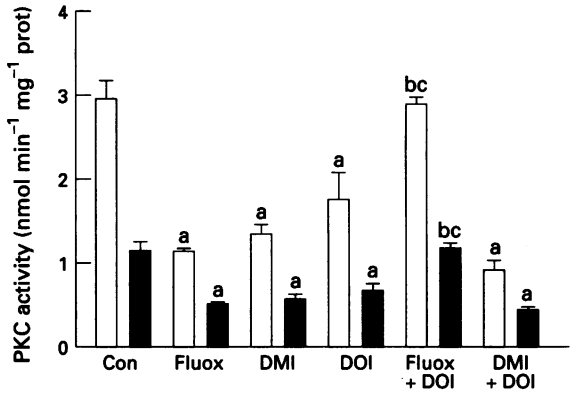

Figure 1 Effect of repeated administration of fluoxetine (Fluox; 5 mg kg^{-1}, i.p. for 21 days) and desipramine (DMI; 10 mg kg^{-1}, i.p. for 21 days) on basal protein kinase C (PKC) activity and PKC responses to a single injection of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 10 mg kg^{-1}, i.p.) in soluble (open columns) and particulate (solid columns) fractions of rat brain cortex. Each column represents the mean value ± s.e.mean from 10 animals in the control group (Con), 9 animals in the DOI group, 5 animals in the Fluox, DMI and DMIO + DOI groups and 4 animals in the Fluox + DOI group. Assays were performed in duplicate. Results were analysed by two-way ANOVA (with single DOI and repeated antidepressant drug administration as the two factors) and by *post-hoc* Neuman-Keuls test for group differences. $^aP<0.01$ vs Con; $^bP<0.01$ vs DOI; $^cP<0.01$ vs Fluox.

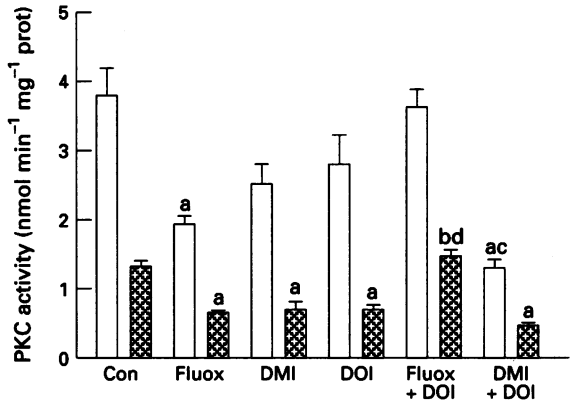

Figure 2 Effect of repeated administration of fluoxetine (Fluox; 5 mg kg^{-1}, i.p. for 21 days) and desipramine (DMI; 10 mg kg^{-1}, i.p. for 21 days) on basal protein kinase C (PKC) activity and PKC responses to a single injection of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 10 mg kg^{-1}, i.p.) in soluble (open columns) and particulate (cross-hatched columns) fractions of rat hippocampus. Each column represents the mean value + s.e.mean from 10 animals in the control group (Con), 9 animals in the DOI group, 5 animals in the Fluox, DMI and DMI + DOI groups and 4 animals in the Fluox + DOI group. Assays were performed in duplicate. Results were analysed by two-way ANOVA (with single DOI and repeated antidepressant drug administration as the two factors) and by *post-hoc* Neuman-Keuls test for group differences. $^aP<0.01$ vs Con; $^bP<0.01$ vs DOI; $^cP<0.05$ vs DOI; $^dP<0.01$ vs Fluox.

autoradiography of [^{3}H]-PDBu binding in rat brain sections has shown a differential distribution of the label among the 7 regions analyzed with highest concentration in CA_{2-3} region of hippocampus and lowest in thalamic nuclei (Table 3). In rats chronically treated with fluoxetine, the [^{3}H]-PDBu binding which reflects the amount of membrane bound enzyme (Worley *et al.*, 1986) was significantly decreased in CA_1 region of hippocampus (by 18%) and paraventricular thalamic nucleus (by 28%; Table 3). *In vitro* exposure of brain sections to 10 μM fluoxetine failed to alter significantly the distribution of [^{3}H]-PDBu binding in the regions examined. However, a higher concentration of the drug (50 μM) produced significant decreases in [^{3}H]-PDBu binding (by 23 to 32%) in all but one region and the highest concentration (100 μM) of fluoxetine markedly reduced (by 36–52%) [^{3}H]-PDBu binding in all regions examined (Table 3). In contrast, exposure of brain sections to 100 μM DMI did not alter the relative amount of specific [^{3}H]-PDBu binding to brain sections (93.7 ± 0.7% vs 94.3 ± 0.3% in controls, $n = 3$).

Discussion

One of the main findings of this study was that repeated administration of fluoxetine, a selective 5-HT uptake inhibitor significantly suppressed the basal activity of PKC in subcellular fractions from rat brain cortex and hippocampus, and significantly altered the PKC response to a 5-$HT_{2A/2C}$ agonist, DOI. The effect of fluoxetine on basal PKC activity could be a part of neuroadaptive changes after repeated administration of this antidepressant since it was not seen after a single dose of the drug. Regulation of 5-$HT_{2A/2C}$ receptor-triggered signalling is associated with PI turnover and the PKC activity (Conn & Sanders-Bush, 1987; Nishizuka, 1988). The decrease in PKC activity observed in the present study could therefore be a result of receptor-mediated downregulation of signalling mechanisms after prolonged exposure of 5-$HT_{2A/2C}$ receptors to increased concentrations of 5-HT subsequent to the inhibition of its neuronal reuptake by fluoxetine. However, the observation that chronic treatment with desipramine which does not appreciably block 5-HT uptake resulted in similar changes in PKC activity would argue against this possibility. On the other hand, desipramine has been reported to downregulate 5-HT_{2A} receptors (Peroutka & Snyder, 1980) and thus the reduction of basal PKC activity and PKC response to DOI after repeated DMI administration could be related to this effect.

The two antidepressant drugs may affect PKC activity by a direct post-receptor action. This notion is supported by our finding that both fluoxetine and desipramine showed similar effects, by a demonstrated inhibitory effect in fluoxetine on Ca^{2+}- and calmodulin-regulated protein kinase system (Silver *et al.*, 1986) and by the finding of an enhanced activity of cyclic AMP-dependent phosphorylation system associated with cerebrocortical microtubule fraction after chronic treatment with desipramine (Perez *et al.*, 1989). It is conceivable that both major phosphorylation systems (PKA- and PKC-linked) are intracellular targets involved in the biochemical mechanism of action of antidepressants drugs.

The reason for the decreases in both the cytosolic and particulate PKC activity after repeated administration of antidepressants is not immediately apparent. The possibilities include a suppression of enzyme synthesis, increased turnover with subsequent degradation, sequestration by endosomes or partial translocation to the nucleus as shown for PKA after chronic imipramine treatment (Nestler *et al.*, 1989). However, a direct effect of the residual drug on PKC cannot be excluded, particularly in the case of fluoxetine which is known to accumulate significantly in cerebral cortex and hippocampus after repeated administration (Caccia *et al.*, 1992). In fact, as shown by these authors, hippocampal fluoxetine levels measured in rats 24 h after 21 day administration at 15 mg kg^{-1} twice daily were 91 nmol g^{-1} and one week later the active metabolite, norfluoxetine was still present at 109 nmol g^{-1}. It is therefore likely that in our experiments fluoxetine was still present in brain in significant concentrations 48 h after the last dose. Due to its long elimination half-life (which is even longer for its

Table 2 Effect of repeated fluoxetine and desipramine (DMI) administration on [^{3}H]-phorbol-12, 13-dibutyrate ([^{3}H]-PDBu) binding in subcellular fractions from rat cortex homogenate

	[^{3}H]-PDBu binding (pmol mg^{-1} protein)	
Treatment	*Soluble*	*Particulate*
Control	57.7 ± 1.3	13.6 ± 1.5
Fluoxetine	58.6 ± 1.2	11.6 ± 0.4
DMI	60.4 ± 1.2	11.6 ± 1.4

Fluoxetine (5 mg kg^{-1}) and desipramine (10 mg kg^{-1}) were given i.p. for 21 days and [^{3}H]-PDBu binding (in triplicate, at 2.5 nM concentration) was determined 48 h and 24 h, respectively after the last dose in subcellular fractions of homogenate from cerebral cortex. $n = 4$ for each group. No significant difference between the groups was found by ANOVA.

Table 3 [^{3}H]-phorbol-12, 13-dibutyrate ([^{3}H]-PDBu) binding in rat brain sections from control and fluoxetine-treated rats and after *in vitro* exposure to fluoxetine

	[^{3}H]-PDBu binding (pmol mg^{-1} protein)				
		Chronic	In vitro *Fluoxetine*		
Region	*Control*	*fluoxetine*	10 μM	50 μM	100 μM
Cortex					
Layer 1-3	17.1 ± 1.6	14.0 ± 0.6	21.3 ± 0.8	13.1 ± 0.5*	7.7 ± 0.7**
Hippocampus					
CA1	19.0 ± 1.1	15.7 ± 0.8*	19.9 ± 1.5	16.7 ± 1.7	12.2 ± 1.5**
CA2-3	22.2 ± 1.4	18.9 ± 0.8	21.6 ± 1.0	15.7 ± 1.1**	11.4 ± 1.2**
Dentate gyrus	14.1 ± 1.4	11.0 ± 0.7	17.4 ± 0.9	10.7 ± 0.8*	6.8 ± 0.5**
PV med.thalamic n.	10.3 ± 0.9	7.4 ± 0.5*	11.3 ± 0.4	7.4 ± 1.0*	5.9 ± 0.7**
Caudate-putamen	16.6 ± 1.6	13.6 ± 0.8	19.7 ± 1.0	11.4 ± 1.2**	8.6 ± 0.8**
Amygdala	18.3 ± 2.1	14.7 ± 0.6	19.4 ± 1.5	12.5 ± 1.9*	9.2 ± 1.1**

Rats were treated with fluoxetine (5 mg kg^{-1}, i.p. for 21 days) or saline (controls). Binding sites were labelled by incubating adjacent sections with 2.5 nM [^{3}H]-PDBu. Values are means ± s.e.mean of measurements from 4 rats in each group with three bilateral determinations made from each of 2–3 sections for each area in each brain. For determination of *in vitro* effect of fluoxetine, the drug was added in 10, 50 or 100 μM concentration to 2–3 adjacent sections from 4 control brains 15 min before exposure to [^{3}H]-PDBu. The anatomical terminology is derived from Paxinos & Watson (1986). *$P < 0.05$; **$P < 0.01$ when values were compared to corresponding values in control rats (Student's t test, two tailed).

metabolite, norfluoxetine) and high (94%) protein binding (Beasley *et al.*, 1992), a significant amount of drug could persist in tissue despite repeated washing. The above evidence and our finding of reduced [^{3}H]-PDBu binding after *in vitro* exposure of tissue sections to fluoxetine (similar in pattern to that seen after repeated administration of the drug) would indicate that residual drug present in the tissue after repeated treatment could have been responsible for the changes in PKC.

In vitro exposure of rat cortical slices to DOI was shown to translocate PKC activity from the cytosolic to membrane fraction (Wang & Friedman, 1990). This effect appears to be due to a selective 5-HT_{2A} receptor stimulation since it is prevented by 5-HT_{2A} receptor antagonists. We found that *in vivo* administration of DOI produced significant decrease of PKC activity in particulate fraction of cortical and hippocampal tissue. We have assayed PKC activity 1 h after DOI injection based on the time course of behavioural effects of DOI (Berendsen & Boekkamp, 1991) and on the fact that maximal translocation of PKC activity *in vitro* was only seen after 20 min of exposure to DOI (Wang & Friedman, 1990). We cannot exclude the possibility that an initial increase in particulate PKC activity after DOI injection did occur but might have terminated due to increased turnover rate and subsequent degradation of the activated enzyme, while the decrease in cytosolic PKC activity persisted. The decrease in membrane-bound PKC activity 1 h after DOI injection was not seen in rats chronically treated with fluoxetine. This might have been due to receptor-mediated desensitization of the signalling system involving PKC. Chronic block of 5-HT uptake by fluoxetine and prolonged availability of the transmitter for action at receptors could have resulted in decreased sensitivity to acute challenge such as DOI injection. Indeed, a substantial down-regulation (by 60–75%) of 5-HT_{2A} receptor number in rat cortex was observed after chronic treatment with the 5-$HT_{2A/2C}$ receptor agonist, DOI (Pranzatelli, 1991). The above suggestion is supported by our observation that no significant alteration of PKC response to DOI challenge was found in rats repeatedly treated with desipramine, a tricyclic antidepressant that does not inhibit 5-HT uptake to a significant degree.

Another possible explanation for the differential effect of fluoxetine and DMI on PKC responses to DOI administration is that fluoxetine may directly interfere with pharmacokinetics of DOI and/or its action at the receptor level. Fluoxetine is a potent inhibitor of cytochrome CPY2D6, a major enzyme catalysing the metabolism of several important drugs including tricyclic antidepressants (Brosen & Skjelbo, 1991). It is however, not known whether fluoxetine influences the disposition of DOI after *in vivo* administration. Furthermore, fluoxetine was found to have an appreciable affinity (pK_i of 6.57) for 5-HT_{2C} receptors in bovine choroid plexus (Wang *et al.*, 1991). It could thus compete with DOI at 5-HT_{2C} receptors and influence the activity of the PI-PKC signalling system in response to DOI, and also modify the basal activity of this system. However, it is not known whether fluoxetine has the same affinity for 5-HT_{2C} receptors in rat brain tissue as for those in bovine choroid plexus.

In conclusion, antidepressant drugs of different pharmacological profile, fluoxetin and desipramine, produce after repeated administration significant effects on PKC activity in subcellular fractions of cortical and hippocampal tissue and differentially affect PKC responses to 5-HT_{2A} receptor stimulation by *in vivo* DOI injection. This novel and possibly post-receptor action of these drugs may be part of an adaptive neuronal changes seen after repeated administration of antidepressants.

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Reduction of inflammation and pyrexia in the rat by oral administration of SDZ 224-015, an inhibitor of the interleukin-1β converting enzyme

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1 The aim of this study was to determine whether a synthetic inhibitor of the interleukin-1β converting enzyme (ICE) displays oral activity in models of inflammation.

2 To this end, the ICE inhibitor, SDZ 224-015, was examined in rat paw oedema, pyrexia and nociception tests.

3 SDZ 224-015 (0.3-300 $\mu g\ kg^{-1}$) potently reduced carrageenin-induced paw oedema, with an oral ED_{50} of approximately 25 $\mu g\ kg^{-1}$. This effect was independent of endogenous glucocorticoid, as shown by retention of activity upon adrenalectomy.

4 Pyrexia induced by lipopolysaccharide (0.1 mg kg^{-1} s.c.) or by interleukin-1β (100 ng i.v.) was also reduced, over a similar dose-range to oedema (oral ED_{50}s 11 $\mu g\ kg^{-1}$ and 4 $\mu g\ kg^{-1}$ respectively).

5 SDZ 224-015 (0.2-5 mg kg^{-1}, p.o.) displayed analgesic activity in the Randall-Selitto yeast-inflamed paw pressure test, significant at a dose of 1 mg kg^{-1}, p.o.

6 Thus, SDZ 224-015 has potent oral activity in several acute models for inflammation, suggesting that ICE inhibitors may constitute a novel type of anti-inflammatory agent.

Keywords: Fever; inflammation; interleukin-1; interleukin-1 converting enzyme; oedema

Introduction

Interleukin-1 (IL-1) appears to be a central mediator of the pathogenesis of acute and chronic inflammation (reviewed by Dinarello, 1991; 1993). Administration of the cytokine has been demonstrated to produce cardinal signs of inflammation such as leukocyte influx, pain and tissue breakdown (Ferreira *et al.*, 1988; Henderson & Pettipher, 1988; Wankowicz *et al.*, 1988). Furthermore, elevated levels can be demonstrated in inflammatory effusions (Bochner *et al.*, 1990; Sim *et al.*, 1994). The suitability of IL-1 as a target for anti-inflammatory therapy has been confirmed by the efficacy in a variety of animal models of the naturally-occurring IL-1 receptor antagonist (IL-1ra), soluble IL-1 receptors or with neutralising antibodies to IL-1. (Jacobs *et al.*, 1991; Thompson *et al.*, 1992; van den Berg *et al.*, 1994).

Of the two IL-1 proteins that exist, α and β, it is the latter that is predominantly released from activated human monocytes (Bailly *et al.*, 1994). IL-1β is synthesized as a cytosolic inactive precursor of 33K, being released only upon cleavage to the mature 17.5K form. The protease (termed interleukin-1β converting enzyme, ICE) responsible for this processing has recently been cloned (Thronberry *et al.*, 1992) and the crystal structure elucidated (Walker *et al.*, 1994; Wilson *et al.*, 1994). ICE is a homodimeric (consisting of two heterodimer subunits p10 and p20) cysteine protease with unique substrate specificity, cleaving proIL-1β between Asp 116 and Ala 117. Since IL-1β release is dependent upon the action of ICE (Young *et al.*, 1988), it follows that inhibition of this enzyme provides a rational therapeutic target for the development of anti-inflammatory agents. Indeed, an example from nature already exists in that cowpox virus produces an inhibitor of ICE, the *crmA* protein, as a means of blocking the inflammatory response; mutants lacking this protein are less pathogenic (Marrack & Kappler, 1994).

Based on the work of Smith *et al.* (1988) who produced acyloxymethyl ketones as selective inactivators of cathepsin B, we have designed irreversible substrate-based inhibitors of ICE. Here we demonstrate specific inhibition of IL-1β *in vitro* and use models of acute inflammation, pyrexia and nociception, in order to show for the first time *in vivo* activity of a synthetic inhibitor of ICE, SDZ 224-015.

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Methods

Animals

For all studies, male Sprague-Dawley rats weighing 140-170 g were used, being supplied by Interfauna AG, Tuttlingen, Germany. They were fed *ad libitum* with a standard diet (Nafag Ecosan AG, Gossau, Switzerland) and allowed at least 4 days acclimatisation before an experiment.

Release of cytokines from THP-1 cells

THP-1 cells 500,000 were cultured for 3 h in 1 ml RPMI with 5% heat-inactivated foetal bovine serum containing 100 u interferon gamma, in 24-well multiwell plates. The acid of SDZ 224-015 (or vehicle) was then added to duplicate wells to achieve the required final concentration, together with 5 μg lipopolysaccharide. After a further 40 h, IL-1β, interleukin-6 (IL-6) or tumour necrosis factor-α (TNF-α) in the cell-free media were determined by ELISA and the amount measured related to total DNA. The latter was determined by the fluorometric method of Kapuscinski & Skoczylas (1977). Lactate dehydrogenase (LDH) was determined in the same media (without freezing which destroys LDH) as described by Schnyder *et al.* (1990).

Carrageenin-induced paw oedema

Rats were divided into groups of 4 and dosed orally by gavage either with the vehicle (Tween 80/1% BSA in 10 mM phosphate buffer pH 6), diclofenac as reference substance, or with various amounts of SDZ 224-015. A zero reading of right paw volume was then taken with an antiphlogmeter according to

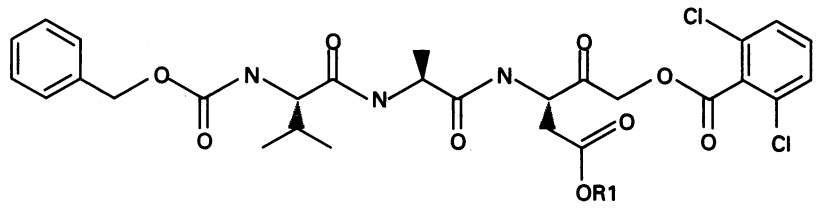

Figure 1 Structure of SDZ 224-015. R1 = Et = SDZ 224-015. R1 = H = active principle.

Kemper & Ameln (1959). This is an electrical method for measuring volumes whereby the paw of the conscious rat is inserted into a tube where a constant high frequency electric field exists; the presence of the paw alters the frequency of the field in proportion to its volume. One hour later, 0.1 ml of a w/v 1% suspension of carrageenin in saline at 37°C was given (without anaesthesia) by subplantar injection into the right paw. Animals were returned to the cage and 3 h later paw oedema measured with the antiphlogmeter. After subtraction of the zero values, the means of the compound-treated groups were compared to the vehicle-treated group to give the percentage inhibition of paw swelling.

Adrenalectomy

Under isoflurane narcosis, a 1 cm lateral incision was made in the dorsal flank and the adrenals exposed. For sham-operated rats the incision was then merely closed, whilst for actual adrenalectomy both adrenals were completely removed with eye forceps. Immediately following surgery all animals received 1 mg 11-deoxycorticosterone acetate i.m. and thereafter were maintained with drinking water containing 0.9% NaCl. Five days after adrenalectomy the standard carrageenin-induced paw oedema test was carried-out as described above.

Lipopolysaccharide (LPS)-induced pyrexia

Rats were placed in single cages and fasted overnight and then divided into groups of three. LPS (0.1 mg kg^{-1}) was administered s.c. and 2 h later, after the initial hypothermic response had occurred, the baseline temperature was measured with a rectal thermocouple probe. Four hours after LPS, compound (or vehicle alone) was given by oral gavage and at 6 h the end temperature recorded. This dose of LPS produces a temperature rise of 2–2.5°C. The difference between the basal and final temperature was calculated and the mean values of the compound-treated groups compared to that of the vehicle-treated in order to determine the percentage inhibition of fever. For pyrexia experiments DuP 697, an inhibitor of prostaglandin E_2 synthesis (Gans *et al.*, 1990), was used as reference compound.

IL-1β-induced pyrexia

Rats were placed in individual cages and fasted overnight and then divided into groups of four. Basal rectal temperature was measured and animals immediately dosed orally with compound (or vehicle alone); 30 min later 100 ng recombinant human IL-1β was administered i.v. via the tail vein. Four hours later the fever was determined (this amount of cytokine induces a temperature rise of 1.5–2°C) and the difference between the basal and final values calculated. Mean values of the compound-treated groups were compared to that of the vehicle-treated to give percentage inhibition of fever.

Inflamed paw pressure test

This was performed according to the method of Randall & Selitto (1957). Rats were divided into groups of 5 and 0.1 ml of a 20% w/v solution of baker's yeast in water at 37°C given by subplantar injection into the hind paw; 2 h later compound (or vehicle alone) was administered by oral gavage. After a further hour, nociception was measured by applying an increasing weight upon the paw with a pressure meter (Ugo Basile, Varese, Italy) until vocalization. The weight applied to each rat was recorded and comparison made between values for the compound-treated and vehicle-treated rats. Diclofenac was used as reference compound.

Statistical analysis

Comparison between vehicle-treated and compound-treated groups was carried-out by analysis of variance and unpaired *t* test, or Dunnett's multiple comparison test (parametric data) or the Mann-Whitney (nonparametric) test.

Materials

THP-1 cells (human monocytic leukaemia) were from the European Collection of Animal Cell Cultures (Porton Down, UK). Bovine serum albumin (fraction V), lipopolysaccharide (*Salmonella abortus equi*) and Tween 80 were obtained from Sigma (St. Louis, MO, U.S.A.). Human recombinant interferon γ was from Boehringer Mannheim (Mannheim, Germany). ELISA kit for human IL-1β was purchased from Cayman chemicals (Paris, France), those for IL-6 and TNF-α were from Innogenetics (Zwijndrecht, Belgium). Carrageenin (satiagum standard) was from Sugro AG (Basel, Switzerland). Baker's yeast was from Fleischmann (Bern, Switzerland). Isoflurane 'Forene' was purchased from Abbot (Cham, Switzerland). 11-Deoxy-corticosterone acetate was from Fluka (Buchs, Switzerland). Corticosterone RIA kit was from ICN Biochemicals (Costa Mesa, CA, U.S.A.). Recombinant human interleukin-1β was supplied by Dr P. Ramage, Biotechnology Department of Sandoz Ltd. (Basel, Switzerland). SDZ 224-015 (Z-valyl-alanyl-3(S)-3-amino-4-oxo-5-(2,6-dichlorobenzoyl-oxypentanoic acid) ethyl ester) was ground to a particle size of 99% < 88 μm diameter (Milling Department, Sandoz, Basel, Switzerland). The structure of SDZ 224-015 and its acid is shown in Figure 1.

Results

The free acid which is the active principle of SDZ 224-015, dose-dependently inhibited the release of IL-1β from the human monocytic cell line THP-1, with an IC_{50} of 0.24 μM (Figure 2a), without affecting release of IL-6, TNFα or lactate dehydrogenase (Figure 2b-d, respectively). This demonstrates that the action of the compound is specific for IL-1β and also that it is not due to cellular toxicity.

SDZ 224-015 was then tested in carrageenin-induced paw oedema, a model for acute inflammation. The results of a series of dose-response experiments are presented in Table 1. It can be seen that the compound is very potent, with significant reduction in paw swelling always apparent with an oral dose of 3 μg kg^{-1}. When these data are combined, a 50% inhibition of total swelling is obtained with a dose of 25 μg kg^{-1} p.o. In-

terestingly, the effect of SDZ 224-105 reaches a plateau at around 60% inhibition, with increasing doses failing to reduce swelling further. Thus, a component of carrageenin-oedema exists that is refractory to this compound class.

Since glucocorticoids potently reduce inflammation, it was important to exclude the possibility that SDZ 224-015 might act indirectly, via induction of endogenous corticosteroid production. Therefore, the carrageenin oedema test was performed on adrenalectomized rats. As shown in Table 2, adrenalectomy did not influence the action of the compound, indicating that its effect was independent of the adreno-pituitary axis. This was subsequently confirmed by radioimmunoassay of circulating corticosterone in intact rats ($n=4$) that had been dosed 2 h earlier with SDZ 224-015 10 mg kg^{-1}, p.o. These animals had a mean (s.e.mean) plasma corticosterone of 25.4 (8.0) ng ml^{-1} compared to 15.1 (1.6) ng ml^{-1} for those dosed with vehicle (values below 50 ng ml^{-1} being normal).

SDZ 224-015 was also examined in LPS-pyrexia, since IL-1 is an endogenous pyrogen induced by LPS. The dose-response curve is presented in Figure 3, the ED_{50} being 11 $\mu g\ kg^{-1}$. Table 3 shows that SDZ 224-015 also reduced pyrexia fol-

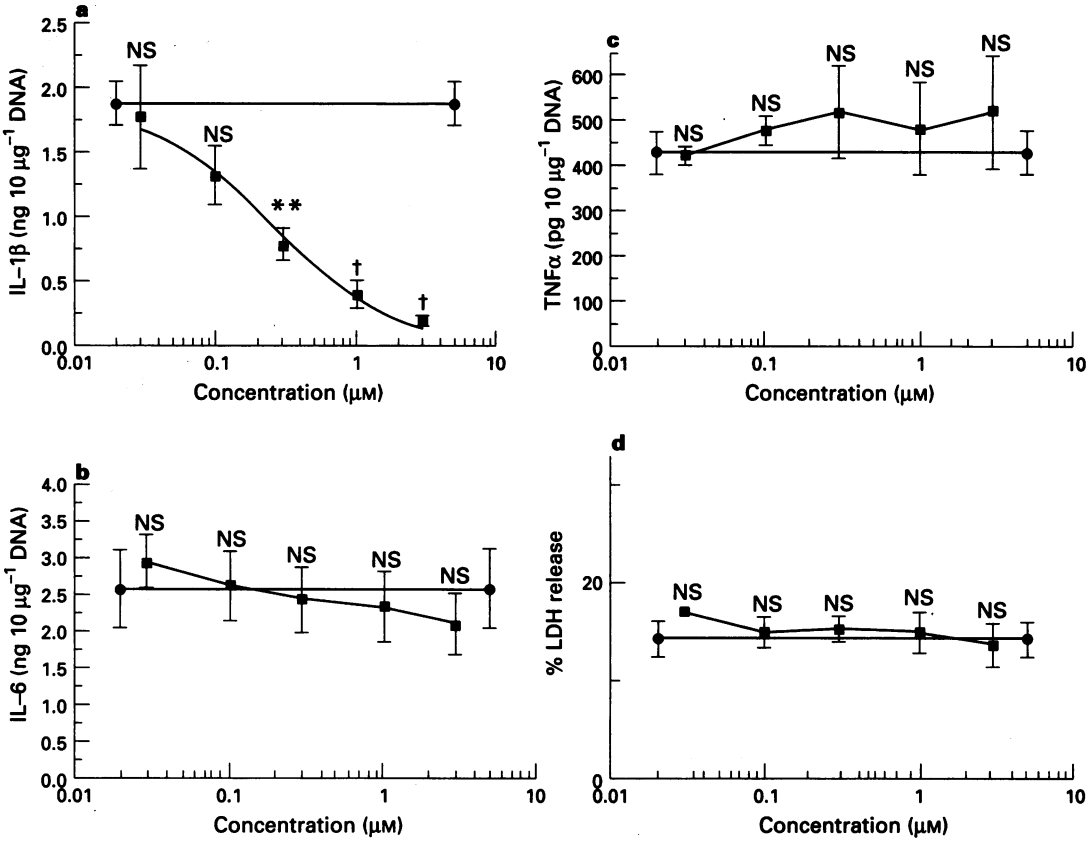

Figure 2 Dose-response for the inhibition of cytokine release from THP-1 cells by the free acid of SDZ 224-015. Following a 43 h culture in the presence of various concentrations of compound (or vehicle), together with interferon γ and LPS as stimulant, cytokines in the media were measured by specific ELISA. The compound inhibited IL-1β release with an IC_{50} of 0.24 μM (a) without causing a change in levels of interleukin 6 (IL-6) (b) and tumour necrosis factor α (TNF-α) (c) or of lactate dehydrogenase (LDH) (d) in the media. Results are the mean ± s.e.mean of 3 experiments. (●) = control in (a), (b), (c) and (d). NS = not significant; $^{**}P<0.01$; $\dagger P<0.001$.

Table 1 Effect of SDZ 224-015 in carrageenin paw oedema

Dose of SDZ 224-015 ($\mu g\ kg^{-1}$ p.o.)	*% inhibition of paw swelling compared to vehicle-treated group*			
	Expt. 1	*Expt. 2*	*Expt. 3*	*Expt. 4*
0.3	33 (2)**	22 (2)*	36 (6)**	30 (5)NS
3	50 (6)**	30 (6)*	38 (3)†	43 (2)*
30	59 (3)†	42 (2)**	59 (7)†	56 (5)*
300	59 (4)†	52 (6)**	59 (2)†	63 (3)**
3000	61 (4)†	ND	ND	ND
Diclofenac 3 mg kg^{-1} p.o.	73 (3)†	64 (7)**	73 (4)†	70 (4)**

Rats were dosed by gavage with SDZ 224-015, or diclofenac as reference and 1 h later paw inflammation induced by injection of carrageenin (see methods); 3 h thereafter paw swelling was measured and compared to that of the vehicle-treated group to ascertain the effect of the substance. The table shows means (s.e.mean) of 4 experiments; ND = not done; NS = not significant; $^{*}P<0.05$; $^{**}P<0.01$; $\dagger P<0.001$.

lowing intravenous administration of IL-1β itself, with ED_{50} of 4 $\mu g\ kg^{-1}$. In this case, it may be that the ICE inhibitor acts by blocking an amplification cascade, since IL-1β is known to stimulate its own production in some *in vitro* systems.

In further studies we tested whether the compound possessed antinociceptive activity, using a model for inflammatory pain, the Randall-Selitto inflamed paw pressure test. As shown in Figure 4, SDZ 224-015 significantly increased the paw pressure threshold required for a nociception response, indicating a probable analgesic action. However, the dose required to produce a significant effect was several orders of magnitude greater than that for oedema, indicating that the mechanism is probably distinct from the reduction of paw swelling induced by lower doses of SDZ 224-015.

Table 2 Effect of adrenalectomy upon the inhibition of carrageenin paw oedema by SDZ 224-015

	% inhibition of paw swelling compared to vehicle-treated control group	
	Sham-operated	*Adrenalectomized*
SDZ 224-015 (50 $\mu g\ kg^{-1}$ p.o.)	48 (4)†	48 (4)†
Diclofenac (3 $mg\ kg^{-1}$ p.o.)	62 (4)†	64 (5)†

Rats were adrenalectomized or merely sham-operated as described in methods; 5 days later the standard carrageenin paw oedema test was performed. Results are means (s.e.mean) using 5 rats per group; 2 such experiments were performed with the same outcome.
†$P<0.001$.

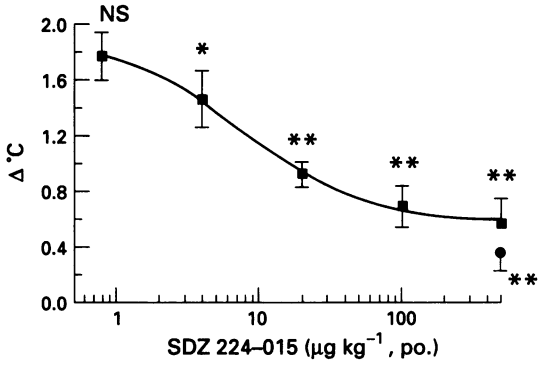

Figure 3 Dose-response to SDZ 224-015 in LPS-pyrexia in the rat. Animals were injected s.c. with 0.1 $mg\ kg^{-1}$ LPS and 2 h later the basal temperature measured. After a further 2 h, substance was administered orally. Six hours after LPS, the final temperature was recorded and values compared to that of the vehicle-treated group. The results are the mean and s.e.mean of 3 experiments. (■) SDZ 224-015; (●) Du697. NS = not significant; *$P<0.05$; **$P<0.01$.

Table 3 Effect of SDZ 224-015 upon pyrexia induced by interleukin-1β (IL-1β)

Dose of SDZ 224-015 ($\mu g\ kg^{-1}$ p.o.)	*% inhibition of fever*
0.1	15 (11) NS
1	46 (14)*
10	54 (15)*
100	77 (13)**
DuP 697 0.5 $mg\ kg^{-1}$ p.o.	62 (10)**

Basal temperature was determined and rats dosed orally with compound or vehicle in groups of 4. 30 minutes later fever was induced by intravenous administration of 100ng IL-1 beta. 4 hours after IL-1 beta, final temperature was recorded and compared to the vehicle treated group. Results are means (s.e.means) from 1 of 3 experiments producing similar results.
NS = not significant; *$P<0.05$; **$P<0.01$.

Discussion

In this study it has been shown that a synthetic peptide inhibitor of ICE reduces parameters of inflammation, consistent with an inhibition of IL-1β production. Although a peptide aldehyde ICE inhibitor has also recently been reported to inhibit specifically IL-1β release from human peripheral blood monocytes *in vitro* (Miller *et al.*, 1993), this to our knowledge is the first report demonstrating therapeutic efficacy of an ICE inhibitor *in vivo*. Although such peptide ICE inhibitors also inhibit cathepsin B, they demonstrate a marked preference for ICE, the acid of SDZ 224-015 displaying 180-fold selectivity for the latter (Dolle *et al.*, 1994). SDZ 224-015 inhibits isolated cathepsin B with an IC_{50} of 0.2 μM; however, other compounds with much lower potency for the latter are also effective in the oedema and pyrexia models. For instance, exchanging the dichlorobenzoic acid moiety for diphenyl acetate removes most activity against cathepsin B (10 μM producing only 7% inhibition), whereas inhibition of IL-1 release from THP-1 cells (IC_{50} 0.4 μM) and of oedema (ED_{50} 20 $\mu g\ kg^{-1}$, p.o.) is retained. The potency of SDZ 224-015 is remarkable, with oral ED_{50}s for inhibition of oedema and fever lower than that of the powerful cyclo-oxygenase inhibitor, diclofenac (which in our laboratory has an oral ED_{50} of 100 $\mu g\ kg^{-1}$ in both tests). ICE is thought to be derived from an autocatalytic precursor, being cleaved at sites with Asp in the P1 position, to form the active subunits p10 and p20 (Wilson *et al.*, 1994). Thus, the remarkable potency of SDZ 224-015 could be due to an amplifying action, through the ability not only to inhibit conversion

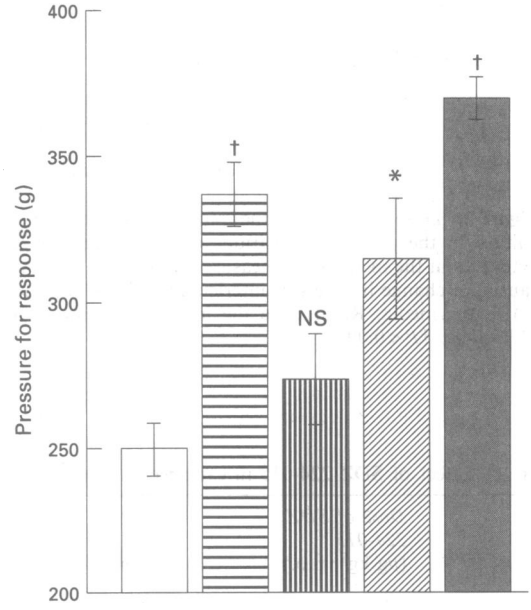

Figure 4 Effect of SDZ 224-015 in the inflammed paw pressure test for antinociception. Two hours after subplantar injection of yeast, compound was given orally and 1 h thereafter the paw pressure required to induce vocalization determined. Data are the mean ± s.e.mean, of 5 rats and are taken from 1 of 2 experiments, the results of which were very similar. NS = not significant; *$P<0.05$; †$P<0.001$. Open column, vehicle; vertically lined column, diclofenac (3 $mg\ kg^{-1}$, p.o.); horizontally lined column, SDZ 224-015 (0.2 $mg\ kg^{-1}$, p.o.); hatched column, SDZ 224-015 (1 $mg\ kg^{-1}$, p.o.); stippled column, SDZ 224-015 (5 $mg\ kg^{-1}$, p.o.).

of proIL-1β but also that of ICE itself, a property of ICE inhibitors also alluded to by others (Ayala *et al.*, 1994).

Part of the paw swelling in the carrageenin oedema model is refractory to SDZ 224-015, dose-response curves reproducibly displaying a plateau of around 60%. It is well established that a variety of mediators are involved in oedema formation (Henson & Murphy, 1989) and therefore it is perhaps not surprising that blockade of a single cytokine does not yield complete inhibition. This might have implications for the efficacy of ICE inhibitors in inflammatory disorders, where elimination only of IL-1β may be counteracted by continued presence of other inflammatory mediators. On the other hand, IL-1 appears to be a crucial player, triggering a cascade of other cytokines, mediators, proteases etc. (Dinarello, 1991); this is corroborated by the efficacy of the soluble IL-1 receptor, receptor antagonist or neutralising antibodies in experimental inflammation models.

Our results extend previous studies using the IL-1 receptor antagonist, which has been reported to be effective in carrageenin-induced pleurisy (Meyers *et al.*, 1993) and IL-1β induced pyrexia (Coceani *et al.*, 1992). In the latter regard, the effectiveness of SDZ 224-015 in IL-1β pyrexia may be related to inhibition of autocrine IL-1β release induced by the cytokine (Sakai *et al.*, 1987).

Endogenous prostaglandins and tachykinins appear to mediate some effects of IL-1, such as hyperalgesic action (Ferreira *et al.*, 1988; Perretti *et al.*, 1993). This may explain the analgesic action of SDZ 224-015, although it is unclear why it appears only at significantly higher doses than those in oedema or pyrexia unless the IL-1 involved in the induction of hyperalgesia is produced in the CNS in which case rather poor penetration of the blood-brain barrier might explain the weaker potency.

Chronic inflammatory disease, such as arthritis, is probably the most obvious disorder where IL-1β blockade could be of benefit. However, it is possible to speculate that therapeutic applications of an ICE inhibitor may be far broader. For example, treatment with the IL-1 receptor antagonist reduces osteoporotic bone loss (Kimble *et al.*, 1994) and ischaemic neuronal damage in rats (Relton & Rothwell, 1992). The viral ICE inhibitor, *crmA*, protects transfected ganglion neurones from apoptosis induced by nerve growth factor depletion (Gagliardini *et al.*, 1994); thus programmed cell death observed in degenerative neuronal diseases may be influenced by inhibitors of ICE.

In conclusion, it has been shown that the synthetic ICE inhibitor SDZ 224-015 potently reduces both acute inflammation and pyrexia. Although it has not actually been proven that this is due to inhibition of IL-1β release, specific blockade of IL-1β by the compound *in vitro*, and the known efficacy of proteinaceous IL-1 inhibitors in inflammatory models, makes this interpretation plausible. ICE inhibitors may constitute a novel type of therapeutic agent, the possible applications of which may well extend beyond inflammatory disease.

Note added in proof

Since submission of the paper, it has been confirmed by Ramage *et al.* (*J. Biol. Chem.*, **270**, 9378–9383) that processing of precursor ICE is autocatalytic and that autocatalysis is inhibited by the free acid of SDZ 224-015.

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The involvement of ATP-sensitive potassium channels in β-adrenoceptor-mediated vasorelaxation in the rat isolated mesenteric arterial bed

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1 We have used the isolated buffer-perfused superior mesenteric arterial bed of the rat to assess the involvement of ATP-sensitive potassium (K_{ATP}) channels in the vasorelaxant responses to β-adrenoceptor agonists.

2 The vasorelaxant potencies of the non-selective β-adrenoceptor agonist, isoprenaline, the β_1-adrenoceptor agonist, dobutamine and the β_2-adrenoceptor agonist, terbutaline were all significantly ($P<0.05$) reduced (isoprenaline, $ED_{50}=265\pm31$ pmol v. 1.05 ± 0.42 nmol; dobutamine, $ED_{50}=294\pm67$ pmol v. 497 ± 115 pmol; terbutaline, $ED_{50}=157\pm26$ nmol v. 452 ± 120 nmol) in the presence of the K_{ATP}-channel blocker, glibenclamide.

3 The presence of glibenclamide only weakly influenced the vasorelaxant properties of salbutamol, a β_2-adrenoceptor agonist, while those of verapamil, a β-adrenoceptor-independent vasorelaxant, were unaffected.

4 In radioligand binding experiments, glibenclamide (1 nM-100 μM) did not displace any specific [^{3}H]-dihydroalprenolol binding from rat β-adrenoceptors. Therefore, glibenclamide does not bind to β-adrenoceptors at the concentration used in the present investigation.

5 Vasorelaxant responses to dibutyryl cyclic AMP, the cell permeable analogue of cyclic AMP, were also unaffected by glibenclamide, indicating that the coupling of β-adrenoceptors to K_{ATP}-channels occurs independently of the elevation of intracellular cyclic AMP.

6 We have shown that a significant element of the vasorelaxant responses to both β_1- and β_2-adrenoceptor activation involves the opening of K_{ATP}-channels. In conclusion, K_{ATP}-channels may play a physiological role in β-adrenoceptor-mediated vasodilatation.

Keywords: ATP-sensitive potassium channels (K_{ATP}-channels); glibenclamide; mesenteric arterial bed; β-adrenoceptors; isoprenaline; salbutamol; dobutamine; terbutaline; dibutyryl cyclic AMP

Introduction

The movement of potassium ions across the cytoplasmic membrane is an important determinant of membrane potential and consequently, cellular activity in excitable tissue such as vascular smooth muscle. Recently, interest has focused on ATP-sensitive potassium channels (K_{ATP}-channels) which are regulated by purine derivatives associated with cellular metabolism (Nichols & Lederer, 1991). Pharmacologically, these channels are known to be the site of action of a novel class of vasodilators, known as potassium channel activators (Edwards & Weston, 1990). These channels are also the site of action of endogenous vasoactive mediators including calcitonin gene-related peptide (Nelson *et al.*, 1990), prostanoids (Bouchard *et al.*, 1994) and adenosine (Kirsch *et al.*, 1990; Merkel *et al.*, 1992; Dart & Standen, 1993). Recent interest has focused on the regulation of vascular tone by K_{ATP}-channels, and in this respect Jackson (1993) has reported that these channels are important regulators of basal microvascular tone in both the hamster cheek pouch and cremaster muscle. Furthermore, Jackson found that, in the cheek pouch, the blockade of K_{ATP}-channels with the sulphonylurea, glibenclamide results in reduced vasodilator responses to adenosine, a prostacyclin analogue and also the non-selective β-adrenoceptors agonist isoprenaline. This raises the possibility that K_{ATP}-channels are involved, at least in part, in mediating vasodilatation to a diverse range of agents. These findings may accord with recent reports that glibenclamide antagonizes relaxant responses to isoprenaline in the mouse ileum (Yeung *et al.*, 1994) and rat aortic rings (Hüsken *et al.*, 1994), while recent electrophysiological evidence has demonstrated that isoprenaline causes hyperpolarization of the canine saphenous vein which is sensitive to glibenclamide (Nakashima & Vanhoutte, 1995). Furthermore, studies on both guinea-pig and bovine isolated trachealis muscle indicate that β-adrenoceptor agonists may activate potassium channels leading to hyperpolarization, which contributes towards the relaxant effects of these agents (Cook *et al.*, 1993; Chiu *et al.*, 1993). Such a hyperpolarizing action of isoprenaline, via potassium channels, has previously been identified in rat myometrial muscle (Kroeger & Marshall, 1973). Furthermore, recent electrophysiological evidence from cat ventricular myocytes has demonstrated that β-adrenoceptors are coupled to the activation of K_{ATP}-channels (Schackow & Ten Eick, 1994). Such findings have challenged the traditional view that β-adrenoceptors are solely coupled to adenylate cyclase and adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation (Torphy, 1994). In addition Gardiner *et al.* (1991a,b) have reported that in the conscious rat, β-adrenoceptor activation is linked to the release of nitric oxide, indicating an alternative, endothelium-dependent, pathway of vasodilatation.

The present investigation was intended to determine whether K_{ATP}-channels contribute towards β-adrenoceptor-mediated vasodilator responses in the isolated perfused superior mesenteric arterial bed of the rat. Specifically, the vasodilator responses to various β-adrenoceptor agonists have been compared in the absence and presence of the K_{ATP}-channel blocker glibenclamide (Sturgess *et al.*, 1985).

A preliminary account of part of this work was communicated to the December 1994 meeting of the British Pharmacological Society (Randall, 1995).

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Methods

Preparation of the isolated buffer-perfused superior mesenteric arterial bed

Male Wistar rats (200–350 g; Bantin & Kingman, Hull, Humberside) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.: Sagatal, Rhône Mérieux, Harlow, Essex) and following a mid-line incision the superior mesenteric artery was cannulated. The arterial vasculature was dissected away from the guts and placed in a jacketed organ bath as previously described (Randall & Hiley, 1988) and perfused at 5 ml min^{-1} with gassed (95% O_2/5% CO_2) Krebs-Henseleit solution (containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2 and D-glucose 10).

Experimental protocol

Perfusion pressure in the superior mesenteric arterial bed was continuously monitored by a pressure transducer coupled to a MacLab 4e recording system (ADInstruments, New South Wales, Australia). Following a 30 min equilibration period, methoxamine (10 μM) was added to the perfusion fluid to increase vascular tone. Once stable tone had been established, bolus doses of the vasodilator agents were administered close-arterially in random order and in volumes less than 100 μl. In order to assess the influence of K_{ATP}-channels on the vasodilator responses, glibenclamide was added to the perfusion fluid to achieve a concentration of 10 μM, in order to block these channels selectively (Randall & Griffith, 1993). Following a further 30 min it was found necessary to add more methoxamine (20–100 μM) to restore vascular tone to a level comparable to that found in the absence of the sulphonylurea. The vasodilator activities of the agents were then assessed in the same preparation.

Radioligand binding studies

Rat cerebral cortex was homogenized in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 8.0 at 25°C) with a polytron disruptor for 30 s on ice. The homogenate was centrifuged at 38,000 *g* (MSE Europa 24) for 10 min at 4°C. The supernatant was discarded and the pellet resuspended and re-centrifuged. The washing procedure was repeated once more and the final pellet was re-suspended to a concentration of 75 mg ml^{-1} (original wet weight of tissue) in 50 mM Tris-HCl buffer (pH 8.0 at 25°C). The homogenate was stored in 2 ml aliquots at −30°C and was thawed immediately prior to the binding assay.

Portions (50 μl) of the homogenate were added, in triplicate, to polystyrene tubes containing 0.2 nM [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) in 50 mM Tris-HCl (pH 8.0 at 25°C). The total volume of the assay was 1 ml. Non-specific binding was defined by the presence of 10 μM (±)-propranolol. Stock solutions (0.1 mM) of glibenclamide were prepared in dimethylsulphoxide. These were diluted in assay buffer immediately before use such that 50 μl aliquots were added to the assay. Isoprenaline was diluted in Tris-HCl buffer. The assay was conducted by incubating the membrane suspension at room temperature for 30 min. The reaction was terminated by rapid filtration over glass fibre filter using a Brandel cell harvesting apparatus. Scintillation cocktail (5 ml) (Packard Scintillator Plus) was added to the filters and after soaking overnight at room temperature, radioactivity was quantified in a LKB rack beta scintillation counter.

Data and statistical analysis

All data are given as the mean ± s.e.mean and were compared by either paired or unpaired Student's *t* tests as appropriate. ED_{50} values for vasodilator responses were obtained from individual dose-response curves as the dose at which the half-maximal relaxant response occurred. These variables were determined by fitting the data to the logistic equation:

$$R = \frac{R_{max} \times A^{n_H}}{ED_{50}^{n_H} + A^{n_H}}$$

where R is the reduction in tone, A the dose of vasorelaxant, R_{max} the maximum reduction of established tone, n_H the slope function and ED_{50} the dose of the vasorelaxant giving half the maximal relaxation. The curve fitting was carried out using KaleidaGraph software (Synergy, Reading, PA, U.S.A.) running on a Macintosh LC III computer. The ED_{50} values were converted to the logarithmic values for statistical analysis.

Drugs

All solutions were prepared on the day of the experiment. (−)-Isoprenaline bitartrate, salbutamol, terbutaline hemisulphate, (±)-propranolol, (±)-verapamil hydrochloride, dibutyryl cyclic AMP and methoxamine hydrochloride were obtained from the Sigma Chemical Company, Poole, U.K., glibenclamide was obtained from Research Biochemicals Incorporated, Natick, MA, U.S.A., dobutamine hydrochloride ('Dobutrex') was obtained from Ely Lilly and Co, Basingstoke U.K. and [^{3}H]-dihydroalprenolol (specific activity 55 Ci $mmol^{-1}$) was obtained from Amersham PLC, U.K. Isoprenaline and salbutamol were dissolved in 0.1 M HCl and then diluted in 0.5 mM ascorbic acid, while terbutaline was dissolved, and further diluted, in 0.5 mM ascorbate. Verapamil and dibutyryl cyclic AMP were dissolved in absolute ethanol and then diluted in 0.9% saline. Glibenclamide was dissolved in dimethylsulphoxide (DMSO) as a 0.2 M stock solution and the final concentration of DMSO in the perfusion fluid was <0.005% (v/v). All other drugs were then diluted to the required concentrations in Krebs-Henseleit solution.

Results

Basal perfusion pressures and established tone

In the 49 preparations used in the present investigation basal perfusion pressure was 22.2 ± 1.8 mmHg. Following the addition of 10 μM methoxamine, tone was raised by 98.6 ± 3.9 mmHg. After the addition of 10 μM glibenclamide, tone was significantly reduced by 23.6 ± 2.2 mmHg but supplementary addition of methoxamine 20–100 μM restored the level of established tone to 78.3 ± 4.1 mmHg above basal perfusion pressure.

Effect of glibenclamide on vasorelaxant responses to isoprenaline

The dose-response curve for the vasodilator effects of isoprenaline (8.3 pmol–27.7 nmol) under control conditions is shown in Figure 1 and is described by an ED_{50} of 265 ± 31 pmol and a maximum relaxation of tone (R_{max}) of 73.7 ± 4.1% (n = 8). In the presence of glibenclamide the dose-response curve for the vasorelaxant effects of isoprenaline was significantly (P < 0.05) shifted to the right and the ED_{50} was 1.05 ± 0.42 nmol and the R_{max} was 64.8 ± 6.1%.

Effect of glibenclamide on vasorelaxant responses to dobutamine

In 12 different preparations, dobutamine (8.8 pmol–30 nmol) gave rise to dose-related relaxations of tone (ED_{50} = 294 ± 67 pmol and R_{max} = 109 ± 2%) (Figure 2a). In the presence of glibenclamide the dose-response curve was significantly (P < 0.05) shifted to the right (ED_{50} = 497 ± 115 pmol and the R_{max} = 106 ± 4%).

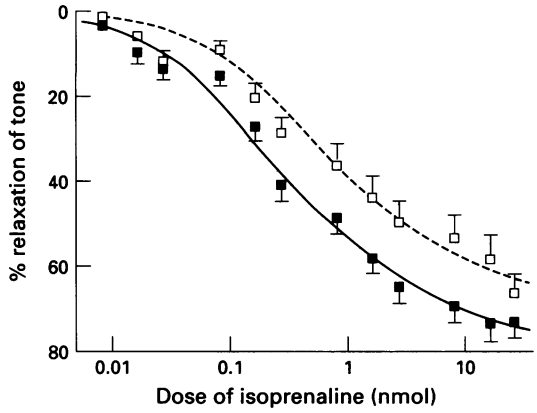

Figure 1 Dose-response curves for the relaxation of established tone in rat isolated perfused superior mesenteric arterial bed by isoprenaline in the absence (■) and presence (□) of 10 μM glibenclamide (both $n=8$). Values are shown as mean ± s.e.mean.

Effect of glibenclamide on vasorelaxant responses to terbutaline

In the 9 preparations to which it was added, terbutaline (10 nmol–22 μmol) gave rise to dose-dependent relaxations of the methoxamine-induced tone ($ED_{50}=157\pm26$ nmol and $R_{max}=100\pm2\%$). Following the addition of glibenclamide the dose-response curve for the relaxant effects of terbutaline was significantly ($P<0.05$) shifted to the right and the ED_{50} was 452 ± 120 nmol and the R_{max} was $99.1\pm2.1\%$ (Figure 2b).

Effect of glibenclamide on vasorelaxant responses to salbutamol

Salbutamol (1.25 nmol–12.6 μmol) caused dose-related relaxations of established tone described by $ED_{50}=142\pm37$ nmol and $R_{max}=96.7\pm4.0\%$ ($n=7-11$). The vasorelaxant effects of salbutamol were unaffected by the addition of glibenclamide ($ED_{50}=237\pm87$ nmol and $R_{max}=86.4\pm4.6\%$) (Figure 2c). However, statistical analysis revealed that at the 1.25 μmol dose, the vasorelaxant response was significantly ($P<0.05$) depressed in the presence of the sulphonylurea ($53.4\pm6.6\%$ v. $77.2\pm3.0\%$).

Effect of glibenclamide on vasorelaxant responses to dibutyryl cyclic AMP

In the 9 preparations to which it was added, dibutyryl cyclic AMP (20 nmol–6.1 μmol) caused dose-dependent relaxations of tone and over the dose-range studied these responses were not affected by the presence of glibenclamide (Figure 3).

Effect of glibenclamide on vasorelaxant responses to verapamil

Verapamil (20 pmol–61 nmol) caused dose-related relaxations of tone ($ED_{50}=1.30\pm0.35$ nmol and $R_{max}=81.7\pm2.9\%$) (Figure 4). Following addition of glibenclamide the vasorelaxant properties of the calcium channel blocker were unaffected ($ED_{50}=1.24\pm0.27$ nmol and the $R_{max}=86.3\pm5.5\%$, all $n=4$).

[^{3}H]-dihydroalprenolol binding assay

Non-specific binding represented $39\pm1\%$ ($n=3$) of total [^{3}H]-DHA binding. A range of concentrations of glibenclamide (1 nM–100 μM) did not displace any [^{3}H]-DHA binding to the rat cerebro-cortical membranes (Figure 5) ($P>0.05$, $n=3$). Under identical experimental conditions, isoprenaline completely displaced all specific [^{3}H]-DHA binding with an IC_{50} of 900 nM.

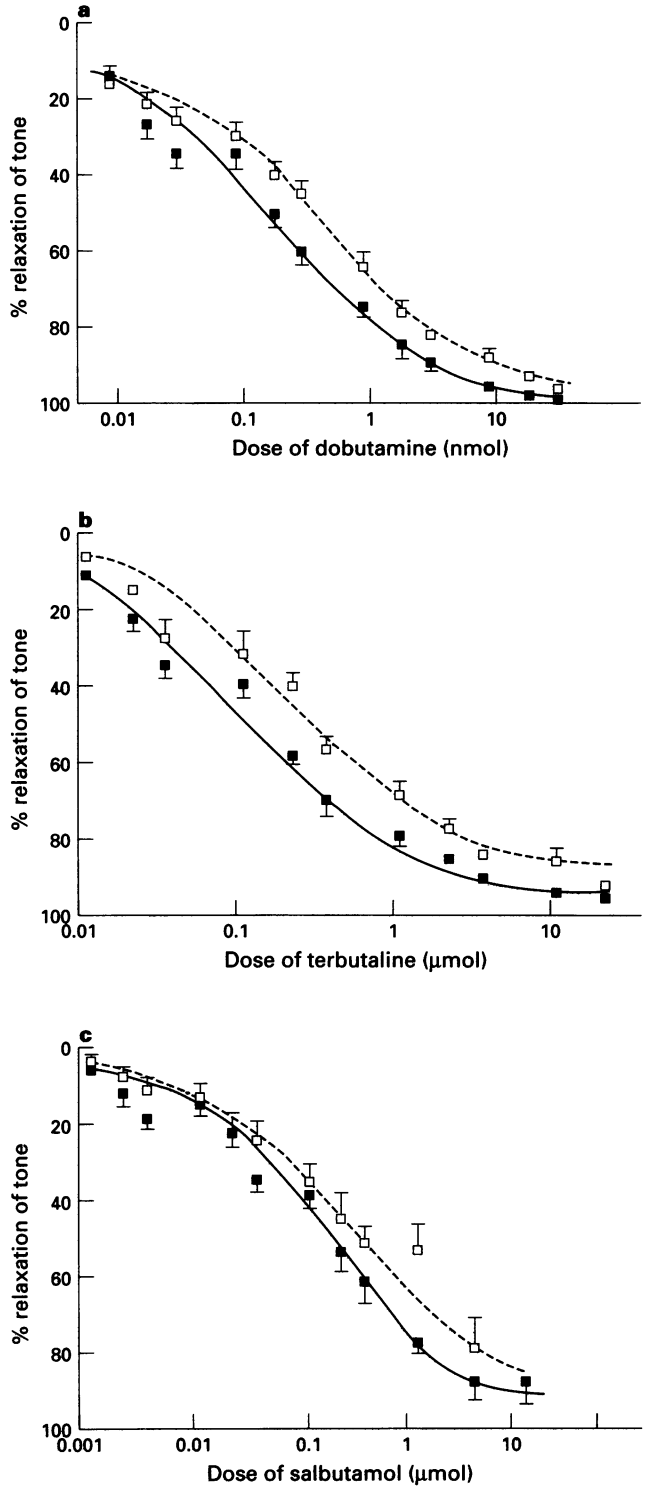

Figure 2 Dose-response curves for the relaxation of methoxamine-induced tone in rat isolated perfused superior mesenteric arterial bed by (a) dobutamine ($n=12$), (b) terbutaline ($n=9$) and (c) salbutamol ($n=7-11$) in the absence (■) and presence (□) of 10 μM glibenclamide. Values are shown as mean ± s.e.mean.

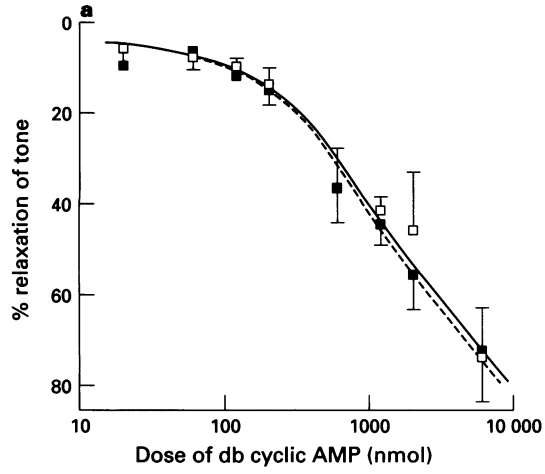

Figure 3 Dose-response curves for the relaxation of methoxamine-induced tone in rat isolated perfused superior mesenteric arterial bed by dibutyryl cyclic AMP in the absence (■) and presence (□) of 10 μM glibenclamide (both $n=9$). Values are shown as mean ± s.e.mean.

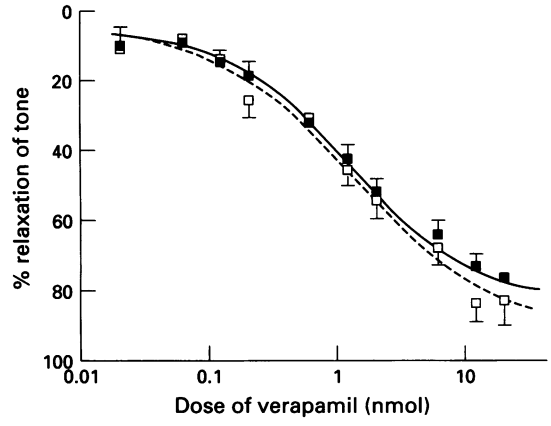

Figure 4 Dose-response curves for the relaxation of methoxamine-induced tone in rat isolated perfused superior mesenteric arterial bed by verapamil in the absence (■) and presence (□) of 10 μM glibenclamide (both $n=4$). Values are shown as mean ± s.e.mean.

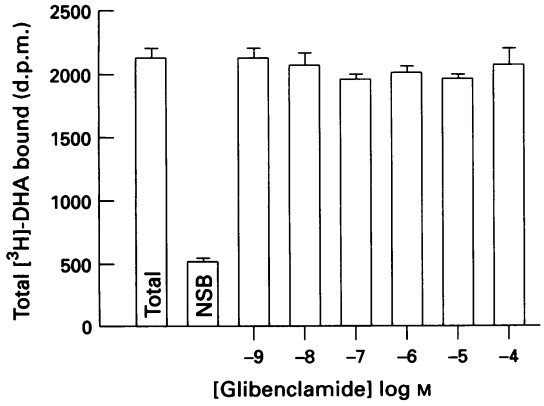

Figure 5 Failure of glibenclamide to compete with [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding to a rat brain total particulate fraction. The figure shows data (mean ± s.e.mean) from a single experiment conducted in triplicate. The data represent [^{3}H]-DHA bound per assay (each assay tube contained approximately 250 μg protein) expressed as disintegrations per minute (d.p.m.). Total = binding in the absence of any competing agent and NSB (non-specific binding) = binding in the present of 10 μM propranolol. Glibenclamide did not significantly affect [^{3}H]-DHA binding in two additional experiments.

Discussion

The results of the present study clearly point to an interaction between β-adrenoceptor activation and K_{ATP}-channels. This interaction contributes towards the vasodilator actions of β-adrenoceptor agonists, but would appear to play a supporting rather than a central role in these responses.

In the present investigation the vasodilator potency of isoprenaline, a non-selective β-adrenoceptor agonist, was significantly reduced by addition of glibenclamide which selectively blocks vascular K_{ATP}-channels (Standen *et al.*, 1989). This observation raised the possibility that β-adrenoceptor activation is coupled to the opening of these potassium channels and accords with reports that glibenclamide reduces responses to isoprenaline in both the hamster cheek pouch (Jackson, 1993) and rat aortic rings (Hüsken *et al.*, 1993). Furthermore, a recent study on the rat basilar artery *in vivo* has demonstrated that noradrenaline acts via β_1-adrenoceptors to cause glibenclamide-sensitive vasodilatation (Kitazono *et al.*, 1993). The coupling of β-adrenoceptors to potassium currents has also been reported in non-vascular tissues including pulmonary smooth muscle, where the responses are linked to calcium-activated potassium channels (Cook *et al.*, 1993; Chiu *et al.*, 1993), and cardiac tissue, where the receptors are coupled to K_{ATP}-channels (Schackow & Ten Eick, 1994). Evidence is therefore accumulating to indicate the involvement of K_{ATP}-channels in β-adrenoceptor-mediated responses.

In the present investigation vasorelaxant responses to the selective β_1-adrenoceptor agonist, dobutamine, and the β_2-adrenoceptor agonist, terbutaline, were also influenced by the addition of glibenclamide. These observations would therefore seem to imply that both β_1 and β_2-adrenoceptors are coupled to the activation of K_{ATP}-channels. This finding contrasts with the observation that only β_1 and not β_2-adrenoceptor-mediated vasodilator responses are coupled to K_{ATP}-channels in the canine coronary vasculature (Narishige *et al.*, 1994). However, in the guinea-pig trachealis preparations, both β_1 and β_2-adrenoceptors are similarly coupled to hyperpolarization (Cook *et al.*, 1993), while in canine saphenous vein smooth muscle only β_2 and not β_1-adrenoceptors appear coupled to K_{ATP}-channel activation (Nakashima & Vanhoutte, 1995).

In the present investigation both a selective β_1- and a β_2-adrenoceptor agonist evoked glibenclamide-sensitive responses but why the β_2-adrenoceptor agonist salbutamol was only weakly influenced is at present unclear. However, this observation parallels the finding in guinea-pig trachealis smooth muscle that isoprenaline, salbutamol, procaterol (a β_2-selective agonist) but not salmeterol (also β_2-selective) activate potassium channels, and the authors ascribe this apparent heterogeneity to salmeterol having a low intrinsic efficacy at β_2-adrenoceptors (Cook *et al.*, 1993). Hence, the participation of K_{ATP}-channels in vasodilator responses may also be agonist- rather than subtype-specific.

The findings from the present investigation point to the involvement of K_{ATP}-channels in β-adrenoceptor-mediated vasorelaxation. By contrast, in human bronchial tissue the relaxant effects of isoprenaline are unaffected by the blockade of K_{ATP}-channels. However, they are sensitive to charybdotoxin, implicating the involvement of large conductance calcium-activated potassium channels (Miura *et al.*, 1992). Hence there may be species and tissue differences in the way in which β-adrenoceptors are coupled to potassium conductances.

An alternative explanation to the above interaction between glibenclamide and β-adrenoceptor agonists could reflect sulphonylurea-binding at adrenoceptors (Cherksey & Altsulzer, 1984). However, in the present investigation glibenclamide, up to 100 μM, did not influence the binding of [^{3}H]-dihydroalprenolol to rat β-adrenoceptors. A non-selective inhibition of vasodilatation by glibenclamide may also be ruled out as vasorelaxant responses to verapamil, a calcium antagonist which acts independently of K_{ATP}-channels, were unaffected by glibenclamide. However, given the consistent reductions in α-

adrenergic tone experienced on addition of glibenclamide it cannot be ruled out that this agent may act as an α-adrenoceptor antagonist in this preparation (Cherksey & Altsulzer, 1984).

How are β-adrenoceptors linked to the activation of K_{ATP}-channels? It is well established that β-adrenoceptors are coupled, via a G-protein, to the activation of adenylate cyclase and a rise in intracellular cyclic AMP. There is also evidence to indicate that protein kinase A links receptor activation to channel opening (Ribalet *et al.*, 1989). In this respect the catalytic subunit of protein kinase A is known to activate glibenclamide-sensitive potassium currents in rabbit mesenteric arterial vascular smooth muscle (Qualye *et al.*, 1994). However, in the present investigation, and in the hamster cheek pouch (Jackson, 1993), K_{ATP}-channel blockade does not influence the vasodilator responses to dibutyryl cyclic AMP, the cell permeable analogue of cyclic AMP. This implies that cyclic AMP is not involved in the activation of K_{ATP}-channels and accords with the observation that relaxant responses due to the adenylate cyclase activator, forskolin, are similarly independent of K_{ATP}-channel activation (Jackson, 1993; Bouchard *et al.*, 1994). Similarly, in cat ventricular myocytes the coupling of β-adrenoceptors and K_{ATP}-channels is unaffected by protein kinase A inhibition (Schackow & Ten Eick, 1994). The precise mechanisms underlying the coupling between receptor and channel activation are not clear from the present investigation, although it is clear that it is independent of cyclic AMP. The activation of K_{ATP}-channels is known to be linked to membranous G-proteins (Brown & Birnbaumer, 1988) and patch clamp studies on guinea-pig ventricular myocytes have shown that both adenosine and acetylcholine may open these channels in a G-protein-dependent manner (Terzic *et al.*, 1994). It is therefore possible that β-adrenoceptor coupling to K_{ATP}-channels is mediated via G-proteins.

The interaction between β-adrenoceptors and K_{ATP}-channels may perhaps account for a previous report of an interaction between these systems. Specifically, salbutamol induces one-way cross tolerance to the potassium channel opener cromakalim in the rat uterus (Downing & Hollingsworth, 1992), with the likely site of interaction being the K_{ATP}-channel.

The findings of the present investigation clearly link a significant element of β-adrenoceptor-mediated vasodilator responses to the activation of K_{ATP}-channels. These findings not only add to the accumulating evidence that β-adrenoceptors are linked to systems other than adenylate cyclase but also to the range of mechanisms by which β-adrenoceptor agonists cause vasodilatation in the cardiovascular system. The observation that β-adrenoceptors are coupled to the activation of K_{ATP}-channels increases the number of endogenous mediators which may interact with these channels and contribute towards vascular regulation in this manner. These findings accordingly increase our knowledge of the physiological significance of K_{ATP}-channels.

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Regional haemodynamic effects of μ-, δ-, and κ-opioid agonists microinjected into the hypothalamic paraventricular nuclei of conscious, unrestrained rats

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1 The cardiovascular effects of bilateral injection into the hypothalamic paraventricular nuclei of selective μ-, δ-, and κ-opioid receptor agonists were investigated in conscious, unrestrained Wistar Kyoto rats, chronically instrumented with pulsed Doppler flow probes for measurement of regional haemodynamics.

2 The selective μ-agonist [D-Ala^2,MePhe^4,Gly^{5}ol]enkephalin (DAMGO), injected bilaterally into the hypothalamic paraventricular nuclei (0.01 - 1.0 nmol), caused increases in blood pressure, tachycardias, vasoconstriction in renal and superior mesenteric vascular beds and substantial vasodilatation in the hindquarter vascular bed.

3 The administration of increasing doses (0.01 - 5.0 nmol) of the selective δ-agonist [D-Phe^{2,5}]enkephalin (DPDPE) or the selective κ-agonist, U50488H into the paraventricular nuclei (PVN) had no significant effect on blood pressure, heart rate, or regional haemodynamics.

4 Together, the present results are further evidence of a role for opioid peptides, especially acting at μ-receptors in the PVN, in the central regulation of the cardiovascular system, whereas a role for opioid peptides, acting at δ- and κ-receptors in the PVN, seems less obvious from the present results.

Keywords: Regional haemodynamics; hypothalamic paraventricular nuclei; blood pressure; heart rate, opioid agonists

Introduction

There is accumulating evidence to suggest a role for endogenous opioid peptides in central cardiovascular control (Holaday, 1983; Feuerstein, 1985; Feuerstein & Sirèn, 1987; Sirèn & Feuerstein, 1992). Mainly, opioid peptides and opiate receptors have been found in specific brain nuclei, with an established role in the regulation of cardiovascular activities (Atweh & Kuhar, 1977; Hokfelt *et al.*, 1977; Fallon & Leslie, 1986; Mansour *et al.*, 1988; Desjardins *et al.*, 1990), and potent cardiovascular effects have been reported following central administration of opioid peptides (Hassen *et al.*, 1983; Pfeiffer *et al.*, 1983a,b; Appel *et al.*, 1986; Kiritsy-Roy *et al.*, 1986; Marson *et al.*, 1989a,b; May *et al.*, 1989; Sirèn *et al.*, 1989; Jin & Rockhold, 1991; Sirèn & Feuerstein, 1991). However, pharmacological studies with opioid ligands have revealed a complex pattern of cardiovascular responses, which has been attributed to the multiple opioid receptors, the type of opioid ligand and its selectivity toward specific opioid receptor, the state of consciousness of the experimental animals, the site(s) of injection and dosage, species, and experimental conditions (i.e., stressed versus resting animals) (Holaday, 1983; Feuerstein, 1985).

The existence of at least three subtypes of opioid receptors, namely, μ-, δ-, and κ-receptors, in the mammalian central nervous system has now been generally accepted (Paterson *et al.*, 1983; Martin, 1984; Goldstein & Naidu, 1989; Reisine & Bell, 1993). Although the functional implications of different opioid receptor subtypes is still difficult to establish, pharmacological studies have shown that activation of different opioid receptor subtypes may have different functional consequences. Unfortunately, some of the opioid ligands used in previous studies are relatively nonselective and have significant affinities for more than one subtype of receptor (Goldstein & James, 1984; James & Goldstein, 1984; Mulder *et al.*, 1989), which makes it difficult to distinguish receptor-specific cardiovascular activity. However, the development of enkephalin analogues with more selective affinities toward each opioid receptor subtype permits further examination of the role of specific opioid receptors in cardiovascular regulation.

Previous studies have demonstrated that i.c.v. or intracisternal injection of the highly selective μ-opioid receptor agonist [D-Ala^2,MePhe^4,Gly^5-ol] enkephalin (DAMGO) (Handa *et al.*, 1981) in conscious animals causes a large increase in blood pressure, a biphasic heart rate response (bradycardia followed by tachycardia) and increases in renal sympathetic nerve activity and plasma catecholamine (Pfeiffer *et al.*, 1982; 1983b; Appel *et al.*, 1986; Marson *et al.*, 1989a,b: May *et al.*, 1989; Matsumura *et al.*, 1992). However, the cardiovascular responses to i.c.v. or intracisternal administration of opioid peptides may reveal little about the discrete functions of the opioid peptides in specific cardiovascular nuclei, as these approaches do not permit accurate localization of the responsible population of neurones. Indeed, activation of opioid receptors in different nuclei in the same region of the brain can induce opposite cardiovascular effects (Morilak *et al.*, 1990; Drolet *et al.*, 1991). The presence of multiple opioid receptors in the brain and their heterogeneous distribution in various brain nuclei (Goodman *et al.*, 1980; Mansour *et al.*, 1988; May *et al.*, 1989; Desjardins *et al.*, 1990) suggest that the cardiovascular effects of centrally administered opioids are highly dependent on the receptor selectivity and the site of drug administration. Therefore, in the present study, we characterized the cardiovascular responses to microinjection of selective opioid receptor agonists into a discrete brain area, the hypothalamus paraventricular nuclei (PVN).

There is considerable evidence to indicate that the PVN play an important role in integrating autonomic control of the cardiovascular system (Sawchencko & Swanson, 1982a,b; Swanson & Sawchencko, 1983). Neuroanatomical and electrophysiological studies have demonstrated that the PVN are reciprocally connected to a number of brain areas thought to be important in cardiovascular regulation (Caverson *et al.*, 1983; Swanson & Sawchencko, 1983; Yamashita *et al.*, 1984; Kannan & Yamashita, 1985; Luiten *et al.*, 1985; Strack *et al.*,

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1989a,b) and important cardiovascular effects have been reported following electrical or chemical stimulation of neurones in PVN (Cireillo & Calaresu, 1980; Jin & Rockhold, 1989; Kannan *et al.*, 1989; Martin *et al.*, 1991; Martin & Haywood, 1992). The PVN have numerous enkephalin-containing neurones and opioid binding sites for μ-, δ-, and κ-receptors (Sar *et al.*, 1979; Goodman *et al.*, 1980; Sawchenko & Swanson, 1982a; Wamsley, 1983; Fallon & Leslie, 1986; Desjardins *et al.*, 1990). Previous studies have shown that microinjection of the selective μ-opioid agonist DAMGO into the PVN produces dose-related increases in blood pressure, heart rate, and plasma levels of catecholamine in conscious rats (Appel *et al.*, 1986; Kiritsy-Roy *et al.*, 1986). Although the effects of μ-opioid receptor activation on blood pressure and heart rate are well known, little is known about the regional haemodynamic mechanisms underlying these actions, and even less is known about the cardiovascular effects resulting from stimulation of PVN δ- and κ-opioid receptors. The study of regional haemodynamics is of major importance, given that a central injection of opioid agonist (or any drug) may fail to produce changes in either blood pressure or heart rate while producing major but opposite changes in vascular resistance in different vascular beds.

The present study was undertaken to investigate the regional haemodynamic effects produced by bilateral injection into the PVN of opioid agonists known to have a high degree of subtype selectivity. Thus, blood pressure, heart rate, and regional haemodynamic responses to PVN administration of DAMGO have been compared with the cardiovascular responses to the δ-opioid receptor agonist [D-Pen^{2,5}]enkephalin (DPDPE) (Mosberg *et al.*, 1983; Gulya *et al.*, 1986; Goldstein & Naidu, 1989) and the κ-opioid receptor agonist U50488H (Von Voigtlander *et al.*, 1983). We used rats that were conscious and unrestrained to avoid interference from anaesthetic agents (Van Loon, 1984).

Methods

Male Wistar Kyoto rats (250–300 g; from Charles River) were anaesthetized with a mixture of ketamine-xylazine (100 and 10 mg kg^{-1}, respectively, i.p., supplemented as required) and then positioned in a stereotaxic frame with the incisor bar set at 3.3 mm below the interaural line. The skull was exposed and cleaned, and two 23-gauge stainless steel guide cannulae targeted 2 mm dorsal to the PVN were obliquely implanted (angle of 10° relative to the vertical) according to the following coordinates: 1.90 mm caudal and ± 1.75 mm lateral to the bregma and 6.3 mm ventral to the surface of the skull. The cannulae were secured to the skull with screws and dental cement. Patency of the guide cannulae was ensured by inserting 31-gauge stainless steel stylets fashioned to extend 0.5 mm beyond the end of the 23-gauge guides and maintained in place with a piece of silastic tubing. The reflected muscles and skin were replaced and sutured. After surgery the animals were treated with ampicillin (Polyflex, Ayerst, 7 mg kg^{-1}, i.m.) and flunixin (Banamine, Schering, 1 mg kg^{-1}, i.m.), housed in individual cages, and allowed to recover.

At least 7 days later, the rats were re-anaesthetized with a mixture of ketamine-xylazine (100 and 10 mg kg^{-1}, respectively, i.p., supplemented as required) and had pulsed Doppler flow probes implanted around the left renal and superior mesenteric arteries and the lower abdominal aorta, as described previously (Haywood *et al.*, 1981; Bachelard *et al.*, 1992; 1994). After operation, the rats were given i.m. injections of ampicillin (7 mg kg^{-1}) and flunixin (1 mg kg^{-1}) and allowed to recover for at least 7 days. After this period, the rats were re-anaesthetized with a mixture of ketamine-xytazine (100 and 10 mg kg^{-1}, respectively, i.p., supplemented as required). The leads of the implanted probes were soldered to a six-way microconnector (Microtech Inc.), which was connected to a pulsed Doppler monitoring system (VF-1 mainframe, Crystal Biotech) to check the quality of the signals. Any animal not showing good-quality signals (signal : noise ratio >20 : 1) from all three probes was rejected from the study. Those that met this criterion had one catheter implanted in the right jugular vein (for drug administration) and one in the distal abdominal aorta via the femoral artery (for measurement of blood pressure and heart rate). The catheters were tunnelled subcutaneously to emerge at the same point as the Doppler probe wires. The microconnector, soldered to the Doppler probe wires, was clamped in a custom-made harness worn by the rat, and the catheters were passed through a flexible, protecting spring attached to the harness. Experiments were begun following an overnight recovery period of 18 to 20 h.

Continuous recordings were made of heart rate, phasic and mean blood pressures, and phasic and mean Doppler shift signals from renal, mesenteric, and hindquarters probes using a pulsed Doppler monitoring system (Crystal Biotech, Holliston, U.S.A.), modified to operate with a pulse repetition frequency of 125 kHz (Gardiner *et al.*, 1990). It has been shown that % change in the Doppler shift signal is a reliable index of change in blood flow (Haywood *et al.*, 1981; Wright *et al.*, 1987). At selected time points (averaged over 20 s) heart rate, mean blood pressure, and mean Doppler shifts were measured and related to the predrug baseline (absolute changes for the former two variables, percentages for the Doppler shifts). In addition, regional vascular conductances were calculated by dividing the appropriate mean Doppler shift by the mean arterial blood pressure. Before every experiment, baseline measurements were made over a 30 min period. The rats were allowed free access to food and water for the duration of the experiment.

Bilateral injections were made directly into the PVN of undisturbed, conscious, freely moving rats through 31-gauge stainless steel injectors that extended 2 mm beyond the previously implanted guide cannulae. The injectors were connected via polyethylene tubing to two Hamilton microsyringes (5 μl) and inserted into the guide cannulae without handling the rats. The animals were allowed to settle following this procedure so that remote injections could be made while they were undisturbed. All solutions for microinjections were freshly prepared. The injection volume was 0.2 μl, delivered by hand simultaneously into both sides for 1 min.

At the end of the experiments, all animals received an injection of 0.2 μl of India ink to mark the placement of the cannula tip. The placements of the microinjection sites were verified histologically in serial coronal sections (40 μm, cut on a freezing microtome), mounted on glass slides, and stained with neutral red (Figure 1). Of a total of 30 rats, six were rejected from the study because one or both cannulae tips were not within the PVN. An animal was considered successfully injected when both cannulae tips were shown to be in the PVN or within 0.5 mm of the PVN according to the atlas of Paxinos & Watson (1986). No apparent anatomical associated differences were observed in individual cardiovascular responses to microinjections of DAMGO, DPDPE, or U50488H within this area. A schematic map showing the mean of sites of injection within the PVN and the sites where failures were observed is illustrated in Figure 1b.

Experimental protocols

Cardiovascular responses to PVN injections of opiate agonists The rats were used on four consecutive days, during which they received eight randomized bilateral injections of DAMGO, DPDPE, or U50488H into the PVN at doses ranging from 0.01 to 1 nmol (for DAMGO) and 0.01 to 5 nmol (for DPDPE and U50488H) per microinjection site. The agonists were dissolved in artificial cerebrospinal fluid (aCSF), pH 7.4 (vehicle), which served as the control injection. On a single day, no rat received more than two bilateral injections, separated by at least 180 min, by which time all monitored variables had returned to control levels. On the first day, agonist injection into each rat was preceded by an injection of vehicle. The composition of the aCSF (in mM) was: NaCl 125,

$NaHCO_3$ 27, KCl 2.5, NaH_2PO_4 0.5, Na_2HPO_4 1.2, Na_2SO_4 0.5, $CaCl_2$ 1.0, $MgCl_2$ 1.0 and glucose, 5.0. Cardiovascular variables were recorded for 60 min following each injection.

Cardiovascular responses to i.v. injections of DAMGO In this experiment, a separate group of rats ($n=14$) received i.v. bolus injections of agonist vehicle (0.1 ml) and DAMGO (2 nmol), in that order, to determine whether the effects of DAMGO were due to leakage into the periphery. Injections were separated by at least 180 min. Measurements were made before, during, and 15 min after i.v. injections of vehicle or DAMGO.

Drugs

The drugs used were DAMGO ([D-Ala^2,MePhe^4,Gly^5-ol]enkephalin; Bachem), DPDPE ([D-Pen^{2,5}]enkephalin; BACHEM), and U50488H (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulphonate; RBI).

Data analysis

Values are expressed as the mean ± s.e. mean; n is the number of observations. Results were analysed for statistical sig-

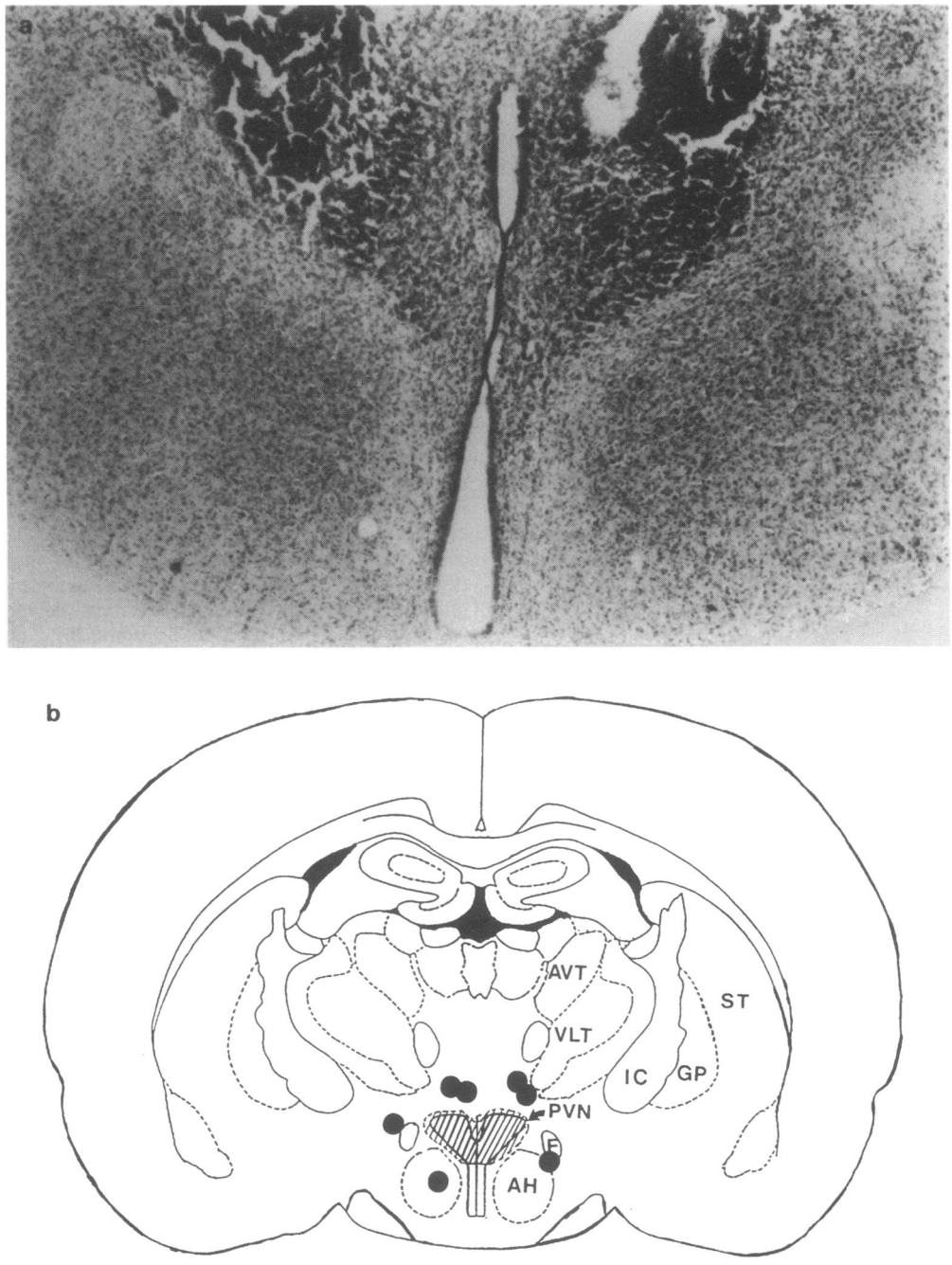

Figure 1 Representative micrograph (a) showing the location of the injection sites in the paraventricular nuclei (PVN) of the hypothalamus. In (b) is the appropriate diagram modified from Paxinos & Watson (1986) showing the mean of sites of injection (shaded area) within the PVN and the sites where failures were observed (●). A rat was considered successfully injected when both cannulae tips were shown to be in the PVN or within 0.5 mm of the PVN. Incisor bar: 3.3 mm below the interaural line; AP = 1.9 mm posterior to the bregma; L = ± 1.75 mm relative to bregma; V = 8.3 mm ventral to the surface of the skull. AH, anterior hypothalamic area; AVT, anteroventral thalamic nucleus; F, fornix; GP, globus pallidus; IC, internal capsule; ST, striatum; VLT, ventrolateral thalamic nucleus.

Table 1 Baseline values for heart rate, blood pressure and regional vascular conductance in conscious, unrestrained Wistar Kyoto rats ($n = 18$)

Heart rate (beats min^{-1})	*Mean blood pressure (mmHg)*	*Doppler shift (kHz)*			*Vascular conductance (kHz mmHg^{-1})10^3*		
		Renal	*Mesenteric*	*Hindquarters*	*Renal*	*Mensenteric*	*Hindquarters*
345 ± 10	108 ± 3	4.1 ± 0.4	11.0 ± 7.3	2.6 ± 1.0	38.2 ± 3.4	103.9 ± 6.6	24.5 ± 2.1

Values are mean ± s.e.mean; n = number of animals.

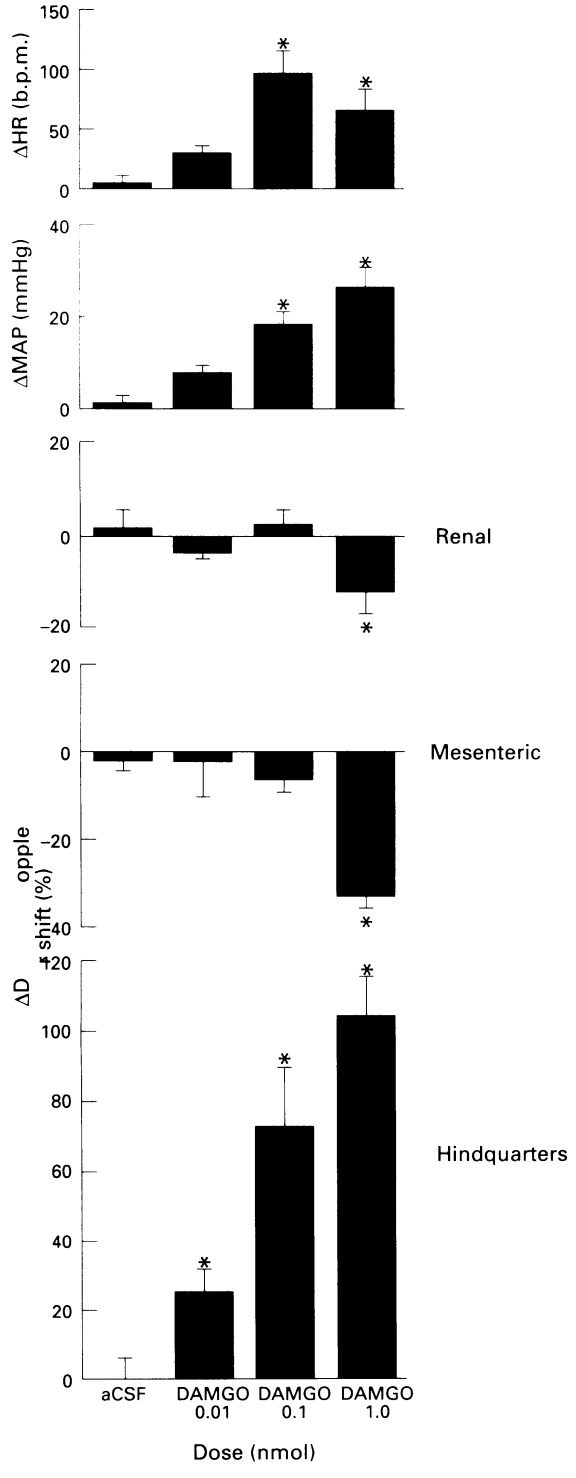

Figure 2 Maximum cardiovascular changes elicited by bilateral microinjection of artificial CSF (aCSF; $n = 14$) or [D-Ala^2,Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) at a dose of 0.01 nmol ($n = 14$), 0.1 nmol ($n = 12$), and 1.0 nmol ($n = 14$) on each side into the paraventricular nuclei of conscious, unrestrained Wistar Kyoto rats. Comparisons are made between vehicle (aCSF) evoked responses and those to DAMGO. Columns are mean with s.e.mean. *$P < 0.05$ for DAMGO-injected group versus aCSF-injected group, analysis of variance followed by Fisher's test.

nificance by an analysis of variance (ANOVA) with repeated measures using the Macintosh Statview 4 programme. *Post-hoc* comparisons were made using Fisher's test. A P value < 0.05 was taken to indicate a significant difference.

Results

Haemodynamic responses to PVN injection of DAMGO in conscious, unrestrained rats

The baseline values (prior to any drug administration) for cardiovascular variables are listed in Table 1. A control bilateral injection of aCSF (0.2 μl) into the PVN had no significant effect on any measured or calculated variables (Figures 2–5). Figures 2 and 3 show that the lowest dose of DAMGO bilaterally injected into the PVN (0.01 nmol on each side) caused a significant increase in hindquarter flow (significant at 2–4 min), but had no significant effect on blood pressure, heart rate, or renal and superior mesenteric flows. Increases in hindquarter vascular conductance (significant at 2–4 min) ocurred, but no significant change was seen in renal and superior mesenteric vascular conductances. However, the PVN bilateral injection of a higher dose of DAMGO (0.1 nmol on each side) elicted a long-lasting increase in blood pressure (significant at 10–60 min) accompanied by tachycardia (significant at 10–60 min) and increases in hindquarter flow (significant at 2–60 min), but no significant change was seen in renal and superior mesenteric flows compared with measurements following aCSF (Figure 2). The maximum rise in mean arterial blood pressure, after the 0.1 nmol dose, was +18 ± 3 mmHg and was reached 15 min after the injection of DAMGO, but blood pressure remained elevated for 1 h after the administration of DAMGO (0.1 nmol). The maximum rise in heart rate was +100 ± 19 b.p.m. and was reached 15 min after the injection of DAMGO, subsiding in 60 min. The maximum increase in hindquarter flow was +72 ± 18% and was achieved 15 min after the injection, but the hindquarter flow remained significantly elevated for 1 h after administration of DAMGO. These responses were associated with falls in renal (significant at 30 min) and superior mesenteric (significant at 5–30 min) vascular conductances and a long-lasting increase in hindquarter (significant at 2–4 and 15–60 min) vascular conductance (Figure 3). The maximum decreases in renal (−12 ± 4%) and superior mesenteric (−20 ± 4%) vascular conductances were observed 30 min after administration of DAMGO (0.1 nmol), and the maximum increase in hindquarter vascular conductance (+48 ± 15%) was observed 15 min after the administration of DAMGO, but it remained high during the entire 60 min observation period.

Bilateral injection of the highest dose of DAMGO tested (1 nmol on each side) into the PVN produced cardiovascular

effects characterized by a long-lasting increase in blood pressure (significant at 3–60 min) and increases in heart rate (significant at 4 and 15–60 min) (Figures 2 and 4). The maximum rise in blood pressure was +26±4 mmHg, achieved 45 min after the injection of DAMGO (1 nmol); the tachycardic response reached a maximum of +66±21 b.p.m. 30 min after the injection. However, both effects remained significantly elevated during the entire 60 min observation period. Furthermore, substantial falls were noted in renal (significant at 5–30) and superior mesenteric (significant at 2–60 min) flows, whereas hindquarter flow increased (significant at 2–60-min) (Figures 2 and 4). The maximum decrease in renal (−12±4%) flow was observed 15 min after the administration of DAMGO; the maximum decreases in superior mesenteric (−33±3%) and maximum increase in hindquarters (+104±11%) flows were reached 30 min after the injection of DAMGO. These blood flow effects in mesenteric and hindquarter vascular beds were still significant during the entire 60 min observation period. The cardiovascular responses to DAMGO (1 nmol) were associated with falls in renal (significant at 2–45 min) and superior mesenteric (significant at 3–60 min) vascular conductances and increases in hindquarter (significant at 2–60 min) vascular conductance (Figures 3 and 5). The maximum decreases in renal (−30±4%) and superior mesenteric (−45±3%) vascular conductances were observed 45 min after the administration of DAMGO, whereas the maximum increase in hindquarter (+73±8%) vascular conductance was reached 30 min after the injection of DAMGO. However, the mesenteric vasoconstrictor and hindquarter vasodilator effects remained significant during the entire 60 min observation period.

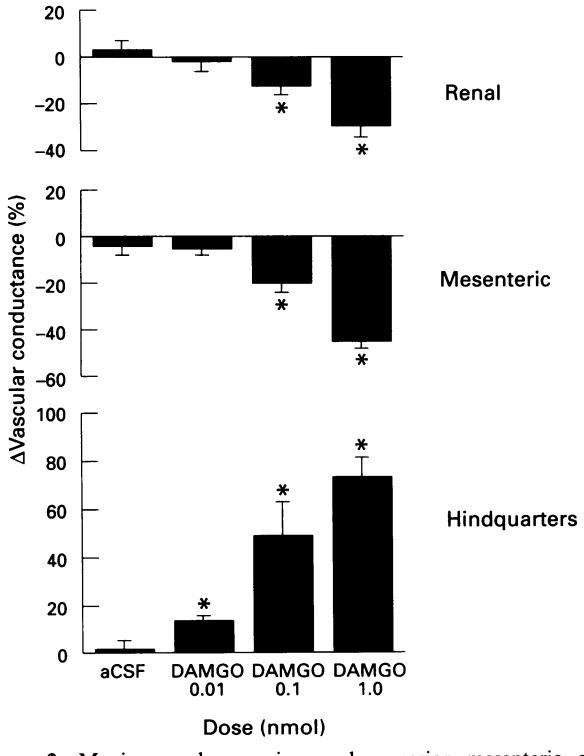

Figure 3 Maximum changes in renal, superior mesenteric and hindquarter vascular conductances produced by bilateral microinjection of artificial CSF (aCSF; $n=14$) or [D-Ala^2,MePhe^4,Gly^5-ol]enkephalin (DAMGO) at a dose of 0.01 nmol ($n=14$), 0.1 nmol ($n=12$) and 1.0 nmol ($n=14$) on each side into the paraventricular nuclei of conscious, unrestrained Wistar Kyoto rats. These data were derived from the data shown in Figure 2. Comparisons are made between vehicle (aCSF) evoked responses and those to DAMGO. Vertical lines represent s.e.mean. *$P<0.05$ for DAMGO-injected group versus aCSF-injected group, analysis of variance followed by Fisher's test.

It is unlikely that the pressor responses induced by DAMGO injection into the PVN were due to changes in regional blood flow and vascular conductance secondary to increased motor activity. Indeed, compared with baseline controls, PVN injection of DAMGO (0.01, 0.1, and 1.0 nmol) produced no significant locomotor activity over the 60 min observation period. Instead, at all doses of DAMGO tested, the rats dis-

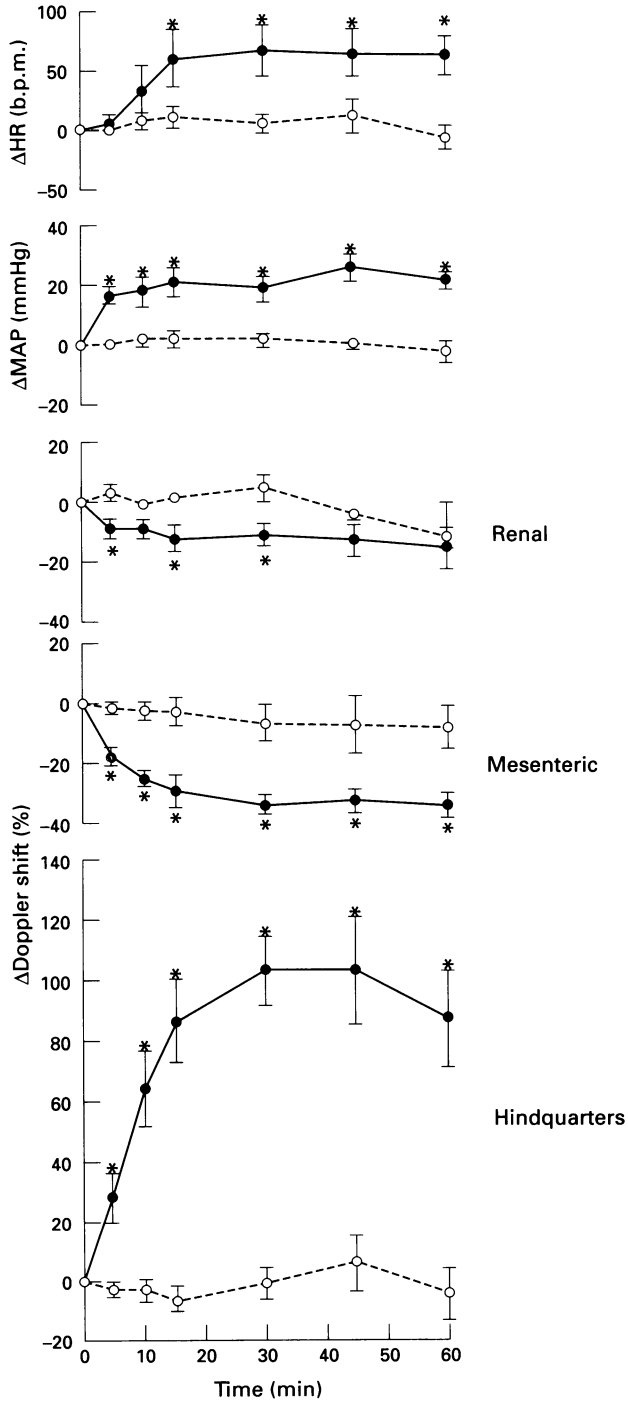

Figure 4 Cardiovascular changes elicited by bilateral administration of 1 nmol of [D-Ala^2,MePhe^4,Gly^5-ol]enkephalin (DAMGO) on each side (●, $n=14$) of aCSF (○, $n=14$) into the paraventricular nuclei of conscious, unrestrained Wistar Kyoto rats. Values are mean with s.e. mean shown by vertical lines. *$P<0.05$ for DAMGO-injected group versus aCSF-injected group, analysis of variance followed by Fisher's test. BP, blood pressure; HR, heart rate; b.p.m. beats per minute.

played signs of sedation; that is, they remained motionless and piloerected in a corner of the test cage, showing little response to external stimuli.

Haemodynamic responses to PVN injection of DPDPE in conscious, unrestrained rats

The effects of bilateral injection of the δ-opiate agonist, DPDPE at the doses of 0.01 ($n=6$), 0.1 ($n=14$), 1.0 ($n=16$), or 5.0 nmol ($n=9$) into the PVN (on each side) were not significant compared with the effects of aCSF injection (data not illustrated).

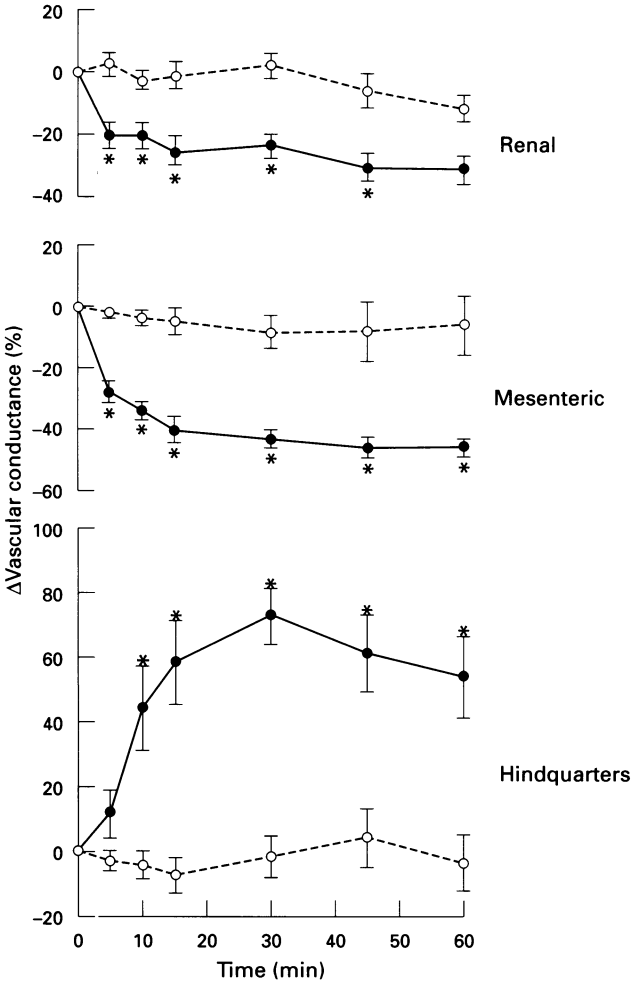

Figure 5 Changes in regional vascular conductances elicited by bilateral administration of 1 nmol of [D-Ala^2,MePhe^4,Gly^5-ol]enkephalin (DAMGO) on each side (●, $n=14$) or aCSF (○, $n=14$) into the paraventricular nuclei of conscious, unrestrained Wistar Kyoto rats. These data were derived from the data shown in Figure 4. Values are mean with s.e. mean shown by vertical lines. *$P<0.05$ for DAMGO-injected group versus aCSF-injected group, analysis of variance followed by Fisher's test.

Haemodynamic responses to U50488H injected into the paraventricular nuclei

The effects of bilateral injection of the κ-opiate agonist, U50488H at the doses of 0.01 ($n=12$), 0.1 ($n=11$), 1.0 ($n=11$) or 5.0 nmol ($n=8$) into the PVN (on each side) were not significantly different from the effects of aCSF (data not illustrated).

Haemodynamic responses to i.v. injection of DAMGO

The effects of i.v. injection of DAMGO (2 nmol, 0.1 ml) were not significantly different from those of saline (NaCl, 0.9%, 0.1 ml) (Table 2).

Discussion

This study focused on the regional haemodynamic responses to PVN administration of specific opioid-receptor agonists in conscious, unrestrained rats. Our results confirm the previous observation that bilateral administration into the PVN of the highly selective and potent μ-opioid receptor agonist, DAMGO, induced a dose-related increase in blood pressure in conscious, freely moving rats (Appel *et al.*, 1986; Kiritsy-Roy *et al.*, 1986). The present study elucidates the peripheral mechanisms of these changes. Thus, bilateral injection of DAMGO into the PVN caused increases in blood pressure accompanied by marked, dose-related decreases in renal and superior mesenteric vascular conductances and increases in hindquarter vascular conductance. Moreover, tachycardia occurred but was less clearly dose-related. The increases in heart rate elicited by the doses of 0.1 and 1.0 nmol of DAMGO in the PVN were not significantly different, although the magnitude of the tachycardic response tended to be smaller at the highest dose tested (1 nmol). This latter result might be due to a direct effect on vagal outflow or to a baroreceptor-mediated reflex response to the rise in blood pressure. Therefore, if incremental doses of DAMGO injected into PVN cause increasing sympathoadrenal activation (see later), a baroreflex-mediated increase in vagal tone might increasingly counteract the effects of the sympathoadrenal stimulation on the heart and thus obscure any dose-relatedness of the latter; this cannot be determined from the present study. However, previous studies have demonstrated that high doses of DAMGO activate parasympathetic outflow when unilaterally injected into the PVN (Kiritsy-Roy *et al.*, 1986) or into the anterior hypothalamus (Pfeiffer *et al.*, 1983a,b).

Intravenous injections of DAMGO (2 nmol) do not cause cardiovascular effects. Thus, the present results were clearly not due to leakage of DAMGO from its central site of injection into the periphery. Moreover, injection of aCSF (0.2 μl) into the PVN had no cardiovascular effects, indicating that dis-

Table 2 Cardiovascular changes produced by intravenous injection of saline (0.1ml) or [D-Ala^2, MePhe^4, Gly^5-ol] enkephalin (DAMGO, 2 mol) in conscious, unrestrained Wistar Kyoto rats

	ΔHR (*beats min^{-1}*)	*ΔMAP* (*mmHg*)	*ΔDoppler shift (%)*			*ΔVasuclar conductance (%)*		
			Renal	*Mesenteric*	*Hindquarters*	*Renal*	*Mesenteric*	*Hindquarters*
Saline ($n=6$)	14±9	3±3	-1.3±2.0	-4.3±4.6	22.3±12.7	-4.2±1.9	-6.5±6.1	17.8±9.4
DAMGO 2.0 nmol (=11)	6±11	1±5	1.5±4.1	-5.8±1.6	38.1±8.8	0.2±5.1	-7.0±3.4	39.2±14.5

Values are mean±s.e.mean; $n=$ number of animals.

placement of the tissue by the volume injected was not a cause of the observed effects. However, we cannot exclude the possibility that the injections could have diffused to regions surrounding the PVN, considering the volume of injection (0.2 μl) we used. Therefore, the effects described in the present paper may have been due to activation of other neighbouring nuclei besides the PVN.

The cardiovascular responses following bilateral injection of DAMGO in the PVN were probably due to an activation of the sympathoadrenomedullary axis. The pattern of changes in regional blood flows (an increase in hindquarter but decreases in the superior mesenteric and renal vascular beds) induced by DAMGO suggests activation of the central sympathetic outflow to the adrenal medulla and sympathetic nerve terminals. The factors underlying the enormous hindquarter vasodilator responses to DAMGO might be due to relatively selective activation of β_2-adrenoceptor-mediated vasodilator mechanisms. This possibility is supported by previous studies showing that central administration of β-endorphin, DAMGO or dermorphin in rats produced a naloxone-reversible increase in plasma catecholamine concentration (Van Loon *et al.*, 1981; Pfeiffer *et al.*, 1983b; Appel *et al.*, 1986; Kiritsy-Roy *et al.*, 1986; Sirén *et al.*, 1989), an increase in sympathetic outflow in peripheral postganglionic sympathetic nerves (Sirén & Feuerstein, 1991), and blockade of the cardiovascular responses by adrenergic neuronal blocking drugs (Jin & Rockhold, 1991; Sirén & Feuerstein, 1991; Pfeiffer *et al.*, 1983b). Plasma adrenaline was far more sensitive to the effects of DAMGO in the PVN, suggesting that the treatment produced a relatively selective activation of the adrenal medulla (Kiritsy-Roy *et al.*, 1986). Moreover, the vascular bed of the hindquarter is particularly well endowed with β_2-adrenoceptors, which mediate vasodilatation (Gardiner & Bennett, 1988). Thus, if DAMGO injected into the PVN causes sympathoadrenal activation, it is feasible that the increase in hindquarter flow and vascular conductance in response to PVN injection of DAMGO was due to relatively selective activation of β_2-adrenoceptor-mediated vasodilator mechanisms, but this cannot be determined from this study.

In the present study, we found no significant cardiovascular effects of PVN administration in increasing doses (0.01–5.0 nmol) of the highly selective δ-opioid receptor agonist DPDPE (Mosberg *et al.*, 1983; Gulya *et al.*, 1986; Goldstein & Naidu, 1989). However, a previous study showed that i.c.v. injection of DAMGO or DPDPE in conscious rats produced dose-related increases in sympathoadrenal outflow and blood pressure, but only DAMGO (5 nmol) caused significant changes in heart rate: an atropine-sensitive bradycardia (Marson *et al.*, 1989a,b). These effects were antagonized by the i.c.v. injection of a μ-selective dose of naloxone (Gordon, 1986; Marson *et al.*, 1989b), but these responses were not reversed by the δ-selective antagonist ICI 174,864 (Marson *et al.*, 1989a,b). Moreover, the concentration of DPDPE used in that study to produce cardiovascular effects was far higher than ours (125 nmol). Therefore, considering the relatively high dose of DPDPE used, and because the δ-antagonist ICI 174,864 was without effect, it was suggested that both DAMGO and DPDPE act on a μ-type brain opioid receptor to modulate cardiovascular responses (Marson *et al.*, 1989a,b). In another study carried out in conscious rabbits, i.c.v. administration of increasing doses (0.01–1.0 nmol kg^{-1}) of DPDPE was associated with increases in blood pressure and heart rate, but not with any change in plasma catecholamine (May *et al.*, 1989). Furthermore, the δ-agonist increased blood pressure more than 10 times less potently than DAMGO (May *et al.*, 1989). However, differences between those results and our present findings might be explained by the different site of administration as well as by the different species used.

In the present study, we found no cardiovascular effects following PVN administration of U50488H, a purported highly selective κ-opioid receptor agonist (Von Voigtlander *et al.*, 1983). These results agree with those of Pfeiffer *et al.* (1982, 1983a) showing that microinjection of a κ-agonist (MR 2034) into the anterior hypothalamic and septal brain regions of conscious rats had no effect on cardiovascular parameters. Similarly, May *et al.* (1989) reported that i.c.v. injection of the highly selective κ-agonist U69593 and dynorphin A(1-13) in conscious rabbits had no cardiovascular effect. However, in conscious rats, dynorphin A(1-13) produced a transient dose-related pressor effect after i.c.v. administration (Glatt *et al.*, 1987). The difference from the present findings may result from the higher doses used, the different site of administration, or the selectivity of the opioid agonists used. Although dynorphin A(1-13) is known to have actions on κ-receptors, other studies would be required to address whether the cardiovascular responses reported in that study were due to κ-receptor activation (Goldstein & James, 1984; James & Goldstein, 1984; Mulder *et al.*, 1989; Rochford *et al.*, 1991).

In conclusion, by using compounds that are highly selective for the various opioid receptor subtypes, we demonstrated that in conscious rats only DAMGO, a highly selective μ-receptor agonist, injected into the PVN, produced significant cardiovascular effects. Thus, bilateral microinjection of DAMGO into the PVN exerts tachycardia and a hypertensive effect through marked vasoconstrictor actions in the renal and superior mesenteric vascular bed and a substantial vasodilatation in the hindquarter vascular bed. According to previous studies, the cardiovascular responses to DAMGO are probably due to activation of the sympathoadrenomedullary axis. On the other hand, we observed no significant cardiovascular effects after central administration of selective δ- and κ-opioid receptor agonists into the PVN. Together with the specific haemodynamic effects of DAMGO, the present results are further evidence of a role for opioid peptides and μ-opioid receptors in the central regulation of cardiovascular function, whereas the involvement of PVN δ- and κ-receptors in cardiovascular regulation are less obvious from the present findings.

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The pharmacology and distribution of human 5-hydroxytryptamine$_{2B}$ (5-HT$_{2B}$) receptor gene products: comparison with 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors

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1 Full length clones of the human 5-HT$_{2B}$ receptor were isolated from human liver, kidney and pancreas. The cloned human 5-HT$_{2B}$ receptors had a high degree of homology (~80%) with the rat and mouse 5-HT$_{2B}$ receptors.

2 PCR amplification was used to determine the tissue distribution of human 5-HT$_{2B}$ receptor mRNA. mRNA encoding the 5-HT$_{2B}$ receptor was expressed with greatest abundance in human liver and kidney. Lower levels of expression were detected in cerebral cortex, whole brain, pancreas and spleen. Expression was not detected in heart.

3 Northern blot analysis confirmed the presence of 5-HT$_{2B}$ receptor mRNA (a 2.4 kB sized band) in pancreas, liver and kidney. An additional 3.2 kB sized band of hybridization was detected in liver and kidney. This raises the possibility of a splice variant of the receptor or the presence of an additional homologous receptor.

4 The human 5-HT$_{2B}$ receptor was expressed in Cos-7 cells and its ligand binding characteristics were compared to similarly expressed human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. The ligand specificity of the human 5-HT$_{2B}$ receptor (5-HT > ritanserin > SB 204741 > spiperone) was distinct from that of the human 5-HT$_{2A}$ (ritanserin > spiperone > 5-HT > SB 204741) and 5-HT$_{2C}$ (ritanserin > 5-HT > spiperone = SB 204741) receptors. On the basis of a higher affinity for ketanserin and a lower affinity for yohimbine the human 5-HT$_{2B}$ receptor also appeared to differ from the rat 5-HT$_{2B}$ receptor.

5 These findings confirm the sequence of the human 5-HT$_{2B}$ receptor and they demonstrate that the receptor has a widespread tissue distribution. In addition, these data suggest that there are differences in ligand affinities between different species homologues of the receptor. Finally, the finding of two distinct bands on the Northern blots of liver and kidney raises the possibility of splice variants or subtypes of 5-HT$_{2B}$ receptors, within these tissues.

Keywords: 5-Hydroxytryptamine; 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$ receptors; human 5-HT receptors

Introduction

5-HT$_{2B}$ receptors are one of three members of the 5-hydroxytryptamine$_2$ (5-HT$_2$) family of 5-HT receptors. 5-HT$_{2B}$ receptors, like 5-HT$_{2A}$ (previously denoted as 5-HT$_2$) and 5-HT$_{2C}$ (previously denoted as 5-HT$_{1C}$) receptors are G-protein linked receptors, positively coupled to phosphoinositide metabolism. This family of 5-HT receptors share sequence homology and have the same pattern of introns and exons. Similarities in the specificity of 5-HT$_{2B}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors for ligands further indicates the commonality of receptors in this family. Recent reviews of this area have been written by Martin & Humphrey (1994) and Hoyer *et al.* (1994).

5-HT$_{2B}$ receptors, initially termed 5-HT$_{2F}$ or serotonin-like receptor, were characterized in rat isolated stomach fundus (Vane, 1959; Clineschmidt *et al.*, 1985; Cohen & Wittenauer, 1987; Cohen, 1989). 5-HT-evoked contractions were found to be mediated by a receptor pharmacologically similar to the 5-HT$_{2C}$ receptor. However, relatively high affinities for 5-HT and yohimbine and relatively low affinity for ketanserin distinguished this receptor from 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors (Baxter *et al.*, 1994a). Recently, the pharmacological specificity of 5-HT$_{2B}$ receptors has been further defined with the high affinity (pK_i = 7.1) 5-HT$_{2B}$ selective, antagonist, SB 204741 (Baxter *et al.*, 1994b).

Little is known about the function of 5-HT$_{2B}$ receptors in tissues other than the stomach fundus but, based on Northern blot analysis or PCR amplification of tissue-specific cDNAs, the rat (Foguet *et al.*, 1992a; Kursar *et al.*, 1992) and mouse (Foguet *et al.*, 1992b) 5-HT$_{2B}$ receptors are expressed in a several peripheral and central tissues including stomach fundus, liver, kidney, muscle, intestine and brain. This distribution, together with the discovery of a 5-HT receptor in vascular tissue with pharmacology similar to that of the 5-HT$_{2B}$ receptor (Glusa & Richter, 1993), suggests a wide ranging role for this receptor in mediating the actions of 5-HT.

Expression of the cloned 5-HT$_{2B}$ receptor has allowed its characterization by radioligand binding. The cloned receptor can be labelled with high affinity (K_i~1 nM) using [^{3}H]-5-HT and it has affinities for 5-HT$_2$ receptor ligands that are similar to those found in the stomach fundus assay. However, several small differences in the affinities of ligands for rat (Wainscott *et al.*, 1993) and mouse (Loric *et al.*, 1992) cloned 5-HT$_{2B}$ receptors suggest that there may be inter-species differences in ligand binding properties of 5-HT$_{2B}$ receptors.

Given the possibility of inter-species differences in 5-HT$_{2B}$ receptor pharmacology and given the paucity of pharmacological information on human 5-HT$_{2B}$ receptors, the goals of the current study were to clone a human 5-HT$_{2B}$ receptor, to assess its expression in a variety of tissues, and to characterize its pharmacological properties. Consequently, the distribution and ligand binding properties of the human cloned 5-HT$_{2B}$ receptor were compared to those of the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. The ligand binding properties of these cloned

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receptors were also compared to their rat homologues and, where possible, to native 5-HT$_2$ receptors in human post-mortem tissue.

Methods

Cloning and expression of human 5-HT$_2$ receptors

The full length human 5-HT$_{2A}$ receptor gene was amplified from human brain cDNA using the PCR primers CTACAAGTTCTGGCTTAGACATG (sense) and CACGGCAACTAGCCTATCACACAC (anti-sense). The receptor was cloned into the PCRII (TA) vector and then sub-cloned into the eukaryotic expression vector pSW104. The clone was then sequenced on both strands.

To clone the human 5-HT$_{2B}$ receptor, PCR primers based on the third exon of the mouse 5-HT$_{2B}$ receptor were used to isolate a 200 base-pair probe from human genomic DNA. This probe was extended in the 3' direction using a two step, nested PCR, procedure and human liver cDNA as a template. The PCR primers, CTATGTCCTGCCTGGTTATTTCT (sense) and ACGAAIACIATGAAGAAIGGGCA (degenerate anti-sense primer) were used for the first amplification step while the PCR primers TATGTCCTGCCTGGTTATTTC (sense nested primer) and ACGAAGCAGATGAAGAAGGGGCACCACAT (anti-sense nested primer) were used for the second amplification step. The resulting 650 base-pair product was used as a probe to screen 1×10^6 plaques of a human liver library. One cDNA clone was identified. This clone encompassed the entire 5' coding region but lacked 50 amino acids from the C terminus. The 3' end of the human 5-HT$_{2B}$ receptor was generated using λgt10 flanking primer and the specific sense primer TGCCATGTACCAGAGTCCAATGAG. Based on the above data, two primers were designed to amplify the complete human 5-HT$_{2B}$ receptor gene. The full length 5-HT$_{2B}$ receptor was then amplified from human liver, kidney and pancreas cDNA using CTCGAGCTCAGCAAATGGCTCTCTCTTACAGAG (sense) and CTTCGCTAGCTATACATAACTAACTTGCTCTTC (anti-sense) primers. These PCR products were sub-cloned into SAC I and Nhe I ends of the poly-linker region of the mammalian expression vector pSW104, a derivative of pCD-SRα (Takebe *et al.*, 1988).

The full length human 5-HT$_{2C}$ receptor gene was amplified from human brain cDNA using the PCR primers TAACACTGAAGCAATCATGG (sense) and GACTGTGCTGTTCTTTCTCACAC (anti-sense). The 1.5 kB amplified product was cloned into PCRII (TA) cloning vector and then sub-cloned into the eukaryotic expression vector pSW104. The clone was sequenced and confirmed to be that of the 5-HT$_{2C}$ receptor.

5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors were transfected into Cos-7 cells using the lipofection method (Flegner *et al.*, 1987). Cells were harvested 65 h after transfection in phosphate buffered saline and centrifuged at 3000 *g* for 15 min. Cell pellets were frozen until required for radioligand binding studies.

Expression of rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors

NIH-3T3 mouse fibroblast cells expressing rat 5-HT$_{2C}$ or 5-HT$_{2A}$ receptors were grown in Dulbecco's Modified Essential Media with 10% foetal calf serum and 250 μg ml^{-1} Geneticin in 5% CO_2 until confluent. Cells were then harvested as described above.

Preparation of membranes from cells and human tissues

Tissues were either homogenized in 0.32 M sucrose using a motor driven (550 rpm. 10 strokes), glass teflon homogenizer (human cortex) or were disrupted with a polytron (human choroid plexus). Tissue homogenates were centrifuged at 900 *g* for 10 min and the pellets were washed once in 0.32 M sucrose. The supernatants were combined and then centrifuged at 12,000 *g* (cortex) or 45,000 *g* (choroid plexus) for 15 min. The resulting pellets were resuspended, incubated at 37°C for 15 min, and then centrifuged at 48000 *g* for 15 min. The final pellet was resuspended in a small volume of 50 mM Tris-HCl, divided into aliquots of 1 ml and stored at −70°C until required.

Cells containing expressed receptors were homogenized in 50 mM Tris-HCl with 5 mM Na_2EDTA (pH 7.4 at 4°C) using a Polytron tissue disrupter. The homogenates were centrifuged at 48,000 *g* for 15 min. The pellets were washed by re-suspension and centrifugation in homogenizing buffer and incubated at 37°C for 15 min. After an additional wash, the membranes were stored in 1 ml aliquots at −70°C.

Receptor binding methods

5-HT$_{2A}$ receptors were labelled with [^{3}H]-ketanserin in human cortex, in Cos-7 cells expressing a cloned human 5-HT$_{2A}$ receptor and in NIH3T3 cells expressing the rat 5-HT$_{2A}$ receptor. For competition binding studies the ligand concentration was approximately 0.1 nM. For saturation binding studies concentrations of radioligand ranged from 0.01 nM to 2.0 nM. Assays were conducted in 0.5 ml of assay buffer containing 50 mM Tris-HCl, 4 mM $CaCl_2$ and 0.1% ascorbic acid (pH 7.4 at 4°C). Non-specific binding was defined with 10 μM unlabelled ketanserin. After a 60 min incubation at 32°C, membranes were harvested onto filters treated with 0.1% polyethylenimine and the bound radioactivity was determined.

Human 5-HT$_{2B}$ receptors were labelled in Cos-7 cells as described above except that the radioligand was [^{3}H]-5-HT and that the assay buffer contained 10 μM pargyline and 0.1% ascorbic acid. For competition binding studies the radioligand concentration was approximately 0.4 nM while for saturation binding studies the concentration of [^{3}H]-5-HT ranged from 0.05 to 8 nM. Non-specific binding was defined with 10 μM 5-HT. Incubations were for 120 min at 4°C.

5-HT$_{2C}$ receptors were labelled in choroid plexus, Cos-7 cells expressing the human 5-HT$_{2C}$ receptor and in NIH3T3 cells expressing the rat 5-HT$_{2C}$ receptor. Assays were conducted as described for the 5-HT$_{2A}$ receptor except that the radioligand was [^{3}H]-mesulergine. The radioligand concentration for competition studies was approximately 0.2 nM while for saturation binding studies the concentration ranged from 0.1 to 18 nM. Nonspecific binding was defined with 10 μM unlabelled mesulergine.

Competition radioligand binding data were analysed with a four parameter logistic equation and iterative curve-fitting techniques to obtain estimates of the IC_{50} and Hill slope. K_d values, determined from saturation binding studies, where then used to calculate inhibition dissociation constants (K_i) according to the method described by Cheng & Prusoff (1973). Data were reported as the negative logarithm of the K_i (pK_i) rather than K_i since pK_i values tend to be normally distributed thus allowing presentation of mean and standard error of the mean.

Saturation binding data were analysed with the iterative nonlinear curve fitting programmes in 'Ligand' (Munson & Rodbard, 1980). Protein concentrations were determined with the Biorad colorimetrical method with bovine gamma-globulin as the standard (Bradford, 1979). Comparison of binding affinities of specific ligands in different tissues was made using independent *t* tests with a *P* value of less than 0.05 used as the criteria for statistically significant differences (CSS statistical package, Tulsa, OK U.S.A.).

Northern and reverse transcription PCR methods for determining receptor distribution

Northern analysis was performed on multiple tissue blots using RNA probes labelled with ^{32}P by random priming. Hybridization was conducted under conditions of medium strin-

gency (42°C, 50% formamide/5 × SSC, 5 × Denhardt's solution, 2% SDS and 100 μg ml^{-1} single strand DNA).

Reverse transcription - PCR was conducted using primers containing specific sequences appropriate for each receptor subtype. cDNA from human cerebral cortex, pancreas, kidney, heart, liver, spleen and whole brain were screened using a two step nested PCR amplification procedure. For the first cycle of PCR the samples were denatured at 94°C for 1 min, annealed at 50°C for 2 min and extended at 72°C for 3 min for 30 cycles. The second step of the PCR reaction was performed using 10 μl from the first step as the template and primers that were internal to those used in the first step to amplification. PCR conditions were identical except that the annealing temperature was increased to 55°C. The PCR products were run on a 1% agarose gel and bands were detected by staining with ethidium bromide. The identities of the PCR products were confirmed by sequencing.

Materials

[^{3}H]-mesulergine (75 Ci mmol^{-1}, 99% pure) and [^{3}H]-5-HT (91 Ci mmol^{-1}, 99% pure) were obtained from Amersham Corporation (Arlington Heights, IL, U.S.A.). [^{3}H]-ketanserin, (62 Ci mmol^{-1}, 99% pure) was obtained from Dupont-NEN (Boston, MA, U.S.A.) Methysergide was a gift from Sandoz Pharma Ltd (Basel, Switzerland). Paroxetine was a gift from SmithKline Beecham Pharmaceuticals (Essex, England). SB 200646A, (N-(1-methyl-5-indolyl)-*N'*-(3-pyridyl)urea hydrochloride (Forbes *et al.*, 1993)), its analogue SB 204741 (Baxter *et al.*, 1994b) and 5-carboxamidotryptamine (5-CT) were synthesized in the Institute of Organic Chemistry, Syntex Discovery Research (Palo Alto, CA U.S.A.). Other chemicals and reagents were purchased from Sigma Chemicals (St. Louis MO U.S.A.) or Research Biochemicals Incorporated (Natick, MA U.S.A.). NIH3T3 cells transfected with rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors were a generous gift from Dr David Julius, Department of Pharmacology, University of California, San Francisco, U.S.A. Post mortem brain tissues were obtained from the Cooperative Human Tissue Network (CHTN) and the National Disease Research Interchange (NDRI) or Dr Gavin Reynolds (University of Sheffield, United Kingdom). Human cDNA libraries and human poly-A RNA were obtained from Clontech, Palo Alto, CA U.S.A.. The TA cloning vector was obtained from Invitrogen, San Diego, CA U.S.A.

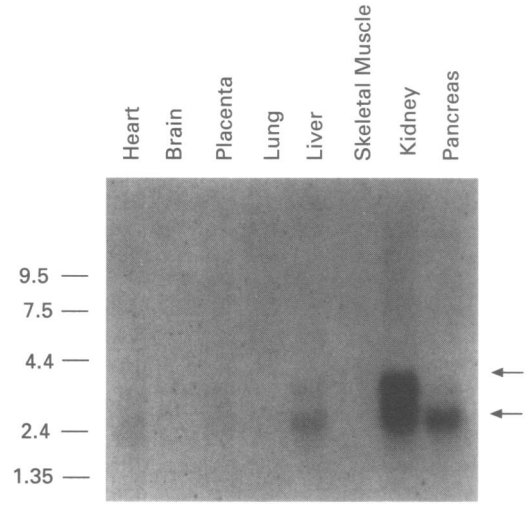

Figure 1 Northern blot of 5-HT$_{2B}$ receptor mRNA hybridization in different tissues. Note the 2.4 kB sized band which corresponds to previously detected 5-HT$_{2B}$ receptor mRNA as well as a second, 3.2 kB sized, band of hybridization in liver and kidney.

Table 1 PCR amplification of 5-HT$_2$ receptor subtypes

Human cDNA	*5-HT$_{2A}$*	*5-HT$_{2B}$*	*5-HT$_{2C}$*
Cerebral cortex	+ +	+ +	+ +
Whole brain	+ +	+ +	+ +
Spleen	+ +	+ +	+ +
Liver	+ +	+ +	+
Pancreas	+	+ +	+ +
Kidney	+	+ +	+
Heart	–	–	+ +

Human tissue cDNA was probed for the presence of 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptor cDNA using two steps of PCR with nested primers. (+ +) Indicates that a PCR product was detected after one round (30 cycles) of PCR. (+) Indicates that a PCR product was detected after two rounds of PCR amplification. (–) Indicates that a PCR product was not detected after two rounds of PCR amplification. PCR products were sequenced to confirm their identity.

Results

Cloning

The sequence of the cloned 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors were identical to those previously published except for a single nucleotide change in the human 5-HT$_{2A}$ receptor and two nucleotide changes in the human 5-HT$_{2C}$ receptor (Saltzman *et al.*, 1991). Sequencing of the appropriate genomic DNA de-

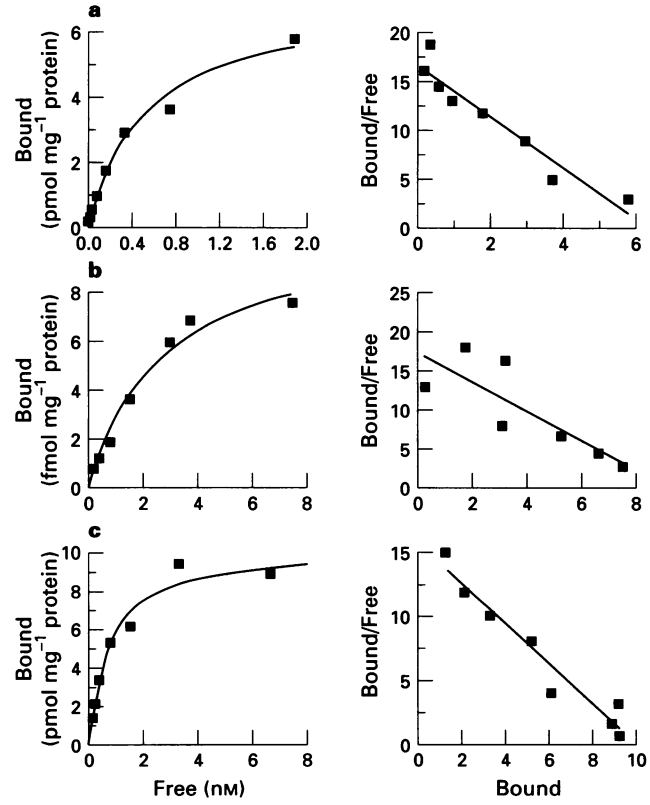

Figure 2 Saturation binding isotherms and Scatchard transformations for cloned human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors: (a) 5-HT$_{2A}$, (b) 5-HT$_{2B}$ and (c) 5-HT$_{2C}$ receptors. The radioligand and the concentrations used in each experiment are given in the Methods section. Note the change in scale and the lower expression for the 5-HT$_{2B}$ receptor.

Table 2 Saturation binding parameters for human 5-HT$_2$ receptors

Tissue	*Radioligand*	n	K_d (nM)	B_{max} (fmol mg^{-1} protein $^{-1}$)
Human 5-HT$_{2A}$ in Cos-7	[^{3}H]-Ketanserin	3	0.45 (0.017)	4870 (1180)
Human 5-HT$_{2B}$ in Cos-7	[^{3}H]-5-HT	2	2.29 (0.04)	10.3 (1.13)
Human 5-HT$_{2C}$ in Cos-7	[^{3}H]-mesulergine	3	0.67 (0.07)	8830 (1620)

Values are the mean, with s.e.mean in parentheses, from 2–3 determinations. Fitting the data to a two site model did not give a statistically better fit than a single site model.

Table 3 Affinities (pK$_i$) of ligands for human 5-HT$_2$ receptors in Cos-7 cells

	5-HT$_{2A}$	*5-HT$_{2B}$*	*5-HT$_{2C}$*
Yohimbine	< 6.0	6.4 (0.11)	< 5.0
mCPP	7.3 (0.17)	7.5 (0.20)	6.6 (0.11)
Mianserin	8.4 (0.07)	7.7 (0.14)	8.3 (0.03)
Spiperone	9.0 (0.15)	5.8 (0.05)	< 6.0
Ketanserin	8.5 (0.11)	6.2 (0.14)	6.7 (0.07)
Methysergide	8.4 (0.04)	8.1 (0.15)	8.8 (0.03)
Ritanserin	10.8 (0.07)	8.3 (0.29)	9.6 (0.02)
SB 200646a	< 5.0	6.2 (0.08)	6.4 (0.06)
SB 204741	< 5.0	7.1 (0.08)	5.7 (0.04)
5-HT	7.2 (0.09)	9.1 (0.16)	6.8 (0.09)

Values are the mean, with s.e.mean in parentheses, from at least three determinations.

monstrated these mutations to be PCR artifacts. These artifacts were corrected by site directed mutagenesis prior to expression of the receptors.

There was no difference between the sequences of the 5-HT$_{2B}$ receptors isolated from liver, kidney or pancreas. The sequences were identical to those previously found for human 5-HT$_{2B}$ receptors (Kursar *et al.*, 1994; Schmuck *et al.*, 1994).

Distribution

The distribution of 5-HT$_{2B}$ receptor mRNA was examined by Northern analysis. The 5-HT$_{2B}$ probe hybridized to a 2.4 kB band in pancreas, liver and kidney. The probe also hybridized to a 3.2 kB band in liver and kidney. In kidney the 3.2 kB band was the major product. 5-HT$_{2B}$ mRNA was not detected in heart, brain, placenta, lung or skeletal muscle (Figure 1).

The distribution of 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors was also examined by PCR amplification of tissue-specific cDNAs. A PCR product corresponding to the 5-HT$_{2A}$ receptor was found after one step of amplification in cerebral cortex, liver, spleen and whole brain. PCR products corresponding to this receptor were also detected in pancreas and kidney following two steps of amplification. PCR products corresponding to the 5-HT$_{2B}$ receptor were detected following one step of PCR amplification in cDNA from cerebral cortex, whole brain, pancreas, liver, kidney and spleen but not heart. PCR products corresponding to the 5-HT$_{2C}$ receptor were detected following one step of amplification in cerebral cortex, whole brain, spleen, pancreas and heart. PCR products corresponding to the 5-HT$_{2C}$ receptor were further detected in kidney and liver following two steps of amplification (Table 1).

Receptor characterization by radioligand binding

Specific, high affinity, saturable binding was detected in Cos-7 cells expressing each of the transiently human 5-HT$_2$ receptors,

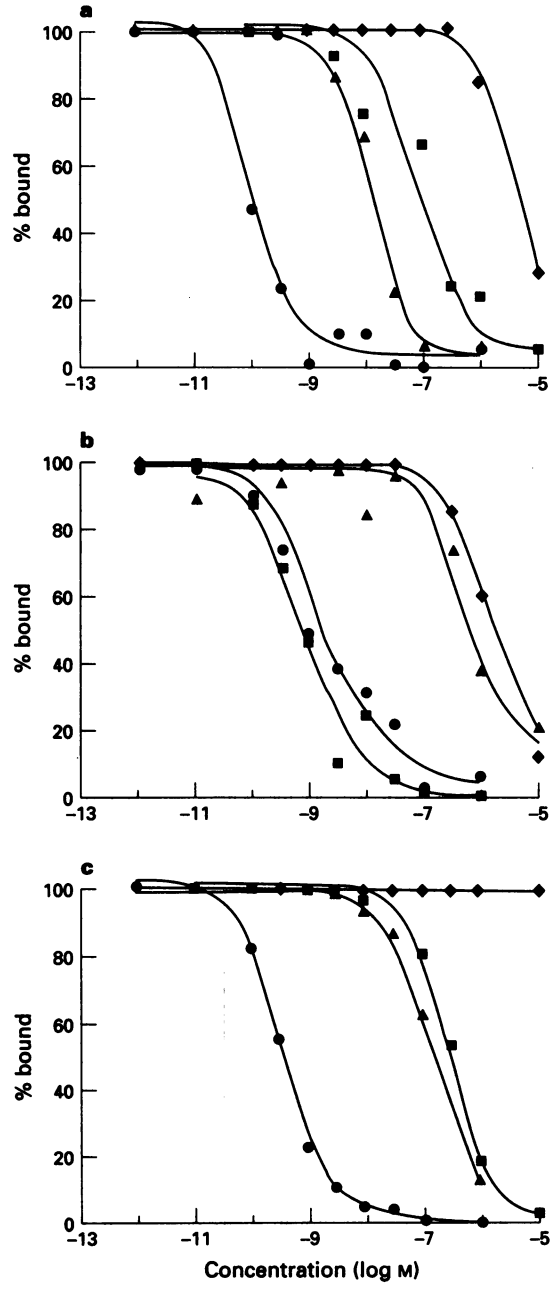

Figure 3 Competition binding curves for cloned human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors: (a) 5-HT$_{2A}$, (b) 5-HT$_{2B}$ and (c) 5-HT$_{2C}$ receptors. In each case, (●) ritanserin, (▲) ketanserin, (■) 5-HT and (◆) yohimbine. In each assay, in the absence of displacing ligand, specific binding accounted for greater than 85% of total binding.

Table 4 Affinities (pK_i) of ligands at native and cloned 5-HT$_{2A}$ receptors

	Human 5-HT$_{2A}$ clone		*Human cortex*		*Rat 5-HT$_{2A}$ clone*	
	pK_i	n_H	pK_i	n_H	pK_i	n_H
Yohimbine	< 6.0		< 5.0		< 5.0	
mCPP	7.3 (0.17)	0.90 (0.16)	6.4 (0.13)	0.84 (0.18)	6.4 (0.08)	0.93 (0.09)
Mianserin	8.4 (0.07)	0.84 (0.22)	8.2 (0.23)	0.49 (0.04)*	8.4 (0.06)	1.04 (0.09)
Spiperone	9.0 (0.15)	1.25 (0.18)	9.0 (0.32)	0.79 (0.06)	9.5 (0.06)	0.91 (0.03)
Ketanserin	8.5 (0.11)	1.10 (0.17)	9.4 (0.30)	0.79 (0.01)	8.9 (0.07)	1.05 (0.08)
Methysergide	8.4 (0.04)	1.22 (0.18)	8.4 (0.01)	0.90 (0.11)	8.8 (0.01)	1.04 (0.12)
Ritanserin	10.8 (0.07)*	1.32 (0.19)	9.0 (0.18)	0.94 (0.17)	9.5 (0.09)	1.21 (0.05)
SB 200646a	< 5.0		< 5.0		< 5.0	
5-HT	7.2 (0.09)	1.23 (0.24)	6.7 (0.04)	0.72 (0.09)	6.9 (0.27)	0.84 (0.07)

Values are the mean pK_i (s.e.mean) and mean Hill (n_H) slope (s.e.mean) of at least three determinations.

Table 5 Affinities (pK_i) of ligands at human and rat 5-HT$_{2B}$ receptors

	Human 5-HT$_{2B}$ clone		*Rat 5-HT$_{2B}$ clone*
	pK_i	n_H	pK_i
Yohimbine	6.4 (0.11)	1.21 (0.17)	7.3
mCPP	7.5 (0.20)	1.19 (0.22)	7.6
Mianserin	7.7 (0.14)	0.95 (0.16)	7.3
Spiperone	5.8 (0.05)	1.13 (0.09)	5.5
Ketanserin	6.2 (0.14)	0.87 (0.14)	5.4
Methysergide	8.1 (0.15)	1.21 (0.31)	8.2
Ritanserin	8.3 (0.29)	1.10 (0.44)	8.3
SB 200646a	6.2 (0.08)	1.17 (0.16)	—
5-HT	9.1 (0.16)	0.90 (0.02)	8.0

Data for binding to the rat 5-HT$_{2B}$ receptor were taken from Wainscott *et al.* (1993).

Table 6 Affinities (pK_i) of ligands at 5-HT$_{2C}$ receptors

	Human 5-HT$_{2C}$ clone		*Human choroid plexus*		*Rat 5-HT$_{2C}$ clone*	
	pK_i	n_H	pK_i	n_H	pK_i	n_H
Yohimbine	< 5.0		< 5.0		< 5.0	
Spiperone	< 6.0		6.3 (0.27)	0.47 (0.06)	6.1 (0.08)	0.91 (0.10)
Ketanserin	6.7 (0.07)	1.03 (0.02)	7.3 (0.01)	0.85 (0.11)	7.2 (0.06)	0.96 (0.14)
Methysergide	8.8 (0.03)	0.97 (0.06)	ND	ND	8.7 (0.10)	1.14 (0.03)
Ritanserin	9.6 (0.02)*	1.03 (0.14)	ND	ND	8.8 (0.11)	1.19 (0.07)
SB 200646a	6.3 (0.06)	1.02 (0.04)	ND	ND	6.6 (0.10)	0.83 (0.04)
Mianserin	8.3 (0.03)	1.04 (0.03)	ND	ND	8.7 (0.02)	0.95 (0.04)
mCPP	6.6 (0.11)	0.94 (0.09)	ND	ND	6.9 (0.12)	0.95 (0.05)

Values are the mean, with s.e.mean in parentheses, of at least three determinations. *The affinity of ritanserin was statistically ($P < 0.05$) greater at the human receptor than at the rat receptor. ND, not determined.

in NIH3T3 cells expressing rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, in human cortex and in human choroid plexus membranes. The saturation binding isotherms for [^{3}H]-ketanserin binding to Cos-7 cells expressing the human 5-HT$_{2A}$ receptors [^{3}H]-5-HT binding to Cos-7 cells expressing the human 5-HT$_{2B}$ receptor and for [^{3}H]-mesulergine binding to Cos-7 cells expressing the human 5-HT$_{2C}$ receptor were best fitted by a single site model (Figure 2). The affinities of [^{3}H]-ketanserin, [^{3}H]-mesulergine were appropriate for the labelling of 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors respectively (Table 2).

Competition binding curves were consistent with homogeneous populations of receptors in the Cos-7 cells expressing the human 5-HT$_2$ receptors in that Hill slopes did not differ from unity and in that the affinities of displacing ligands were consistent with the labelling of 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors in the appropriate tissues (Figure 3). The 5-HT$_{2A}$ receptor could be distinguished from 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors on the basis of a relatively high affinity for spiperone and ketanserin. The 5-HT$_{2B}$ receptor could be distinguished from the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors on the basis of a lower affinity for ritanserin and higher affinities for 5-HT and yohimbine. The compound with the highest selectivity for the 5-HT$_{2B}$ receptor was SB 204741 (Table 3). The 5-HT$_{2C}$ receptor could be distinguished from the 5-HT$_{2A}$ receptor on the basis of a low affinity for spiperone and ketanserin, and from the 5-HT$_{2B}$ receptor on the basis of a low affinity for yohimbine.

The affinities of ligands at the cloned human 5-HT$_{2A}$ receptor were compared to those at the cloned rat 5-HT$_{2A}$ receptor and to a binding site in human cortex labelled with [^{3}H]-ketanserin (Table 4). The affinities of most ligands at the human and rat receptors were similar. However, ritanserin and mCPP exhibited 3–10 fold higher affinity for the human receptor than for the rat receptor. Similar affinities were found for a site labelled with [^{3}H]-ketanserin in human cortex. However, ketanserin had a 10 fold higher affinity for the site in cortex than for the human cloned 5-HT$_{2A}$ receptor while ri-

tanserin had ~60 fold lower affinity in human cortex. Hill slopes of displacement curves generated with mianserin, in the cortical preparation, were less than unity.

The affinities of ligands at the human 5-HT$_{2B}$ receptor in Cos-7 cells were compared to those values previously reported for the rat 5-HT$_{2B}$ receptor (Table 5). The affinities of most ligands for the human receptor were similar to those at the rat binding site. However, the human 5-HT$_{2B}$ receptor had about a 10 fold lower affinity for yohimbine and about a 10 fold higher affinity for ketanserin and 5-HT.

The affinities of ligands at the human 5-HT$_{2C}$ receptor in the Cos-7 cells were compared to those at the cloned rat 5-HT$_{2C}$ receptor and to those at a [^{3}H]-mesulergine binding site in human choroid plexus (Table 6). The affinities of ligands at the human and rat receptor were similar. However, ritanserin had about 10 fold higher affinity for the human receptor. Values in the human choroid plexus were similar to those at the cloned receptor.

Discussion

In the present study, human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors were cloned, expressed in Cos-7 cells, and characterized. The distribution of the 5-HT$_2$ receptor gene products in a variety of tissues was then examined by both Northern blot hybridization and reverse transcription PCR.

The human 5-HT$_{2B}$ receptors cloned in the current study had nucleotide sequences identical to those previously reported (Saltzman *et al.*, 1991; Kursar *et al.*, 1994; Schmuck *et al.*, 1994). There is a high degree of homology between human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors and their counterparts in rats and mice (Cohen, 1989; Yang *et al.*, 1992; Foguet *et al.*, 1992a,b; Kursar *et al.*, 1992; Martin & Boess, 1994). However, the homology between subtypes of 5-HT$_2$ receptors is only about 40%, a somewhat surprising finding, given the similarities in the ligand binding and signal transduction properties of these receptors.

The expression of 5-HT$_{2B}$ receptor mRNA in different tissues was examined by both Northern analysis and the more sensitive reverse transcription PCR amplification method. Northern analysis resulted in the detection of 5-HT$_{2B}$ receptor mRNA (a 2.4 kB band) in pancreas, liver and kidney but not heart, brain or placenta. PCR amplification of 5-HT$_{2B}$ receptor cDNA confirmed the expression of this receptor in these tissues. PCR amplification also indicated the presence of 5-HT$_{2B}$ receptor mRNA in human cerebral cortex, whole brain and spleen but not heart. While estimating the level of mRNA expression by PCR is qualitative, these findings suggest relatively higher levels of expression of 5-HT$_{2B}$ receptor mRNA in liver, kidney and pancreas than in cortex and spleen with little or no expression in heart. This pattern of receptor expression is similar to that previously reported by Schmuck *et al.* (1994) or Kursar *et al.* (1994) with the exception that we failed to detect PCR product for this receptor in heart cDNA. Taken together, these findings suggest that 5-HT$_{2B}$ receptors are expressed in a wide range of tissues including brain.

The pattern of tissue distribution of the 5-HT$_{2B}$ receptor was different from that of the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors since 5-HT$_{2C}$ receptor cDNA was detected in heart cDNA and since 5-HT$_{2A}$ receptor cDNA was only detected following two cycles of PCR amplification in either the pancreas or the kidney. These findings suggest that human heart tissue may be enriched in 5-HT$_{2C}$ receptors while human kidney may be enriched in 5-HT$_{2B}$ receptors (assuming some correspondence between mRNA levels and receptor expression). The reason for the apparent differences in 5-HT$_{2B}$ receptor expression in heart found here, as compared to prior studies (Kursar *et al.*, 1994; Schmuck *et al.*, 1994), will need to be examined further.

In addition to the expected 2.4 kB band of hybridization found on Northern blots from liver and kidney, a 3.2 kB band was also detected in these tissues. Indeed, in the kidney this larger band was the most prominent band of hybridization. The identity of the 3.2 kB band was not determined although, given the stringency of conditions used in the hybridization assays, it probably had at least moderate homology with the 2.4 kB band. One possibility is that there are splice variants of the 5-HT$_{2B}$ receptor. Alternatively, these findings raise the possibility that human liver and kidney contain a novel receptor having homology with the 5-HT$_{2B}$ receptor.

Comparison of the receptor binding properties of the human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors confirmed the identities of these receptors and further defined their pharmacological characteristics. The human 5-HT$_{2B}$ receptor was distinguished from the human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors on the basis of a low affinity for ritanserin and a higher affinity for yohimbine. The receptor also had a higher affinity for 5-HT, although agonist-mediated conformational changes may have accounted for this higher affinity. The rank order of affinities of ligands binding to the human 5-HT$_{2B}$ receptor, as expressed in Cos-7 cells, was similar to that previously reported by Kursar *et al.* (1994); however, in the current study a 10 fold higher affinity of the receptor for 5-HT was detected. The reason for this difference was not determined but differences in the cell lines used to express the receptor may be one explanation. The most selective 5-HT$_{2B}$ receptor ligand to be tested was SB 204741 with approximately 20 fold selectivity for the human 5-HT$_{2B}$ receptor as compared to the human 5-HT$_{2C}$ receptor. These data confirm preliminary reports by Baxter *et al.* (1994b) and further define the binding pharmacology of human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors.

Comparison of the affinities of ligands at the cloned human and rat 5-HT$_2$ receptors extend previous indications of interspecies heterogeneity in 5-HT$_2$ receptor pharmacology (Pazos *et al.*, 1985; Kao *et al.*, 1992; Nelson *et al.*, 1993; Choi *et al.*, 1994). Thus for 5-HT$_{2A}$ receptors, mCPP and ritanserin were shown to have 10–20 fold higher affinity for the cloned human receptor in comparison to the cloned rat receptor. Whereas, for 5-HT$_{2C}$ receptors, ritanserin was nearly 10 fold selective for the human receptor, in general, comparison of affinities of ligands at rat and human 5-HT$_{2B}$ receptors revealed no striking differences in the affinities of antagonists, although yohimbine tended to have a higher affinity for the rat receptor while ketanserin had a higher affinity at the human receptor. 5-HT also had about 10 fold higher affinity for the human 5-HT$_{2B}$ receptor but this may be a consequence of the effect of different temperatures on agonist binding affinity (Wainscott *et al.*, 1993).

To validate data obtained with the cloned human receptors, binding assays were established in human cortex tissue using [^{3}H]-ketanserin and human choroid plexus using [^{3}H]-mesulergine (Pazos *et al.*, 1984). The affinity of displacing ligands for human choroid plexus were consistent with the labelling of a 5-HT$_{2C}$ receptor but the difficulty in obtaining sufficient quantities of this tissue precluded a more extensive evaluation. The affinity values for ligands in human cortex membranes, in general, matched those obtained with the cloned human 5-HT$_{2A}$ receptor. However, one striking difference was found with ritanserin in that this compound was nearly 100 fold more potent for the cloned receptor than for the receptor labelled in human membranes. Conversely, ketanserin was found to have approximately 10 fold higher affinity for the cloned receptor. Whether these differences reflect an artifact of the cloned receptor or the binding of the ligand to heterogeneous binding sites in cerebral cortical membranes remains to be determined. The observation that Hill slopes of the competition curves, obtained in human cortex, tended to be less than unity supports the latter idea. Nonetheless, these discrepancies between affinity estimates in human cortical membranes and human receptors expressed in cell lines raise a cautionary note to the reliance on only cloned receptors for predicting ligand affinity at endogenous receptors.

In summary, these studies further characterize the distribution and receptor binding properties of human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors. Based on the expression of mRNA, these receptors have unique and heterogeneous pat-

terns of distribution in peripheral and central tissues. The 5-HT$_{2B}$ receptor was found to be widely distributed in numerous tissues with a distribution pattern distinct from that of 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors. The ligand binding specificity of these receptors generally matched that of their counterparts cloned from rat tissues although differences in the affinities of several ligands support previous indications of pharmacologically distinct species homologues. Finally, the finding of two distinct bands of hybridization on the Northern blots raises the possibility of splice variants or subtypes of 5-HT$_{2B}$ receptors.

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Polydeoxyribonucleotides and nitric oxide release from guinea-pig hearts during ischaemia and reperfusion

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1 Two polydeoxyribonucleotides, produced by the controlled hydrolysis of DNA of mammalian lung (defibrotide and its lower molecular weight fraction, P.O. 085 DV), were studied for their ability to modify the release of nitrite and the coronary flow in perfusates collected from isolated, normally perfused hearts of guinea-pigs and from hearts subjected to regional ischaemia and reperfusion.

2 In guinea-pig normally perfused hearts, both defibrotide (DFT) and its fraction, P.O. 085 DV, increase the amount of nitrite appearing in perfusates in a concentration-dependent fashion. At the highest concentration studied (10^{-6} M), P.O. 085 DV was more effective than DFT. A concomitant increase in the coronary flow was observed.

3 The increase in nitrite in perfusates and the increase in coronary flow induced by both DFT and P.O. 085 DV were significantly reduced by N^G-monomethyl-L-arginine (L-NMMA, 10^{-4} M), an inhibitor of nitric oxide synthase (NOS).

4 The endothelium-dependent vasodilator, acetylcholine (ACh), enhances the formation of nitrite and the coronary flow. Both the increase in coronary flow and in the formation of nitrite were significantly reduced by L-NMMA (10^{-4} M).

5 In guinea-pig hearts subjected to ischaemia and reperfusion, the effect of both compounds in increasing the amount of nitrite in perfusates was more evident and more pronounced with P.O. 085 DV.

6 Reperfusion-induced arrhythmias were significantly reduced by both compounds to the extent of complete protection afforded by compound P.O. 085 DV.

7 The cardioprotective and antiarrhythmic effects of DFT and P.O. 085 DV are discussed.

Keywords: Defibrotide; P.O. 085 DV; guinea-pig hearts; coronary flow; ischaemia-reperfusion; N^G-monomethyl-L-arginine (L-NMMA)

Introduction

Vascular endothelial cells synthesize prostacyclin (PGI_2) and nitric oxide (NO), which are involved in maintaining the surface of the normal, healthy endothelium in a non-thrombogenic state (Lefer *et al.*, 1978; Schrör *et al.*, 1981; Simpson *et al.*, 1987; Palmer *et al.*, 1987; Botting & Vane, 1989). PGI_2 and NO are released together following stimulation of endothelial receptors and act synergistically to defend endothelial cells against blood-borne cells and chemicals. The breakdown of this balance may result in endothelial damage and subsequent myocardial injury.

Ischaemia and reperfusion can modify the generation of both PGI_2 and NO from vascular endothelial cells. However, no clear cut evidence is available on the generation of PGI_2 in the regional, ischaemic, stunned myocardium of experimental animals (Schrör & Hohlfeld, 1993), even though PGE_1 has been found to improve tissue survival after ischaemic injury (Berti *et al.*, 1987; 1991). As far as NO is concerned, in the guinea-pig isolated heart the amount of nitrite (one of the breakdown products of NO) released during local ischaemia was significantly lower than in control, sham-operated hearts (Masini *et al.*, 1992b).

Reperfusion of the ischaemic heart is associated with the formation of oxygen free radicals which facilitate calcium overload and excerbate ischaemic injury (Hearse *et al.*, 1977). It has also been speculated that superoxide may form a harmful radical, peroxynitrite, by interacting with NO (Beckman *et al.*, 1990; Sato *et al.*, 1993). Therefore, ischaemia reperfusion may entail an alteration in the production of eicosanoids, NO, and the generation of potentially harmful free radicals (Kaul *et al.*, 1993).

Defibrotide (DFT) is the sodium salt of a single stranded polydeoxyribonucleotide obtained by controlled depolymerization of deoxyribonucleic acid of bovine lungs; it has profibrinolytic and antithrombotic activity. In animal models of acute myocardial ischaemia and reperfusion, DFT exerts considerable beneficial effects on the biochemical and functional parameters of post-ischaemic tissue injury (Thiemermann *et al.*, 1985; 1989a, b). However, the mechanism by which DFT protects the ischaemic-reperfused myocardium is still largely unknown. We have recently reported that DFT decreases the release of histamine and lactate dehydrogenase (LDH) in an *in vitro* guinea-pig model of regional cardiac ischaemia reperfusion, offering significant protection against tissue damage by reducing the calcium overload (Lupini *et al.*, 1992). In the same experimental model, we have also shown that the release of histamine and LDH, the generation of malonyldialdehyde, and calcium overload which occur during ischaemia reperfusion were increased by blockers of nitric oxide synthase (NOS) and decreased by NO donors (Masini *et al.*, 1992b). These results suggest that NO, which is known to inhibit the release of histamine from isolated and residential mast cells (Masini *et al.*, 1992a,b; 1994), could influence post-ischaemic myocardial injury by modulating the release of histamine during ischaemia-reperfusion.

The aim of the present study was to evaluate whether DFT could influence the coronary flow and generation of NO in

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guinea-pig isolated hearts normally perfused or subjected to local ischaemia-reperfusion, and to compare its effects with those produced by a lower molecular weight oligonucleotide, with more pronounced antithrombotic and profibrinolytic activity.

Methods

Animal preparation and coronary artery ligation

Male guinea-pigs were anaesthetized with fentanyl (5 mg kg^{-1}, i.p.), intubated and ventilated with room air using a Palmer pump with a stroke volume of 20 ml kg^{-1} at a rate of 50 strokes min^{-1}. After thoracotomy, the pericardium was opened and 00 braided silk sutures (Ethicon) were placed in two different places around the left anterior descending (LAD) coronary artery together with small silicone rings to permit the release of ligation. The heart was quickly excised and then set up as a Langendorff preparation. The heart was perfused with Tyrode buffer at a constant pressure of 40 cm water. The solution was kept at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2 giving a final pH of 7.48. After 60 min of stabilization, ischaemia was produced by tying the two silk sutures around the LAD coronary artery for 20 min. After this period, the two ligatures were released to allow reperfusion of the ischaemic tissue. Heart rate and contraction were recorded by means of pressure transducers connected to a clip on the apex of the heart and recorded on a thermic writing oscillograph. The onset and type of arrhythmias were monitored by means of a bipolar surface electrogram. Coronary perfusates were collected over 5 min intervals in graduated tubes, to evaluate coronary flow rates. All the perfusates were lyophilized and then resuspended in a fixed volume of water for nitrite (NO_2^-) determination.

Nitrite analysis

Release of NO_2^- in perfusates from the hearts was measured spectrophotometrically by the Griess reaction. A determined volume of each perfusate was lyophilized and resuspended in 4 ml of water. The samples were centrifuged and each supernatant allowed to react with Griess reagent (1% sulphanilamide/0.1% naphthylethylene diamide dihydrochloride/2.5% H_3PO_4) to form a stable chromophore which absorbs at 546 nm. The NO_2^- concentration was determined with sodium nitrite as a standard. Results were expressed as nmol of NO_2^- ml^{-1} (Salvemini *et al.*, 1992). Incubation of both compounds with the Griess solution failed to yield any positive reaction.

Classification of arrhythmias

The heart was considered to be in ventricular fibrillation (VF) if an irregular modulating baseline was present on the electrocardiogram. Supraventricular tachycardia (VT) was defined as three or more consecutive premature ventricular contractions. This classification included repetitive monomorphic VT and ventricular flutter, which is difficult to differentiate from rapid VT.

The heart was considered to be in normal sinus rhythm if normal sinus complexes occurring at a regular rate were present on the electrocardiogram, including ventricular tachycardia and sinus bradycardia (Gelvan *et al.*, 1991). The values are reported as percentage of arrhythmic hearts out of the total number.

Drugs and solutions

The composition of the modified (Dieterich & Löffelholz, 1977) Tyrode solution was (mM): Na^+ 149.3, K^+ 2.7, Ca^{2+} 1.8, Mg^{2+} 1.05, Cl^- 145.4, HCO_3^- 11.9, H_3PO_4 0.3 and (+)-glu-

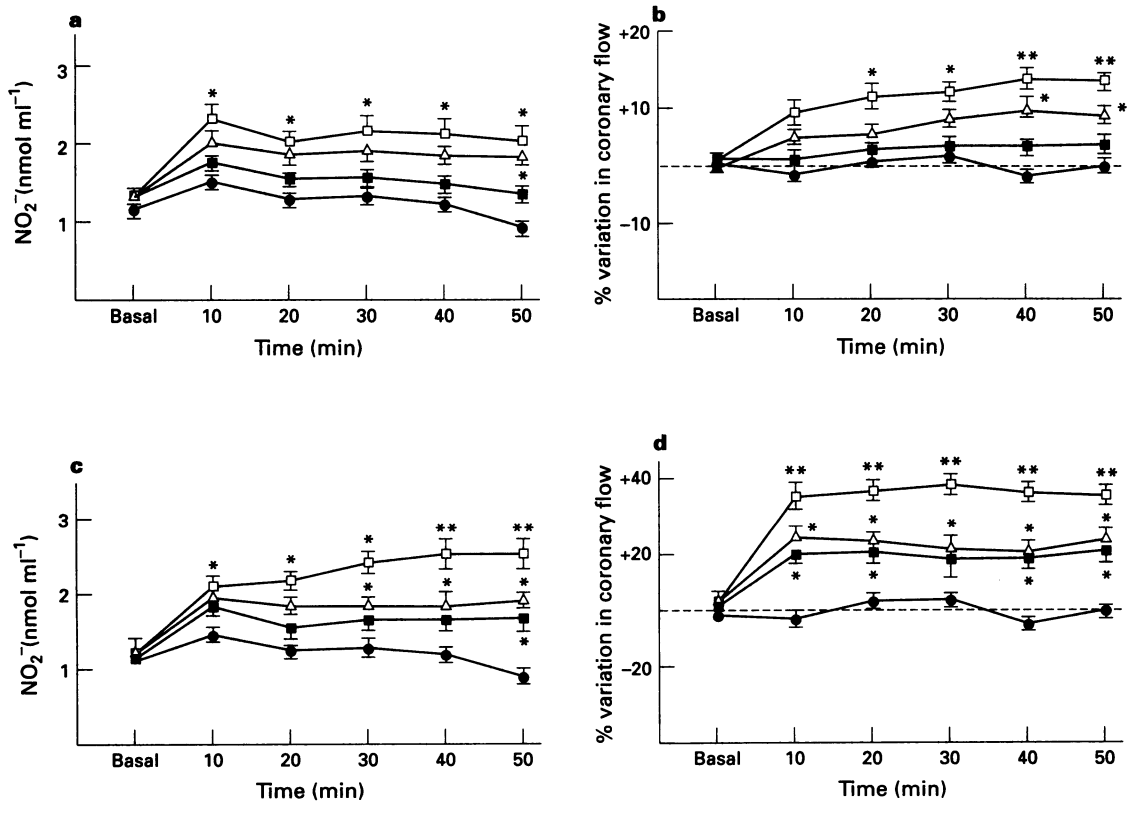

Figure 1 Effect of defibrotide (DFT) and of P.O. 085 DV on the release of nitrite (NO_2^-) in perfusates (a and c) and on % variation of coronary flow (b and d) in guinea-pig isolated hearts. (a and b): (●) Control hearts; (■) DFT 10^{-8} M; (△) 10^{-7} M; (□) 10^{-6} M; (c and d): (■) P.O. 085 DV 10^{-8} M; (△) 10^{-7} M; (□) 10^{-6} M. All points are a mean ± s.e.mean, $n=10$. *$P<0.05$; **$P<0.01$.

cose 5.6. Sulphanilic acid, N-1-naphthylethylene diamine dihydrochloride, sodium nitrite, physostigmine sulphate and acetylcholine chloride (batch number: 2340078) were obtained from Sigma (Poole, Dorset, U.K.). DFT, molecular wt. 29,400, and a lower molecular weight oligonucleotide (P.O. 085 DV, molecular wt. 6,690) were obtained from Crinos Research Lab., Villa Guardia, Italy.

Statistical analysis

Results are presented as the mean ± s.e.mean. For statistical comparisons, ANOVA, followed by Student's unpaired *t* test, were used. The Chi-squared test was used to detect statistically significant differences between arrhythmias in control and drug-treated preparations. Differences were regarded as significant if $P<0.05$.

Results

When DFT was added to the perfusion fluid in guinea-pig isolated perfused hearts it significantly increased the release of nitrite in the perfusates. The increase in nitrite release was dependent upon the concentration of DFT, ranging from a 15% increase over the control values at a DFT concentration of 10^{-8} M, to a 35% increase at 10^{-7} M, to a maximum increase of 60% at a DFT concentration of 10^{-6} M (Figure 1). The time course of the release of nitrite induced by DFT was steady at concentrations of 10^{-8} and 10^{-7} M. At the highest concentration studied (10^{-6} M), the most pronounced effect was detected within the first 10 min, followed by a small decline during the following 10 min, and by a steady increase during the subsequent observation time (Figure 1a). A corresponding increase in the coronary outflow was observed to run in parallel with the augmented release of nitrite in perfusates (Figure 1b).

The lower molecular weight oligonucleotide (P.O. 085 DV) was significantly more active. In fact, P.O. 085 DV increased the amount of nitrite appearing in perfusates almost to the same extent as DFT, at concentrations of 10^{-8} and 10^{-7} M (Figure 1c). However, at the highest concentration studied (10^{-6} M), the nitrite release reached a 95% increase over the control values. The time-course of the release of nitrite induced by P.O. 085 DV was steady at any given concentration (Figure 1c). A corresponding increase in the coronary outflow was observed to run in parallel with the augmented release of nitrite in perfusates (Figure 1d). Neither DFT nor P.O. 085 DV produced any changes in cardiac rate or contractility. When the hearts were perfused for 30 min with N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS devoid of any antagonistic effect on muscarinic receptors (Buxton *et al.*, 1993), a significant diminution in the formation of nitrite and of coronary flow was observed (Figure 2). Under these circumstances, the increase in nitrite in the perfusates and the increase in coronary flow induced by both DFT (Figure 2a and b) and P.O. 085 DV (Figure 2c and d) were significantly reduced. The effects of DFT and of P.O. 085 DV on nitrite formation and coronary flow were compared with those of an endothelium-dependent vasodilator, acetylcholine. Acetylcholine was perfused in the presence of physostigmine (10^{-7} M) to avoid prompt inactivation by acetylcholinesterases. At the concentration used (10^{-7} M), physostigmine was devoid of any significant action on both the coronary flow and nitrite formation. Under these circumstances, acetylcholine increased the amount of nitrite appearing in the perfusates and the coronary flow, at concentrations of the same order as those of DFT and P.O. 085 DV (10^{-7}–10^{-8} M; Figure 3a and b). At high con-

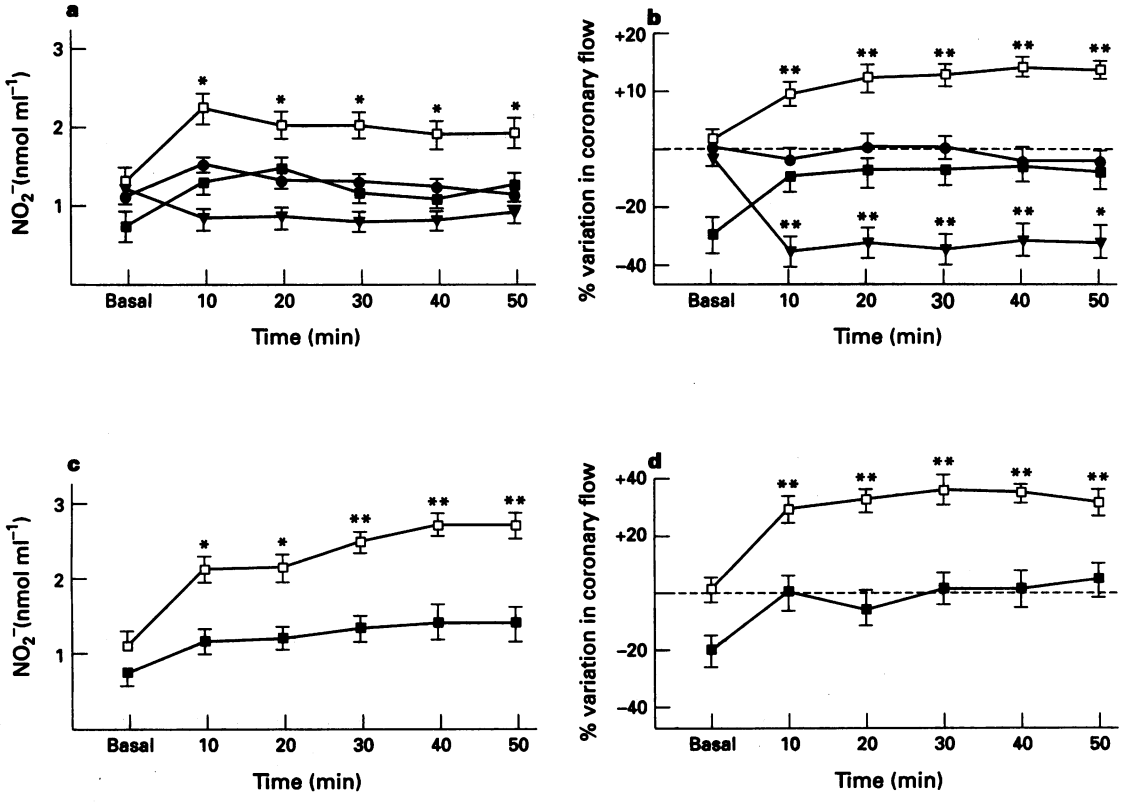

Figure 2 Effect of N^G-monomethyl-L-arginine (L-NMMA, 10^{-4} M) on defibrotide (DFT,' 10^{-6} M, a and b) and P.O. 085 DV (10^{-6} M, c and d)-induced variation of nitrite (NO_2^-) release in the perfusates (a and c) and of coronary flow (b and d) in guinea-pig isolated hearts. (a and b): (●) Control hearts; (□) DFT 10^{-6} M; (▼) L-NMMA 10^{-4} M; (■) DFT 10^{-6} M in the presence of L-NMMA 10^{-4} M; (c and d): (□) P.O. 085 DV 10^{-6} M; (■) P.O. 085 DV 10^{-6} M in the presence of L-NMMA 10^{-4} M. All the points are a mean ± s.e. mean, $n=4$. $^*P<0.051$ $^{**}P<0.01$.

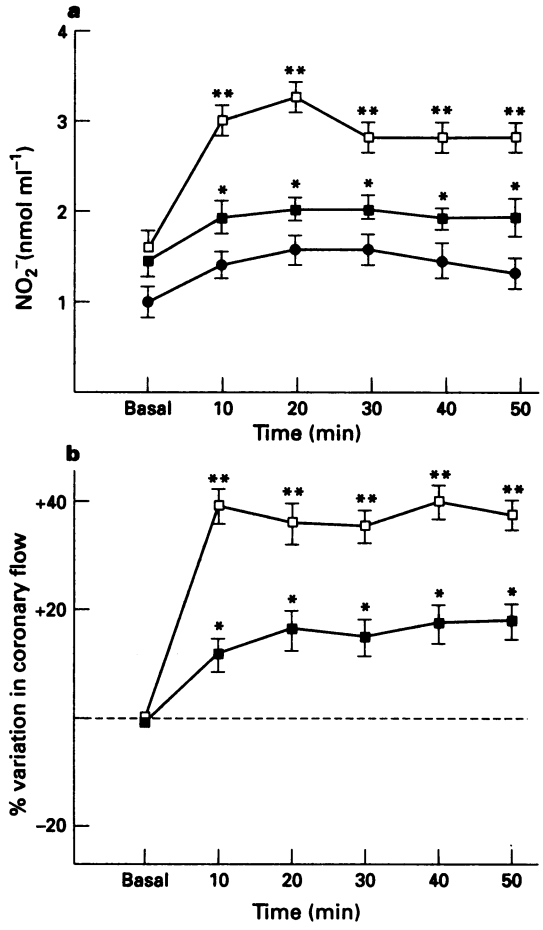

Figure 3 Effect of acetylcholine (ACh) in the presence of physostigmine 10^{-7} M on the release of nitrite (NO_2^-) in perfusates (a) and on % variation of coronary flow (b) in guinea-pig isolated hearts. (●) Control hearts; (■) ACh 10^{-8} M; (□) ACh 10^{-7} M. All the points are the mean ± s.e. mean, $n=5$. $^*P<0.05$; $^{**}P<0.01$.

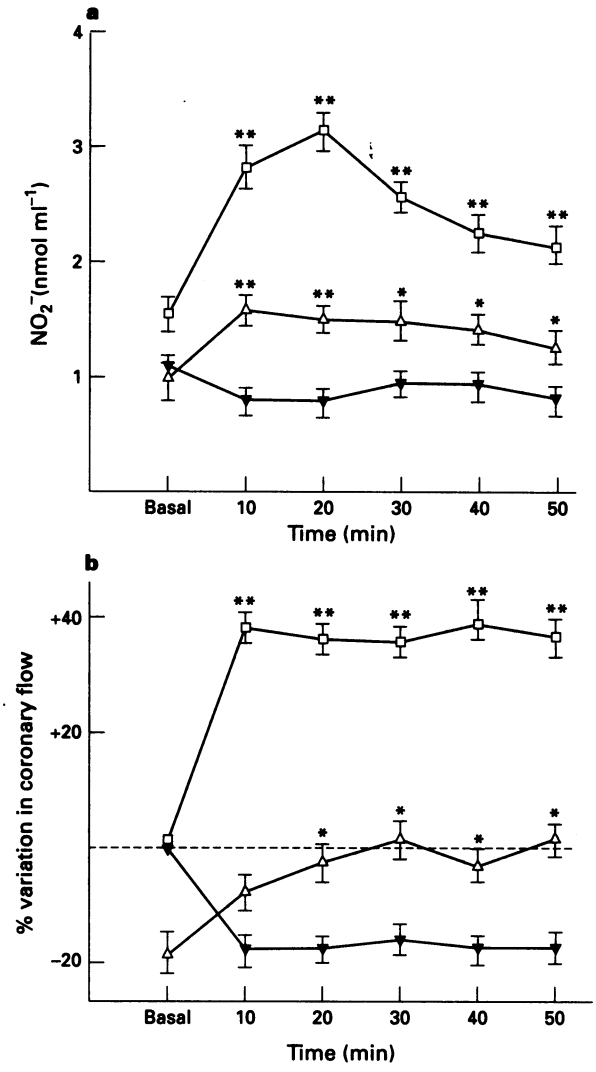

Figure 4 Effect of NG-monomethyl-L-arginine (L-NMMA, 10^{-4} M) on acetylcholine (ACh, 10^{-7} M)-induced variation of nitrite (NO_2^-) release in the perfusates (a) and of coronary flow (b) in guinea-pig isolated hearts. (□) ACh 10^{-7} M in the presence of physostigmine 10^{-7} M; (▼) L-NMMA 10^{-4} M; (△) ACh 10^{-7} M plus physostigmine 10^{-7} M in the presence of L-NMMA 10^{-4} M. All the points are the mean ± s.e. mean, $n=4$. $^*P>0.05$; $^{**}P>0.01$.

centrations (10^{-6} M), acetylcholine produced the expected inhibition of frequency and strength of contraction. As in the case of DFT and P.O. 085 DV, the acetylcholine-induced increase in nitrite formation was significantly reduced by L-NMMA (10^{-4} M; Figure 4a). The acetylcholine-induced increase in coronary flow was also reduced by prior perfusion with L-NMMA (Figure 4b).

In guinea-pig isolated perfused hearts submitted to local ischaemia-reperfusion, the present experiments have confirmed our previous observations (Masini *et al.*, 1992b) of the decrease in the amount of nitrite appearing in perfusates during ischaemia-reperfusion (Figure 5). It is unlikely that the observed diminution of the nitrite release during ischaemia-reperfusion could be accounted for by changes in coronary outflow. In fact, in our experimental model of regional ischaemia-reperfusion, no changes occurred in overall coronary outflow during ischaemia-reperfusion. Under these circumstances, both DFT and P.O. 085 DV produced a substantial increase in the amount of nitrite appearing in perfusates (Figure 5a and c). The percentage increase in nitrite release induced by DFT and P.O. 085 DV in perfusates collected from ischaemic and reperfused hearts was significantly higher than that observed in normally perfused hearts. No substantial differences were observed in the potency of DFT and P.O. 085 DV in restoring the nitrite overflow in ischaemic-reperfused hearts. As in the case of normally perfused hearts, a corresponding increase in the coronary outflow was observed (Figure 5b and c) to run in parallel with the augmented release of nitrite in the perfusates. No changes in cardiac contractility were detected.

Severe reperfusion arrhythmias were a characteristic feature of our experimental model (Mannaioni & Masini, 1988). A powerful and concentration-dependent antiarrhythmic effect was observed with both DFT and P.O. 085 DV (Table 1). P.O. 085 DV was significantly more active than DFT in abating reperfusion arrhythmias.

Discussion

The present experiments show that two polydeoxyribonucleotides of different molecular weight increase the nitrite overflow in guinea-pig isolated perfused hearts. The effect is amplified in guinea-pig hearts submitted to ischaemia-reperfusion due to the decrease in the amount of nitrite appearing in the perfusates during the ischaemic-reperfusion phase (Masini *et al.*, 1992b). Heart rate and contraction are left unchanged, while reperfusion arrhythmias are significantly abated. In terms of potency, the lower molecular weight P.O. 085 DV is more active than the higher molecular weight DFT in increasing the nitrite overflow and has a more significant cardioprotective effect, as shown by the more pronounced antiarrhythmic activity.

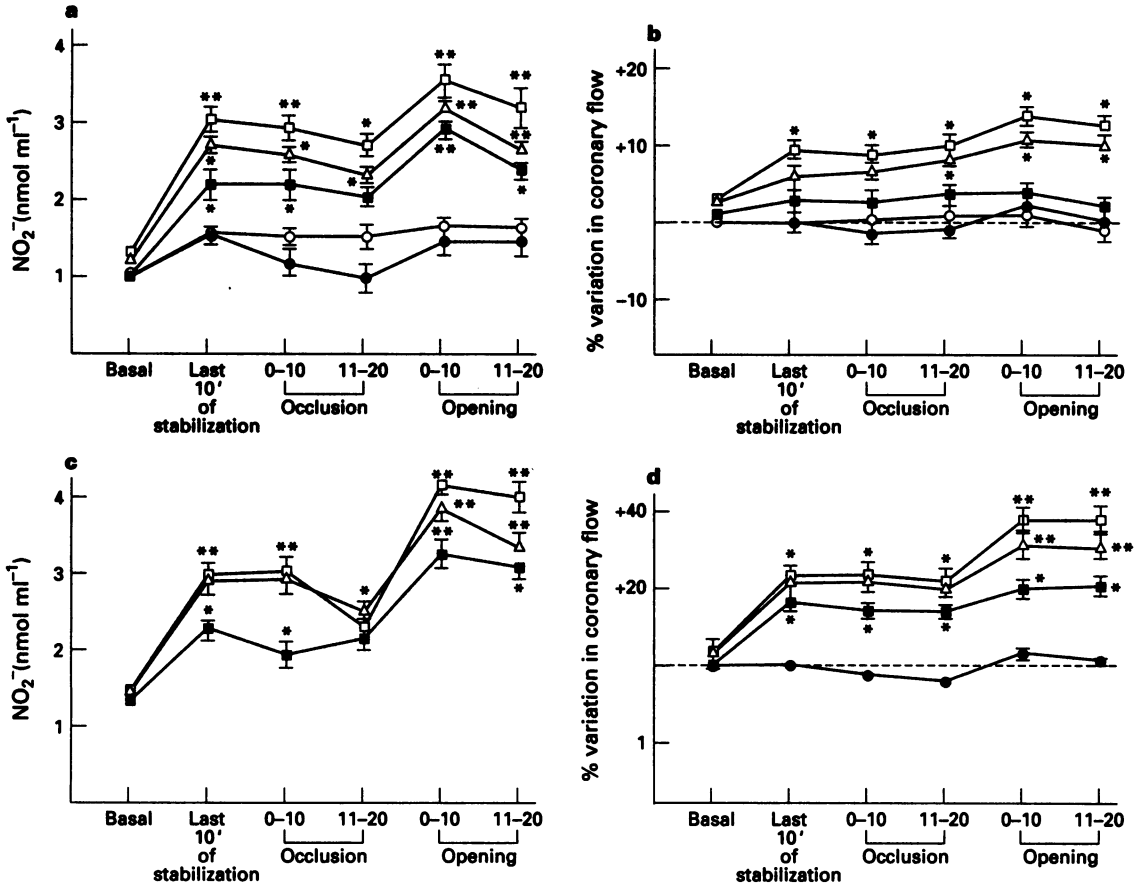

Figure 5 Effect of defibrotide (DFT) and P.O. 085 DV on the release of nitrite (NO_2^-) in the perfusates (a and c) and on % variation of coronary flow (b and d) in guinea-pig isolated hearts, during ischaemia and reperfusion (●) and sham-operated hearts (○). DFT 10^{-8}M (■); 10^{-7}M (△); 10^{-6}M (□). P.O. 085 DV 10^{-8}M (■); 10^{-7}M (△); 10^{-6}M (□). All the points are the mean ± s.e. mean, $n = 10$. $^*P < 0.05$; $^{**}P < 0.01$.

Table 1 Effect of defibrotide (DFT) and its fraction P.O. 085 DV on ischaemia-reperfusion induced ventricular arrhythmias in guinea-pig isolated perfused hearts

	Concentration of drugs (M)	*Incidence of arrhythmias (%)* *Ischaemic phase*	*Reperfusion phase*
Control (6)		67	100
DFT (8)	10^{-8}	37*	62
DFT (8)	10^{-7}	25*	37*
DFT (8)	10^{-6}	25*	37*
P.O. 085 DV (8)	10^{-8}	25*	25**
P.O. 085 DV (8)	10^{-7}	12*	0**
P.O. 085 DV (8)	10^{-6}	0**	0**

The number of experiments is given in parentheses.
$^*P < 0.05$; $^{**}P < 0.01$.

The observed changes in the amount of nitrite detected in the perfusates are actual changes in nitrite production. In fact, the inhibition of synthesis of NO by L-NMMA, resulted in a significant diminution of the effects of both compounds on the coronary flow and on the generation of NO. Moreover, the effects observed with the two oligonucleotides were almost identical to those of acetylcholine, an endothelium-dependent vasodilator, which induces similar changes in the coronary flow and NO formation. These effects were decreased by the NOS inhibitor L-NMMA, to the same extent as those induced by the two oligonucleotides. Considering nitrite as the end product of the metabolic oxidation of NO, it is conceivable that the two polydeoxyribonucleotides increase the generation of NO from the heart, both in normally perfused preparations and in hearts submitted to ischaemia-reperfusion, with a concomitant beneficial effect on reperfusion arrhythmias.

The present experiments cannot determine whether the increase in nitrite overflow is accounted for by the inducible or constitutive NOS. In any case, many reports indicate that NO can function as a protective agent against the ischaemic damage to both brain and heart (Morikawa *et al.*, 1992; Siegfried *et al.*, 1992; Wink *et al.*, 1993). Therefore, the cardioprotective/antiarrhythmic effect of DFT and P.O. 085 DV may be mediated by increased generation of NO, tentatively acting via a histamine-related mechanism. In fact, in the same experimental model we have recently demonstrated that DFT sig-

nificantly decreases both histamine and lactate dehydrogenase release during ischaemia-reperfusion; DFT also reduces cardiac mast cell degranulation and calcium overload in the ischaemic area (Lupini *et al.*, 1992). Taken together, these observations suggest that polydeoxyribonucleotides increase the generation of NO which in turn decreases the release of histamine from resident cardiac mast cells induced by ischaemia-reperfusion (Mannaioni & Masini, 1988), producing a salvage of the heart from the arrhythmogenic action of histamine. The arrhythmogenic effects of histamine are well known both in experimental animals (Mannaioni, 1972; Wolff & Levi, 1986) and in man (Lorenz & Doenicke, 1978), and NO donors significantly inhibit the release of histamine from mast cells, which is increased by NOS inhibitors (Masini *et al.*, 1994).

The two polydeoxyribonucleotides may increase the nitrite overflow in normally perfused and in ischaemic guinea-pig hearts by direct stimulation of cardiac NO-generating cells, or by an indirect mechanism, mediated by an increase in the release of prostanoids. Recently, DFT has been classified as a prostacyclin-mimetic drug (Gryglewski *et al.*, 1989; Schrör & Hohlfeld, 1993; Gross, 1993) because of its ability to stimulate PGI_2 formation in mini pigs subjected to coronary artery occlusion-reperfusion (Hohlfeld *et al.*, 1993). DFT reduces the ischaemic contracture due to low perfusion of rabbit isolated hearts by releasing PGE_2 and PGI_2, since indomethacin infusion completely abolishes the anti-ischaemic activity of DFT, its ability to increase the generation of prostaglandin, and its effect in preventing calcium overload (Berti *et al.*, 1992). The enhanced prostanoid synthesis induced by DFT has been recently accounted for by a mechanism possibly linked to the stimulation of adenosine receptors (Bianchi *et al.*, 1991; 1993). Our recent results are in keeping with these hypotheses (Lupini *et al.*, 1992), as they show that indomethacin abolishes the protection afforded by DFT from the release of histamine and LDH, calcium overload, degranulation of cardiac mast cells and reperfusion arrhythmias in guinea-pig ischaemic reperfused hearts.

In conclusion, DFT is a new profibrinolytic, antithrombotic substance, showing a definite pharmacological and clinical pharmacological profile (Ulutin *et al.*, 1990), which reduces the infarct size in a variety of models of experimental myocardial ischaemia and reperfusion (cats, pigs and rabbits; Thiemermann *et al.*, 1989a, b). Justification for seeking more active compounds among the oligonucleotide family is supported by the more pronounced effect of the PO 085 DV fraction obtained in our experiments. Thus, the oligonucleotide family may represent a new strategy for treatment of myocardial infarction (Schrör *et al.*, 1989), due to their effects in reducing oxygen demand via the adenosine receptors, by increasing coronary flow through the release of PGI_2 and NO, and by protecting the heart from the cardiac toxicity of histamine.

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Contribution of P_2-purinoceptors to neurogenic contraction of rat urinary bladder smooth muscle

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1 The contribution of P_2-purinoceptors to neurogenic contraction was investigated in rat urinary bladder smooth muscle by measurement of isotonic tension.

2 Contraction of rat urinary bladder smooth muscle induced by electrical stimulation was decreased to $84.19 \pm 3.90\%$ of the control ($n=16$) in the presence of atropine (1 μM), which was further decreased to $38.80 \pm 2.75\%$ of the control ($n=49$) in the presence of both atropine and 10 μM α,β-methylene adenosine 5'-triphosphate (α,β-Me ATP).

3 The contractile response induced by electrical stimulation in the presence of atropine and α,β-Me ATP was decreased to $27.81 \pm 4.07\%$ ($n=23$) and $26.63 \pm 5.01\%$ ($n=15$) of the control, by the addition of 100 μM cibacron blue 3GA and 100 μM suramin, respectively. The application of 100 μM adenosine 5'-*o*-2-thiodiphosphate (ADPβS) in the presence of atropine and α,β-Me ATP decreased the contractile response induced by electrical stimulations to $17.15 \pm 3.71\%$ ($n=15$) of the control.

4 Pretreatment of muscle strips with 100 μM ADPβS significantly reduced the response to either 200 μM α,β-methylene adenosine 5'-diphosphate or 200 μM ADPβS.

5 Uridine 5'-triphosphate (100 μM to 1 mM) concentration-dependently contracted muscle strips, and this contraction was significantly antagonized by desensitization of P_2-receptors with α,β-Me ATP (10 μM), and completely antagonized by pretreatment of muscle strips with both α,β-Me ATP and ADPβS (100 μM).

6 Di(adenosine-5') tetraphosphate (30 and 100 μM) contracted muscle strips, whereas it failed to contract after desensitization of P_2-receptors.

7 It is suggested that about 20% of the neurogenic contraction of rat urinary bladder smooth muscle is mediated via ADPβS-sensitive purinoceptors.

Keywords: P_2-purinoceptors; urinary bladder smooth muscle; α,β-methylene adenosine 5'-triphosphate; adenosine 5'-*o*-2-thiodiphosphate; cibacron blue 3GA; suramin

Introduction

It is well known that there is a variety of receptors in urinary bladder smooth muscles (Hisayama *et al.*, 1988; Iacovou *et al.*, 1990). The existence of P_{2X}-receptors was confirmed in guinea-pig urinary bladder (Burnstock & Kennedy, 1985; Iacovou *et al.*, 1988); in mouse urinary bladder it was reported that P_{2X}- and P_{2Y}-receptors coexist (Boland *et al.*, 1993) and in rat urinary bladder, P_1- and P_{2X}-receptors (Bhat *et al.*, 1989; Nicholls *et al.*, 1992) were shown to exist.

Recently, we reported that a P_2-purinoceptor other than P_{2X}, which mediates contraction, exists in rat urinary bladder (Suzuki & Kokubun, 1994). This type of purinoceptor responds to either adenosine 5'-*o*-2-thiodiphosphate (ADPβS) or α,β-methylene adenosine 5'-diphosphate (α,β-Me ADP) but not to α,β-Me ATP and 2-MeSATP (ADPβS-sensitive purinoceptor), and is, not completely but significantly, antagonized by cibacron blue 3 GA (CB3GA). However, the physiological role of this receptor in rat urinary bladder, i.e. the role of the receptor in neurogenic contraction of the bladder, has not yet been investigated. Therefore, the first aim of this study was to investigate whether ADPβS-sensitive purinoceptors mediate the neurogenic contraction of rat urinary bladder.

Since various P_2-purinoceptors are known to induce contraction of various smooth muscles or increase intracellular Ca^{2+} concentration in various tissues (Fredholm *et al.*, 1994), the second aim of this study was to investigate whether purinoceptors other than P_{2X} and ADPβS-sensitive purinoceptors mediate contraction in rat urinary bladder smooth muscle.

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Methods

Male Wistar strain rats (weighing 200 to 250 g) were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}), and the urinary bladder was rapidly removed. The bladder was transferred into Tyrode solution and then cut into small tissue strips of 7.5×2 mm. The strip was suspended between two platinum-plate electrodes in an organ bath which contained 10 ml of Tyrode solution with the following composition (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0 and glucose 5.0 (pH = 7.4). The solution was aerated with O_2 and maintained at 37°C. Responses to drugs as well as electrical stimulations were monitored by measuring isotonic tension under a resting load of 0.5 g, after the strip had been equilibrated for 90 min. After each application of the drug, it was washed out with more than 100 ml of Tyrode solution; the next application of the drug was given at least 30 min later. When the concentration-response curves were performed, each dose was added separately. To desensitize P_{2X} receptors, 10 μM α,β-Me ATP was added 8 min before the experiments, and to desensitize P_{2X} and ADPβS-sensitive purinoceptors simultaneously, both 10 μM α,β-Me ATP and 100 μM ADPβS were added 18 min before the experiments. Guanethidine (5 μM) and atropine (1 μM) were present in experiments shown in Figure 3.

Electrical field stimulation was with pulses of 0.5 ms duration at supramaximal voltage (30-35 V) from a bath drive amplifier SEG-3104 (Nihon Kohden). The strips were stimulated at a frequency of 10 Hz for 1 s in every 2 min. In preliminary experiments, it was established that contractile responses to electrical stimulations were fully abolished by tetrodotoxin, 0.5 μM; such responses were therefore deemed to be wholly neurogenic. Guanethidine (5 μM) was always present in electrical stimulation experiments. When we investigated the

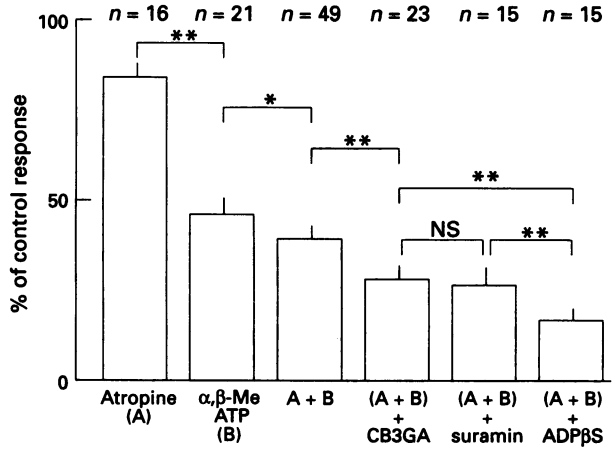

Figure 1 Contractile responses of rat urinary bladder smooth muscle to electrical stimulations in the presence of various agents. Each column represents the mean contractile response and s.e.mean, expressed as a percentage of the control response. Concentrations of agents are: atropine 10^{-6} M; α,β-Me ATP 10^{-5} M; CB3GA 10^{-4} M, suramin 10^{-4} M and ADPβS 10^{-4} M. A+B on the abscissa scale indicate the presence of atropine (10^{-6} M) and α,β-Me ATP (10^{-5} M). CB3GA, suramin and ADPβS were applied in the presence of atropine and α,β-Me ATP. The number of experiments is shown above each column. $*P<0.05$; $**P<0.01$. For abbreviations, see text.

effects of various agents on the contraction induced by electrical stimulation, responses at the steady state were obtained at least 30 min after the application of agents.

Drugs

α,β-Methylene adenosine 5'-diphosphate (α,β-Me ADP), α,β-methylene adenosine 5'-triphosphate (α,β-Me ATP), adenosine 5'-*o*-2-thiodiphosphate (ADPβS), uridine 5'-triphosphate (UTP), uridine 5'-diphosphate (UDP), di(adenosine-5') tetraphosphate (Ap_4A), atropine, guanethidine and cibacron blue 3GA were purchased from Sigma Chemical Company. Acetylcholine chloride (ACh) was purchased from Daiichiseiyaku. Germanin (suramin) was a gift from Bayer. All drugs were prepared freshly before each experiment by dissolving them in Tyrode solution.

Statistical analysis

Results are expressed as mean ± s.e.mean. One-way analysis of variance (one-way anova) was used to test for statistical significance. A probability of 0.05 or less was considered significant.

Results

Inhibition of contractile responses to electrical stimulation by various agents

We investigated the contribution of purinergic regulation in neurogenic contraction of rat urinary bladder (Figure 1). In the presence of atropine (1 μM) the contractile response was decreased to 84.19 ± 3.90% of the control ($n=16$), whereas in the presence of α,β-Me ATP (10 μM) it was decreased to 43.25 ± 3.16% of the control ($n=21$). The inhibitory potency of these drugs was significantly different ($P<0.01$). When we applied both agents simultaneously, the contractile response was decreased to 38.80 ± 2.75% ($n=49$). In the presence of both atropine and α,β-Me ATP, either CB3GA (100 μM) or suramin (100 μM) significantly decreased contraction ($P<0.01$). The contraction observed in the presence of atropine, α,β-Me ATP and CB3GA was 27.81 ± 4.07% ($n=23$), and that in the presence of atropine, α,β-Me ATP and suramin 26.63 ± 5.01% ($n=15$) of the control. They were not significantly different. When we applied ADPβS (100 μM) in the presence of atropine and α,β-Me ATP, the contractile response to electrical stimulation was potently inhibited, being 17.15 ± 3.71% of the control ($n=15$), which was significantly smaller than that observed in any other conditions ($P<0.01$).

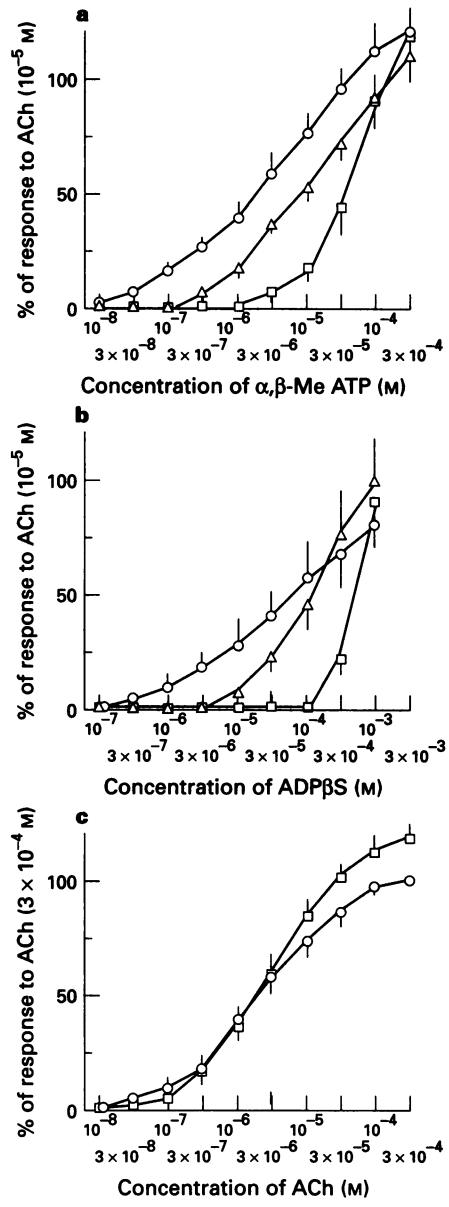

Figure 2 Effects of suramin on concentration-response curves to α,β-Me ATP (a), ADPβS (b) and ACh (c). (a) Responses to α,β-Me ATP in control (○), in the presence of 10^{-5} M suramin (△) and of 10^{-4} M suramin (□) are plotted against the concentration of α,β-Me ATP (M). Each point represents mean ± s.e.mean ($n=5$), expressed as percentage of response to 10^{-5} M ACh. (b) Responses to ADPβS in P_{2X}-desensitized tissues in control (○), in the presence of 10^{-5} M (△) and of 10^{-4} M suramin (□) are plotted against the concentration of ADPβS (M). Each point represents mean ± s.e.mean ($n=6$), expressed as percentage of response to 10^{-5} M ACh. (c) Responses to ACh in control (○) and in the presence of 10^{-4} M suramin (□) are plotted against the concentration of ACh (M). Each point represents mean ± s.e.mean ($n=6$), expressed as a percentage of the response to 3×10^{-4} M ACh. For abbreviations, see text.

Effects of suramin on contractile response to α,β-Me ATP, ADPβS and ACh

Suramin had similar inhibitory effects on the neurogenic contraction of rat urinary bladder to CB3GA in the presence of

α,β-Me ATP and atropine. Since suramin is an antagonist of P_2-purinoceptors (Dunn & Blakely, 1988; Voogd *et al.*, 1993), we investigated the effects of suramin on the concentration-response relations of α,β-Me ATP and ADPβS (Figure 2).

Suramin (10 μM) significantly decreased the contraction induced by α,β-Me ATP at concentrations lower than 100 μM ($P<0.01$), though at 100 and 300 μM, it did not significantly decrease α,β-Me ATP-induced contractions (Figure 2a); 100 μM suramin further decreased contractions. At concentrations of α,β-Me ATP lower than 100 μM, 100 μM suramin was significantly more potent than 10 μM ($P<0.01$ at concentrations lower than 10 μM; $P<0.05$ at 30 μM), though at 100 and 300 μM α,β-Me ATP, 100 μM suramin, like 10 μM, did not significantly inhibit the contraction.

We examined the effect of suramin on ADPβS-induced contraction in P_{2X}-desensitized strips, since ADPβS is known to contract muscle strips not only via ADPβS-sensitive purinoceptors but also via P_{2X}-receptors (Suzuki & Kokubun, 1994) which were relatively antagonized by suramin as shown in Figure 2a (Figure 2b). P_{2X}-receptors were desensitized by pretreatment with α,β-Me ATP (10 μM). Suramin 10 μM significantly inhibited contractions induced by ADPβS at concentrations lower than 100 μM ($P<0.01$ at concentrations lower than 30 μM; $P<0.05$ at 30 μM). At 300 μM and 1 mM ADPβS, suramin slightly augmented contraction rather than inhibited it, though the difference was not significant. Suramin 100 μM completely antagonized contractile effects of ADPβS lower than 300 μM. The mean contractile response to 300 μM ADPβS in the presence of 100 μM suramin was 27.27% of that in the absence of the antagonist, which was significantly smaller ($P<0.01$). At 1 mM ADPβS, 100 μM suramin did not significantly inhibit contraction.

We also examined the effect of suramin (100 μM) on the concentration-response relation of ACh (Figure 2c). Though suramin did not affect the contractile effect of ACh at concentrations lower than 30 μM, it augmented the contraction between 30 and 300 μM ($P<0.05$ at 30 and 100 μM, $P<0.01$ at 300 μM). In the presence of 10 μM suramin, the concentration-response relation of ACh was not significantly affected in four experiments (data not shown).

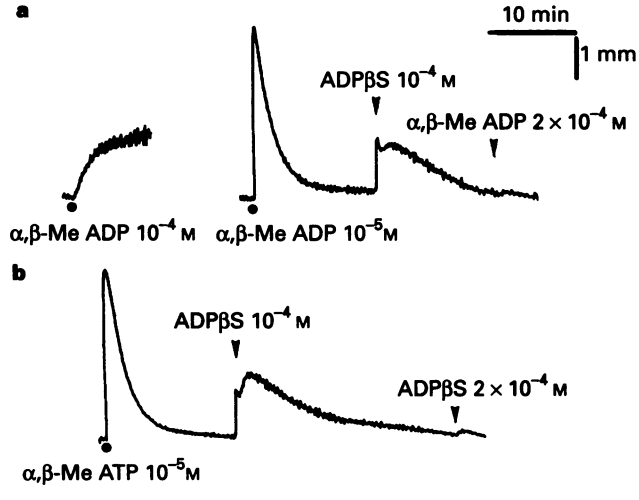

Figure 3 Contraction of rat urinary bladder smooth muscle induced by α,β-Me ADP (a) and ADPβS (b) in the absence and the presence of 10^{-5} M α,β-Me ATP and 10^{-4} M ADPβS. Experiments were done in the presence of atropine (1 μM) and guanethidine (5 μM). At the left side of (a), the control contraction induced by 10^{-4} M α,β-Me ADP is shown. At the right side the contraction induced by 2×10^{-4} M α,β-Me ADP after pretreatment of the muscle strip with 10^{-5} M α,β-Me ATP and 10^{-4} M ADPβS. In (b) the contraction by cumulative application of ADPβS (final concentration $=2\times10^{-4}$ M) in the presence of α,β-Me ATP (10^{-5} M) is shown. All records in this figure were obtained from the same strip. Drugs were applied at (●) or (▼). For abbreviations, see text.

Contraction by ADPβS and α,β-Me ADP in ADPβS-pretreated muscle strips

Since addition of ADPβS in the presence of atropine and α,β-Me ATP most significantly decreased contractile response to electrical stimulation as shown in Figure 1, we examined whether ADPβS desensitized the drug-sensitive purinoceptors. We first examined the contractile response to α,β-Me ADP (100 μM) (Figure 3a) 35 min after the washout of the drug; we treated the muscle strip with both α,β-Me ATP and ADPβS. We applied α,β-Me ATP (10 μM) to desensitize P_{2X}-receptors, and then applied ADPβS (100 μM); 14 min after the application of ADPβS, α,β-Me ADP (200 μM) was applied. The response to 200 μM α,β-Me ADP in the presence of α,β-Me ATP and ADPβS was 5.56% of that to α,β-Me ADP (100 μM) in the absence of these agents in this particular experiment. In 9 experiments the contractile response to α,β-Me ADP in the presence of α,β-Me ATP and ADPβS was 11.31 ± 5.65% of the control. In Figure 3b we examined the contractile response to ADPβS in the muscle strip pretreated with α,β-Me ATP and ADPβS. The experiment was done with the same muscle strip as that used in Figure 3a. We first applied α,β-Me ATP (10 μM), and then applied ADPβS (100 μM). ADPβS (final concentration in the bath: 200 μM) was cumulatively applied 24 min after the first application of the drug. The contractile response to successive applications of ADPβS was 5.75% of

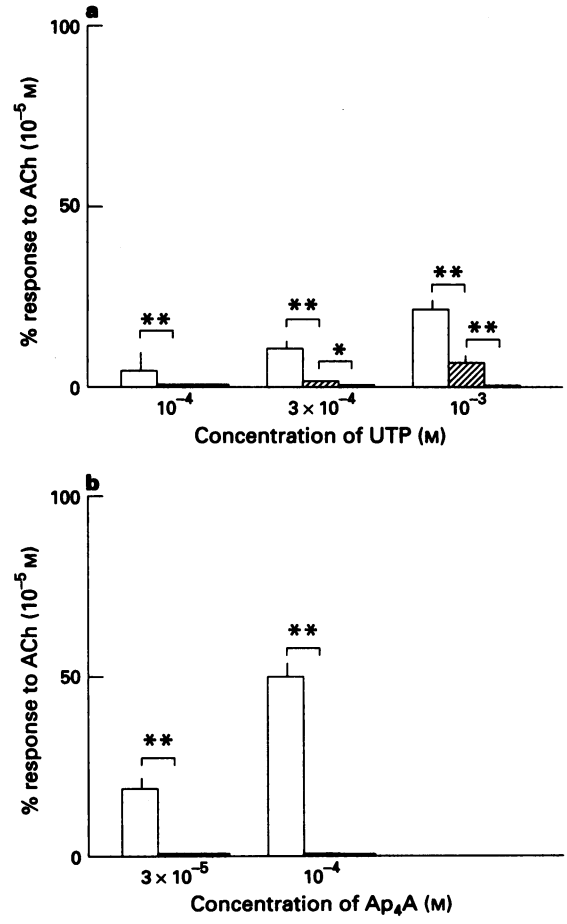

Figure 4 Contractile responses induced by UTP (a) and Ap_4A (b) in the control (open columns), the presence of 10^{-5} M α,β-Me ATP (hatched columns) and the presence of 10^{-5} M α,β-Me ATP and 10^{-4} M ADPβS (solid columns). Each column indicates the mean contractile response and s.e.mean, expressed as a percentage of the response to 10^{-5} M ACh. Abscissae indicate concentrations of drugs (M). The number of experiments in (a) was 13 in all concentrations of UTP, whereas in (b) it was 4 in 3×10^{-5} M and 5 in 10^{-4} M of Ap_4A. $^*P<0.05$, $^{**}P<0.01$. For abbreviations, see text.

that to the first application of the drug. In 9 experiments the response to the second application of ADPβS was 11.17 ± 5.00% of that to the first application.

Effects of UTP, UDP and Ap_4A on urinary bladder smooth muscle

Since ADPβS almost, though not completely, desensitized the drug-sensitive purinoceptors as has been shown in the previous section, we considered the possibility that purinoceptors such as P_{2U} and P_{2D} mediated the neurogenic contraction which still remained in the presence of atropine, α,β-Me ATP and ADPβS. Therefore, we investigated the effects of UTP and Ap_4A on rat urinary bladder smooth muscle. Figure 4a shows the contractile effect of UTP on muscle strips in the control, the presence of α,β-Me ATP (10 μM) and the presence of both α,β-Me ATP (10 μM) and ADPβS (100 μM). UTP concentration-dependently induced contraction; 1.15 ± 0.28% of response to ACh (10 μM) at 30 μM (not included in Figure 4a), 5.00 ± 6.00% at 100 μM, 10.7 ± 1.40% at 300 μM and 22.05 ± 2.09% at 1 mM ($n = 13$). These effects were significantly antagonized by pretreatment with α,β-Me ATP (10 μM). In the presence of α,β-Me ATP UTP did not produce contraction at concentrations lower than 300 μM. At 300 μM and 1 mM UTP the response was 2.05 ± 0.22% and 6.62 ± 1.23% of that to ACh (10 μM), respectively. When we pretreated muscle strips with both α,β-Me ATP (10 μM) and ADPβS (100 μM), UTP at any concentration induced no obvious contraction of muscle strips. We also examined the effects of UDP on urinary bladder smooth muscle. In 11 experiments UDP (< 5 mM) did not induce contraction (data not shown).

We next examined whether Ap_4A induced contraction after desensitization of P_{2X}-receptors as well as ADPβS-sensitive purinoceptors. As shown in Figure 4b, Ap_4A contracted urinary bladder smooth muscle producing 18.08 ± 2.82% of the response to ACh (10 μM) at 30 μM ($n = 4$) and 49.22 ± 3.15% at 100 μM ($n = 5$). In the presence of α,β-Me ATP (10 μM), or α,β-Me ATP (10 μM) plus ADPβS (100 μM), Ap_4A did not contract muscle strips.

Discussion

The contribution of muscarinic receptors to neurogenic contraction was significantly smaller than that of P_{2X}-receptors. About 15% of the neurogenic contraction was via muscarinic receptors, while about 50% of that was via P_{2X}-receptors. After inhibition of both muscarinic receptors and P_{2X}-receptors, about 34% of the control contraction remained. It was interesting to know which receptors mediated the contraction under these conditions.

As previously reported (Suzuki & Kokubun, 1994), ADPβS-sensitive purinoceptors mediating contraction, which respond not to α,β-Me ATP but to either ADPβS or α,β-Me ADP and which are significantly inhibited by CB3GA, exist in rat urinary bladder smooth muscle in addition to P_{2X} receptors. Therefore, in this study we examined whether CB3GA inhibited the neurogenic contraction in the presence of both atropine and α,β-Me ATP. CB3GA significantly inhibited the neurogenic contraction under these conditions, though about 28% of control response still remained.

Suramin, which is a specific P_{2X}-purinoceptor antagonist (Dunn & Blakely, 1988; Voogd *et al.*, 1993), inhibited the neurogenic contraction by a similar extent to CB3GA. We, therefore, examined whether suramin antagonized ADPβS. Though suramin had a more potent inhibitory effect than CB3GA on P_{2X}-mediated contraction, it showed almost the same potency as CB3GA in inhibiting the response to lower concentrations of ADPβS and was less potent than CB3GA in inhibiting the response of ADPβS at higher concentrations (> 100 μM). Suramin did not inhibit but did augment the response to ACh at concentrations higher than 10 μM, though CB3GA did not affect it at all (Suzuki & Kokubun, 1994). These results suggest that CB3GA is a more specific antagonist than suramin at ADPβS-sensitive purinoceptors in rat urinary bladder smooth muscle.

The neurogenic contraction was most potently inhibited by the application of ADPβS in the presence of atropine and α,β-Me ATP. This suggested the possibility that, just as α,β-Me ATP acts as a specific agonist to P_{2X}-receptors as well as a specific antagonist by desensitizing the receptors (Kasakov & Burnstock, 1983), ADPβS also acted as an agonist to ADPβS-sensitive purinoceptors as well as an antagonist by desensitizing them. If this were the case, the contraction inhibited by the application of ADPβS in the presence of atropine and α,β-Me ATP was via ADPβS-sensitive purinoceptors. Indeed, pretreatment of P_{2X}-desensitized muscle strips by ADPβS significantly inhibited the contractions induced by either ADPβS or α,β-Me ADP. These effects were not due to P_{2X}-desensitization by α,β-Me ATP, since we previously found that α,β-Me ATP by itself did not inhibit responses induced by either ADPβS or α,β-Me ADP (Suzuki & Kokubun, 1994). Therefore, the neurogenic contraction inhibited by the application of ADPβS in the presence of atropine and α,β-Me ATP, which was about 20% of the control response, is suggested to be via ADPβS-sensitive purinoceptors.

Which receptor mediated the neurogenic contraction observed in the presence of atropine, α,β-Me ATP and ADPβS, which was about 17% of the control response? While atropine (1 μM) may abolish all cholinergic responses, α,β-Me ATP (10 μM) reduces but does not abolish responses mediated via P_{2X}-purinoceptors in this tissue, as previously reported (Suzuki & Kokubun, 1994). Similarly, ADPβS (100 μM) reduced but did not abolish responses mediated via ADPβS-sensitive purinoceptors. Therefore, the remaining response in the presence of atropine, α,β-Me ATP and ADPβS could be mediated via either P_{2X} and/or ADPβS-sensitive purinoceptors. Alternatively, the remaining response in the presence of atropine, α,β-Me ATP and ADPβS could be mediated by other P_2-purinoceptors, such as P_{2U}, P_{2D}, P_{2Z} or P_{2T}.

Since P_{2U}-receptors respond to UTP and ATP but not to α,β-Me ATP and 2-MeSATP (O'Connor *et al.*, 1991), we examined the effect of UTP on rat urinary bladder smooth muscle. In this preparation, UTP induced contraction, which was significantly inhibited by pretreatment of the muscle with α,β-Me ATP and completely inhibited by pretreatment with both α,β-Me ATP and ADPβS. This indicates that UTP mediated contraction of this tissue was not via P_{2U}-receptors but via P_{2X} and ADPβS-sensitive purinoceptors. We also examined the effect of the P_{2D} agonist, Ap_4A (Hilderman *et al.*, 1991; Castro *et al.*, 1992). The drug induced a stronger contraction than UTP, which was almost completely inhibited by pretreatment with α,β-Me ATP. This indicates that Ap_4A mediated contraction mainly via P_{2X}-receptors. These results suggest that in rat urinary bladder smooth muscle, the neurogenic contraction was mediated by neither P_{2U}- nor P_{2D}-receptors.

P_{2Z}- and P_{2T}-receptors respond to ATP^{4-} and ADP, respectively. The former was reported to exist in macrophages (Steinberg & Silverstein, 1987), mast cells (Dahlqvist & Diamant, 1974) and vas deferens (Fedan *et al.*, 1990), while the latter exists only in platelets (Gordon, 1986). In order to investigate whether P_{2Z}-receptors exist in rat urinary bladder smooth muscle, we would have to examine the dependency of contractile effects of ATP on the concentration of divalent cations, such as extracellular Mg^{2+} concentration, as has been done in guinea-pig vas deferens (Fedan *et al.*, 1990). However, we experienced difficulties in performing quantitative experiments on the effect of ATP, since in rat urinary bladder smooth muscle, adenosine produced by hydrolysis of ATP relaxed muscle strips via A_{2b}-receptors (Suzuki & Kokubun, 1994). Similarly, quantitative experiments on ADP to investigate the existance of P_{2T}-receptors were difficult, since ADP is also hydrolyzed. Therefore, in this study we have not investigated whether the neurogenic contraction in the presence of atropine, α,β-Me ATP and ADPβS is mediated by P_{2Z}- and/or P_{2T}-receptors.

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Mechanism of bradykinin-induced plasma extravasation in the rat knee joint

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1 We have investigated the mechanism of bradykinin (BK)-induced plasma extravasation into the knee joint of the anaesthetized rat. Accumulation of [^{125}I]-human serum albumin within the synovial cavity was used as a marker of increased vascular permeability.

2 Perfusion with BK (1 μM) produced significant plasma extravasation into the knee which was inhibited by co-perfusion of the selective bradykinin B_2 receptor antagonist D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe 140, 200 nM).

3 The bradykinin B_1 receptor agonist, [des-Arg^9]-BK (up to 100 mM), did not induce plasma extravasation into the knee joint over this time period.

4 Chemical sympathectomy by chronically administered 6-hydroxydopamine (6-OHDA) did not inhibit bradykinin-induced plasma extravasation. Acute intra-articular perfusion with 6-OHDA (to stimulate transmitter release from sympathetic nerve terminals) at concentrations up to 50 mM did not induce significant plasma extravasation. Intra-articular perfusion of 100 mM 6-OHDA induced significant plasma extravasation but produced severe systemic toxicity.

5 The selective neurokinin$_1$ (NK_1) receptor antagonist, RP67580 (230 nmol kg^{-1}), or receptor antagonists for the mast cell products histamine and 5-hydroxytryptamine did not significantly inhibit BK-induced plasma extravasation.

6 Co-perfusion of the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (1 mM) did not significantly inhibit the response to BK. ^{133}Xe clearance from L-NAME (1 mM)-injected joints was significantly ($P < 0.05$) reduced compared to D-NAME injected joints, suggesting a reduction in blood flow as a result of decreased basal NO production. Systemic administration of L-NAME at doses sufficient to produce significant and sustained elevation of blood pressure (5 or 30 mg kg^{-1}, i.v. 15 min prior to BK perfusion) also failed to significantly inhibit the BK-induced response.

7 We conclude that, in normal joints, BK induces plasma extravasation by acting on bradykinin B_2 receptors and that this response is not dependent on secondary release of mediators from sympathetic nerve terminals, sensory nerves, mast cells or on generation of NO.

Keywords: Bradykinin; joint; plasma protein extravasation

Introduction

Kinins are generated from plasma and tissue precursors at sites of tissue injury and contribute to many aspects of both acute and chronic inflammation including oedema formation, vasodilatation and pain (Hall, 1992). Bradykinin (BK) has diverse actions on vascular endothelium, smooth muscle and cellular function and is a powerful algesic agent which can both sensitize and directly stimulate sensory nerve terminals. Its actions are mediated both by activation of BK receptors on target tissues and, indirectly, by release or amplification of nitric oxide or other inflammatory agents including neuropeptides and prostaglandins. In a previous study BK was found to produce a dose-dependent increase in plasma extravasation into the rat knee joint without producing systemic oedema (Cambridge & Brain, 1992). It was the most potent of the inflammatory mediators tested in this assay and, unlike histamine, was not potentiated by vasodilators. BK may act via several pathways to produce extravasation into joints. These include receptor-mediated effects on endothelial cells to increase vascular permeability and activation of sensory nerves with subsequent release of pro-inflammatory peptides including substance P, neurokinin A (NKA) and calcitonin gene-related peptide (CGRP). Recently, considerable evidence has been presented to support a role for sympathetic nerves in mediating the response to BK (Green *et al.*, 1993a,b). In addition BK may also activate synovial mast cells to release histamine (and 5-hydroxytryptamine (5-HT) in rodents). Local release of NO from endothelial cells and sensory or perivascular neurones may also contribute to the effects of BK by increasing blood flow in the synovial membrane.

In our study modulation of the response to BK was measured in the perfused knee model preparation of the rat. The release of pro-inflammatory peptides from sensory nerves was mimicked by co-perfusion of a selective NK_1 tachykinin receptor agonist, GR73632 with CGRP. GR73632 was used in preference to substance P as, in a previous study, intra-articular perfusion of substance P at sufficiently high concentrations to induce plasma extravasation, also produced systemic oedema (Cambridge & Brain, 1992). To assess the role of sympathetic nerve activation, BK-induced plasma extravasation was measured in rats which had been chemically sympathectomized by chronic treatment with 6-hydroxydopamine (6-OHDA). In addition the effects of acute sympathetic terminal activation by intra-articular perfusion of 6-OHDA on plasma extravasation were also measured. The contribution of BK-stimulated production of NO to plasma extravasation was measured indirectly by use of the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME). Local effects of L-NAME on blood flow within the synovium were measured by use of the ^{133}Xe clearance method (Cambridge & Brain, 1992).

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Methods

Animals

Male Wistar rats (250–300 g) kept in a temperature-controlled environment and fed standard laboratory food and water *ad libitum* were used in this study. For the joint perfusion and blood flow studies and for the initial sympathectomy treatment with 6-OHDA, animals were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p., additional doses as required).

Joint perfusion and quantification of plasma extravasation

Plasma extravasation was measured by accumulation of radiolabelled albumin in the synovial cavity of the rat knee after perfusion of test agents through the joint space (Cambridge & Brain, 1992). Animals were injected via the tail vein with 1.5 μCi [^{125}I]-human serum albumin, mixed with Evans Blue (0.2 ml, 25 mg ml^{-1}) as a visible marker. In each animal one knee joint was perfused with 100 μl min^{-1} Tyrode solution (composition, mM: NaCl 136.9, KCl 2.7, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $MgCl_2$ 1.0 and glucose 5.6) via 27G needles placed within the synovial cavity and connected via a polythene cannula to a roller pump (Watson-Marlow, Falmouth, U.K.). To establish a stable baseline the joint was perfused with Tyrode solution for 20 min. The solution was then changed to Tyrode (or other vehicle solution where appropriate) containing test agents and perfusion continued for 5 min. The pump was then stopped and the test agents were allowed to remain in the joint for a further 10 min after which time perfusion was recommenced with Tyrode solution alone and the perfusate (1 ml) collected and radioactivity counted in a gamma counter. Plasma extravasation was expressed as μl/joint by comparison of counts in the synovial perfusate to counts in a plasma sample.

Blood pressure measurement

Blood pressure was continuously monitored in animals which received either intravenous L-NAME (or saline) or intra-articular perfusion of 6-OHDA at both 50 mM and 100 mM. A cannula was placed in the right carotid artery and connected to a pressure transducer and chart recorder. Data are expressed as mean arterial pressure (MAP) in mmHg.

Chemical sympathectomy

Animals received either 6-OHDA or an equivalent volume of vehicle (1% ascorbic acid in saline) following the sympathectomy protocol of Green *et al.* (1993b). 6-OHDA was administered by i.p. injection on days 1, 2, and 3, (50 mg kg^{-1} daily) and on days 6 and 7 (100 mg kg^{-1} daily). Joint perfusion experiments were carried out on day 8.

To assess the functional effects of the sympathectomy regimes, the pressor response to tyramine was measured in a separate group of 6-OHDA-treated ($n=5$) and normal rats ($n=4$). Tyramine (dissolved in normal saline) was injected as a bolus via a butterfly cannula placed in the tail vein.

^{133}Xe clearance from synovial cavity

Estimation of changes in knee joint blood flow produced by L-NAME was carried out using a ^{133}Xe clearance method as previously described (Cambridge & Brain, 1992). Briefly ^{133}Xe (100 μCi) was mixed with 1 ml of L-NAME (1 mM), D-NAME (1 mM) or saline. One hundred μl of either D- or L-NAME was rapidly injected into one knee joint and 100 μl of saline injected into the contralateral joint. After a 5 min clearance period the animals were killed, the joints removed and radioactivity counted in a gamma counter. Results are expressed as percentage difference in clearance between L- or D-NAME-injected joints and saline-injected joints.

Materials

BK, [des-Arg^9]-bradykinin, 6-OHDA (hydrobromide), mepyramine, N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-D-arginine methyl ester (D-NAME) were obtained from Sigma (Poole, U.K.), GR73632 (δ-Ava-Phe-Phe-Pro-MeLeu-Met-NH_2) was a gift from Dr D. Beattie, Glaxo (Ware, U.K.) and human αCGRP, a gift from Dr U. Ney, Celltech (Slough, U.K.). The BK B_2 receptor antagonist D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK (Hoe140) was obtained from Peninsula Laboratories, (St Helens, U.K.). [^{125}I]-human serum albumin (2.5 μCi mg^{-1}) and ^{133}Xe were obtained from Amersham International (U.K.). Agents for intra-articular perfusion were dissolved in Tyrode solution except for 6-OHDA which was dissolved in Tyrode solution containing 1% ascorbic acid (to prevent oxidation). The specific NK_1 tachykinin receptor antagonist, RP67580 ([3a**R**, 7a**R**]-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)-ethyl] perhydroisoindol-4-one), a gift from Dr C. Garret, Rhone-Poulenc Rorer (France) was dissolved in saline and injected (230 nmol kg^{-1}) into the tail vein 10 min before the start of the BK perfusion, a protocol which virtually abolishes neurogenic oedema induced by stimulation of the saphenous nerve (Garrett *et al.*, 1991). The 5-HT antagonist, methysergide (a gift from Sandoz, U.K.), and histamine H_1 antagonist, mepyramine were dissolved in saline and injected (both 10 mg kg^{-1}, i.p.) 15 min before the start of the BK infusion. This treatment has been shown to inhibit oedema formation in rat skin induced by the mast cell degranulating agent compound 48/80 (Brain & Williams, 1985). L-NAME was given locally (as a 1 mM solution co-perfused with BK) or systemically via a tail vein injection (5 mg kg^{-1} or 30 mg kg^{-1}) 15 min before the intra-articular perfusion with BK.

Statistical analysis

Except where indicated in the figure legend, results are expressed as mean ± s.e.mean and n refers to the number of animals in each group in all cases. One-way ANOVA, followed by Tukey's multiple comparisons test was used to assess the significance of differences between group means. Where variances were different, nonparametric analysis of variance (Kruskal-Wallis test), followed by a Dunn's multiple comparison test, was used. Blood pressure responses before and after L-NAME and 6-OHDA were compared by Student's paired t test and differences in ^{133}Xe clearance from L-NAME- and D-NAME-treated joints by Student's unpaired t test.

Results

Modulation of BK-induced plasma extravasation

As previously reported (Cambridge & Brain, 1992) perfusion of 1 μM BK induced significant ($P<0.01$, $n=10$) plasma extravasation in the knee joint when compared to joints which were perfused only with Tyrode solution ($n=8$) (Figure 1). Co-perfusion of the B_2 receptor antagonist Hoe140 (200 nM; $n=12$) significantly inhibited BK-induced plasma extravasation compared to BK alone whilst at a lower dose (20 nM; $n=13$) Hoe140 was without significant effect. The selective NK_1 receptor antagonist, RP67580 (230 nmol kg^{-1}, i.v.) did not inhibit BK-induced plasma extravasation. By contrast, RP67580 (230 nmol kg^{-1}, i.v.) significantly attenuated the response induced by co-perfusion of the specific NK_1 agonist GR73632 (1 μM) with CGRP (100 nM). Methysergide and mepyramine, the selective antagonists of the mast cell mediators 5-HT and histamine, respectively, did not significantly inhibit the response to BK (Figure 1). Co-perfusion of the NO synthase inhibitor L-NAME (1 mM; $n=6$) with BK did not significantly decrease plasma extravasation although a trend towards attenuation of the response was seen (Figure 1). The selective BK B_1 receptor agonist, [des-Arg^9]-BK, at doses up to

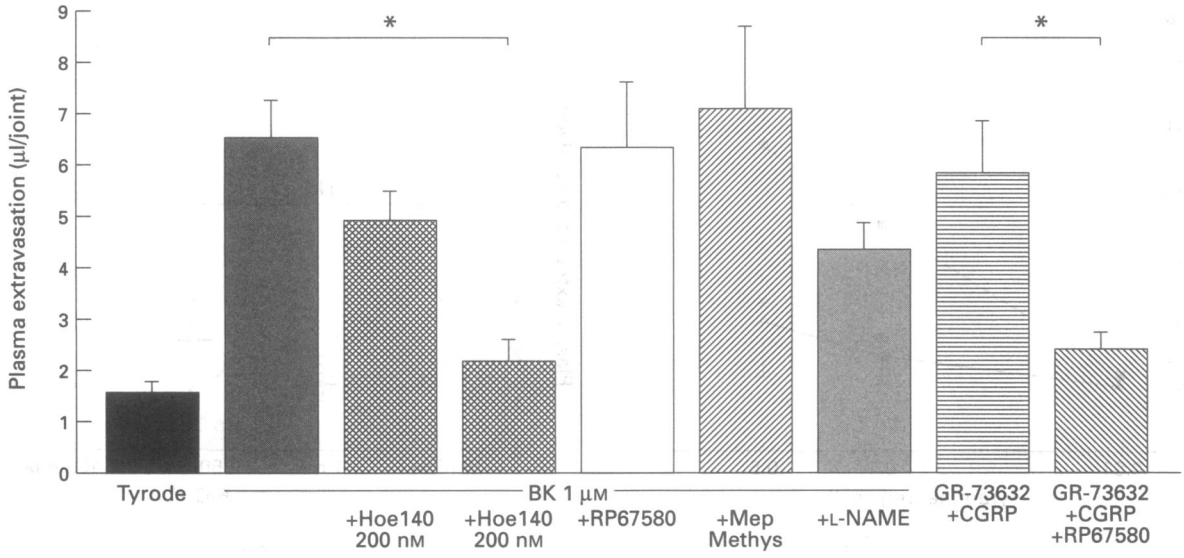

Figure 1 Modulation of BK-induced plasma extravasation into the rat knee joint. Results are shown as follows: BK (1 μM) alone (solid column); BK (1 μM) co-perfused with Hoe140 (20 nM and 200 nM), (cross-hatched columns); BK (1 μM) perfused 10 min after i.v. injection of RP67580 (230 nmol kg^{-1}), (open column); BK (1 μM) perfused 15 min after i.p. injection of methysergide and mepyramine (both 10 mg kg^{-1}), (hatched column); BK (1 μM) co-perfused with L-NAME (1 mM) (stippled column). Responses induced by co-perfusion of GR73632 (1 μM) plus CGRP (100 nM) (horizontal lined column) and 5 min after i.v. injection of RP67580 (230 nmol kg^{-1}), (hatched column) are also shown. Results are mean ± s.e.means. $n = 6-13$ animals. *$P < 0.05$, Tukey's multiple comparison test. For abbreviations, see text.

100 μM, failed to elicit significant plasma extravasation (100 μM, 2.4 ± 5.0 μl/joint, $n = 7$).

Intravenous administration of L-NAME at 5 mg kg^{-1} produced a significant ($P < 0.05$, paired t test) rise in blood pressure in all animals (mean basal, 77.5 ± 6.1 mmHg vs mean L-NAME, 101.7 ± 4.8, $n = 4$), but no significant effect on BK-induced plasma extravasation (Figure 2). At the higher dose of L-NAME (30 mg kg^{-1}) a greater increase in MAP was seen (mean basal 97.8 ± 4.0 mmHg vs L-NAME, 141.3 ± 5.0, $n = 9$, $P < 0.0001$, paired t test). However, consistent inhibition of the BK response was not evident (Figure 2). Blood pressure was unchanged over the experimental period in the control group which received i.v. saline ($n = 7$).

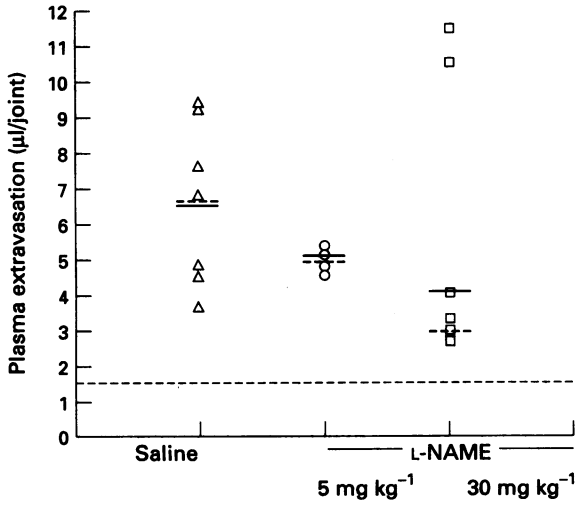

Figure 2 Effect of systemic N^G-nitro-L-arginine methylester (L-NAME) on bradykinin (BK)-induced plasma extravasation. BK-induced plasma extravasation (measured by accumulation of labelled albumin) into the knee joint of animals treated with intravenous L-NAME (5 mg kg^{-1}, $n = 4$, or 30 mg kg^{-1}, $n = 9$) and a control group ($n = 7$) which received an equivalent volume of saline. Perfusion of BK was started 15 min after L-NAME injection. Solid bars represent mean response and dashed bar represents median response in each group. No significant differences were found between groups. Dashed line represents mean response in Tyrode-perfused joints of untreated animals ($n = 8$).

Effect of L-NAME on ^{133}Xe clearance from synovial cavity

^{133}Xe clearance was reduced in L-NAME (0.1 μmol)-injected joints compared to contralateral saline-injected joints by 39.3% ± 3.6, ($n = 5$). This was significantly different ($P < 0.05$, unpaired t test) from D-NAME (0.1 μmol)-injected joints where ^{133}Xe clearance was 10.4% ± 7.0 less than in contralateral joints.

Effect of chemical sympathectomy

Pressor effect of intravenous tyramine To establish the effectiveness of the sympathectomy treatment the pressor response to i.v. tyramine, an indirect sympathomimetic (0.01 to 0.5 mg kg^{-1}), was measured in additional groups of untreated and 6-OHDA-treated animals. In untreated animals ($n = 4$) tyramine produced a dose-dependent increase in mean arterial blood pressure which was absent in 6-OHDA-treated animals ($n = 5$), demonstrating that sympathectomy was successful in this group (Figure 3a).

BK-induced plasma extravasation In animals chronically treated with 6-OHDA, BK (1 μM)-induced plasma extravasation into the knee joint was significantly ($P < 0.05$) increased compared to vehicle (1% ascorbic acid) controls (Figure 3b).

Effect of acute perfusion of 6-OHDA on plasma extravasation into the joint

Intra-articular perfusion with 6-OHDA (1 mM, $n = 6$, and 50 mM, $n = 10$), did not cause significant plasma extravasation compared to vehicle ($n = 10$) (Figure 4a). At the highest dose tested (100 mM), 6-OHDA produced a significant response ($P < 0.05$) (Figure 4a), although the results were highly variable. In the 50 mM 6-OHDA group, signs of systemic sympathetic activation including piloerection and tachycardia were observed in all rats during intra-articular perfusion. At 100 mM 6-OHDA produced obvious toxic effects, including pronounced hypersecretion in the airways, tachycardia and

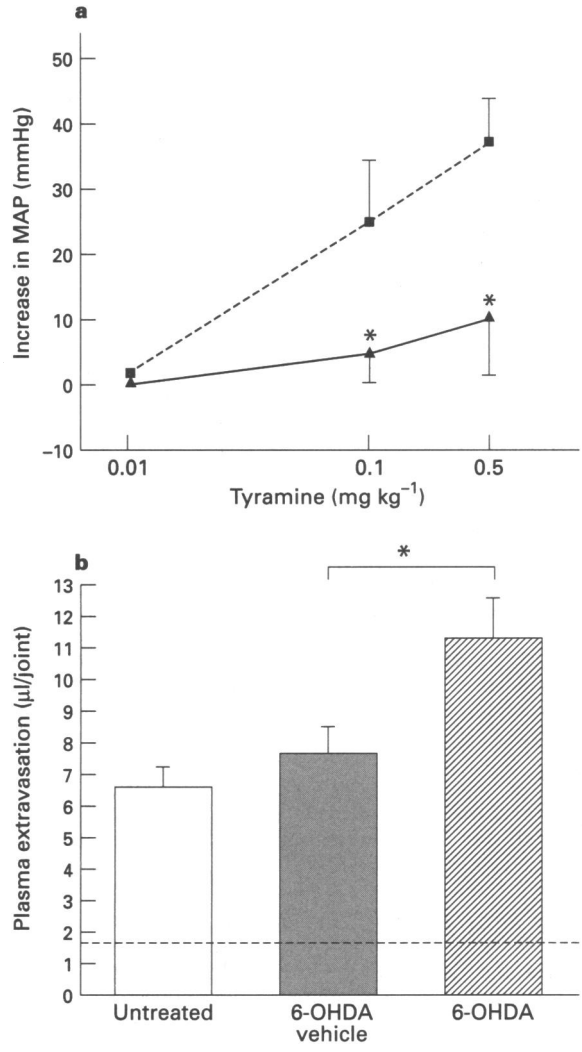

Figure 3 (a) Pressor response (mean ± s.d.) to i.v. tyramine (0.01–0.5 mg kg^{-1}) in (■) untreated rats, $n=4$; (▲) 6-hydroxydopamine (6-OHDA)-treated rats, (50 mg kg^{-1}, i.p. on days 1,2,3, 100 mg kg^{-1}, i.p. on days 6,7) $n=5$. *$P<0.05$, untreated vs 6-OHDA-treated rats, unpaired t test. (b) Effect of chemical sympathectomy on bradykinin (BK)-induced plasma extravasation. Plasma extravasation (measured by accumulation of labelled albumin in the knee joint) in response to intra-articular perfusion of BK (1 µM) in untreated rats (open columns, $n=10$); 6-OHDA vehicle (normal saline with 1% ascorbic acid) treated rats (stippled column, $n=15$); 6-OHDA-treated (as in [a] above) rats (hatched column, $n=11$). Dashed line represents the response to perfusion of Tyrode solution alone in a separate group of 8 untreated animals. Mean ± s.e.mean *$P<0.05$.

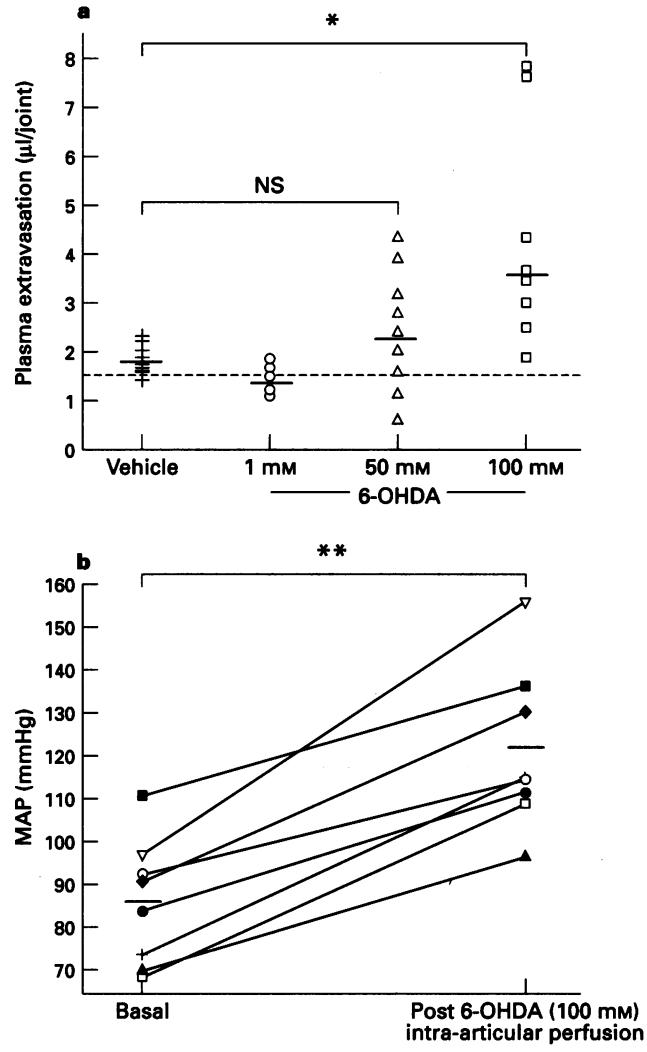

Figure 4 (a) Plasma extravasation into the knee joint induced by intra-articular perfusion of 6-hydroxydopamine (6-OHDA). Data from individual animals are shown at each dose; vehicle, $n=10$; 1 mM, $n=6$; 50 mM $n=10$; 100 mM, $n=8$. Bar represents the median response. *$P<0.05$. (b) Effect of intra-articular perfusion of 6-OHDA (100 mM) on MAP in 8 rats. Basal = MAP during perfusion of joint with vehicle (Tyrode solution with 1% ascorbic acid). Post = maximum MAP reached during perfusion of joint with 6-OHDA (100 mM for 5 min). Same animals as in (a). Bar represents mean response. **$P<0.01$.

cardiac arrhythmias. All animals in this group developed significant and prolonged hypertension with mean blood pressure increasing from a pretreatment mean of 82.4 ± 5.4 mmHg to 119.3 ± 5.9 mmHg ($P<0.0001$, $n=8$), indicating leakage of the drug into the systemic circulation. Data for individual animals are shown in Figure 4b.

Discussion

As BK-induced plasma extravasation was inhibited by co-perfusion with Hoe140, a selective B_2 antagonist (Hock *et al.*, 1991), these results show that in the normal joint, BK produces plasma extravasation via activation of the BK B_2 receptor. The selective B_1 agonist, [des-Arg^9]-bradykinin did not produce plasma extravasation at concentrations up to 100 times greater than that for BK. A recent report (Cruwys *et al.*, 1994) has described B_1 plasma extravasation in normal rat knees measured 3 h after intra-articular injection, however [des-Arg^9]-bradykinin was much less potent than BK and produced a smaller maxium response. Our findings are consistent with *in vitro* and *in vivo* studies (Boutillier *et al.*, 1987; Perkins & Dray, 1993) and *ex vivo* studies (Farmer *et al.*, 1991) where the presence of tissue damage, inflammation or the action of some cytokines (e.g. interleukin-1β) has been necessary to induce the B_1 receptor and thus allow effects of B_1 receptor activation to be measured. For example, hyperalgesia associated with adjuvant arthritis and thermal injury in rats is more effectively attenuated by B_1 than B_2-selective antagonists (Perkins *et al.*, 1993) but the effects of B_1 receptor activation on the local vascular processes of inflammation have not been extensively investigated. The significant inhibition of the response by Hoe140 provides further evidence for the importance of B_2 receptors in producing acute plasma extravasation into the joint.

A possible mode of action of BK in the joint is via activation of sensory nerves, with subsequent release of pro-inflammatory peptides and induction of neurogenic oedema. In some tissues, notably the rabbit iris, the response to BK is mediated indirectly by neuropeptides including substance P and CGRP (Ueda *et al.*, 1984; Wahlestedt *et al.*, 1985). To

mimic the release of sensory neuropeptides, the selective NK_1 agonist (GR73632), which induces oedema formation in rat skin (Richards *et al.*, 1993), and the vasodilator CGRP were co-perfused. We have previously shown that CGRP does not induce plasma extravasation at vasodilator doses (Cambridge & Brain, 1992) but it has been well established that CGRP acts synergistically with mediators of increased vascular permeability (Brain & Williams, 1985). GR73632 and CGRP produced significant plasma extravasation into the joint which could be totally inhibited by RP67580, a non-peptide NK_1 receptor antagonist. RP67580 is selective for the rodent NK_1 receptor and inhibits neurogenic oedema in many tissues (Garrett *et al.*, 1991; Beaujouan *et al.*, 1993; Shepheard *et al.*, 1993) but was without effect on BK-induced plasma extravasation in the joint. Thus, although the synovium has many nerves which show positive immunohistochemical staining for these and other peptides (Mapp *et al.*, 1990), our findings do not support a significant role for sensory neuropeptide release in BK-induced plasma extravasation. In addition, previous studies of plasma extravasation in joints showed substance P to be much less potent than BK whilst CGRP, although a potent vasodilator, could only act to potentiate oedema induced by other agents (Cambridge & Brain, 1992; Cruwys *et al.*, 1992).

NO released from endothelial cells, and possibly also from neural structures, may produce vasodilatation and thus enhance vascular leakage induced by BK. In a rat skin blister base model, an NO synthase inhibitor, N^G-nitro-L-arginine (L-NOARG), attentuated both vasodilatation and plasma extravasation to BK (Khalil & Helme, 1992). In the joint, however, co-perfusion of L-NAME, at a concentration shown to inhibit substance P-induced oedema formation by a local vasoconstrictor effect (Hughes & Brain, 1990), did not significantly depress plasma extravasation. A significant decrease in ^{133}Xe clearance produced by L-NAME (at the same concentration used for the plasma extravasation studies) suggests that perfusion of the joint is decreased by local inhibition of basal NO release and this may account for the observed trend towards attenuation of the BK response. In the rabbit knee, close arterial perfusion of L-NAME has also been shown to produce a significant drop in blood flow (Najafipour & Ferrell, 1993). Without simultaneous measurement of local blood flow or NO production it is not possible to differentiate between effects of L-NAME on basal NO release or on enhanced NO production stimulated by BK. As the effects of local L-NAME were inconclusive, the effect of intravenous administration was also measured. At a dose of 5 mg kg^{-1}, i.v., L-NAME did not significantly attenuate the BK response, although the individual data points are at the lower end of the control range. The response to BK after the higher dose of L-NAME (30 mg kg^{-1}) was highly variable but remained statistically unchanged from saline controls. Insufficient inhibition of the enzyme cannot account for these observations as a significant pressor response was observed in both treatment groups, indicative of decreased basal NO production by endothelial cells, and possibly NANC nerves (Rees *et al.*, 1989; Toda *et al.*, 1993). Thus the data obtained with intravenous L-NAME, shows clearly that NO production is not essential for BK-induced plasma extravasation in the joint. Gardiner and co-workers (1990a) demonstrated prolonged vasoconstriction in rat hindquarters and a pronounced fall in cardiac output following L-NAME (10 mg kg^{-1}, i.v.); thus it is likely that blood flow to the synovium was also decreased in treated rats and may account for the variability, and also the trend towards inhibition of plasma extravasation, seen in our study. A component of the vasodilator response induced by BK (3.2 nmol, i.v.) in rat hindquarters was found to be unaffected by L-NAME (Gardiner *et al.*, 1990b) indicating that some vascular actions of BK are also independent of NO synthesis. The lack of effect of antagonists for the important mast cell amines, histamine and 5-HT suggests that, although BK has been shown to stimulate mast cells directly, this is not an important mechanism of its action in this model.

In recent years a large body of experimental evidence has pointed to a key role for sympathetic terminals in enhancing BK-induced plasma extravasation into the joint in arthritis (Coderre *et al.*, 1991; Green *et al.*, 1993a,b). This appears to be highly specific to this property of BK as another major physiological effect of BK, nociceptor sensitization, is not dependent on sympathetic nerve activation (Koltzenberg *et al.*, 1992). This effect also seems confined to the joint as in some other sites, the pro-inflammatory effects of sympathetic nerve terminal activation have been more difficult to demonstrate (Donnerer *et al.*, 1991). Therefore sympathetic nerve activation may be of particular significance to arthritis and further investigation of this process is warranted. However, in our study chemical sympathectomy did not inhibit the response to BK and surprisingly a small increase was observed. The reason for this is unlikely to be inadequate sympathectomy as chronic 6-OHDA treatment is a well-established method for selective neurotransmitter depletion (Thoenen & Tranzer, 1968). The dosing protocol has been shown to deplete tyrosine hydroxylase containing nerves (Sulakvildze *et al.*, 1994) and has been used in previous studies in the joint (Green *et al.*, 1993a,b). In the present study the significant decrease in response to intravenous tyramine points to effective functional impairment of sympathetic nerve endings. A possible reason for the discrepancy between our results and previous findings is the use of different joint perfusion protocols. As in rat skin (Brain & Williams, 1985), the response to BK in the joint is rapid and, to minimize the possibility of systemic effects due to leakage from the joint, a relatively short (5 min) perfusion time is used. Green *et al.* (1993a,b) perfused the joints with a lower concentration of BK but at a higher flow rate and for up to 100 min, which may be sufficient time for both indirect and direct actions of BK on inflammatory cells to be of importance (Bjerknes *et al.*, 1991). In broad agreement with our results, Donnerer *et al.* (1991) found sympathectomy to have little inhibitory effect on neurogenic (saphenous nerve stimulation) or non-neurogenic inflammation (carrageenin paw oedema). The increased response to BK in sympathectomized animals may reflect loss of vasoconstrictor tone of noradrenaline and NPY, both of which inhibit plasma extravasation (Coderre *et al.*, 1989) although non-specific effects of 6-OHDA treatment cannot be ruled out.

Despite good evidence that BK can stimulate sympathetic nerves to release stored neurotransmitters (Weiss *et al.*, 1990; Green *et al.*, 1993b) and a recent report of an electrophysiological mechanism for the excitatory action of BK on cultured sympathetic ganglia (Jones *et al.*, 1995) the mechanism of the pro-inflammatory effect of sympathetic nerve stimulation is not well understood. Acute activation of sympathetic nerves by intra-articular perfusion with 6-OHDA has been reported to produce plasma extravasation in a similar fashion to BK (Coderre *et al.*, 1989); however, in our study, this was not observed. Due to drug leaking into the systemic circulation, a marked pressor response was observed in all animals in the 100 mM 6-OHDA group and severe toxic side effects of the drug were observed in several animals. From these results it is thus difficult to ascribe selective local actions of the drug on sympathetic nerves within the joint, despite significant plasma extravasation. Recently 6-OHDA has been shown to produce plasma extravasation in the rat trachea, but via a mechanism involving sensory rather than sympathetic nerves (Sulakvilidze *et al.*, 1994). Therefore further work, using alternative tools to 6-OHDA, is required to elucidate the mechanism of sympathetic nerve oedema which is clearly non-adrenergic in origin (Khalil & Helme, 1989). The effect on the BK-response of several vasoactive mediators (including ATP, adenosine, prostaglandins and NO) known to be released from sympathetic terminals has been measured (Coderre *et al.*, 1991; Green *et al.*, 1991). Each of these may modulate plasma extravasation by effects on local blood flow, however, none is a potent mediator of increased vascular permeability. The role of purines requires additional investigation as Evans Blue, used as a marker, is a purinoceptor antagonist (Bültmann & Starke, 1993).

We conclude from this study that, in the normal joint, BK mediates plasma extravasation by direct stimulation of B_2 type BK receptors which are likely to be located on vascular endothelial cells and/or smooth muscle. Although neural structures may be activated by BK there is no evidence that specific release of stored sensory neuropeptides, sympathetic neurotransmitters or activation of mast cells contribute significantly to the oedema response.

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Contribution of P_1- (A_{2b} subtype) and P_2-purinoceptors to the control of vascular tone in the rat isolated mesenteric arterial bed

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1 The direct vascular effects of adenosine and ATP were compared in the isolated and perfused mesenteric arterial bed of the rat. The actions of analogues of adenosine and ATP were also examined.

2 In preparations at basal tone, adenosine lacked vasoconstrictor actions, while ATP elicited dose-dependent vasoconstrictor responses. When the tone of preparations was raised by adding methoxamine to the perfusate, adenosine and its stable analogue, 2-chloroadenosine (2-CADO) elicited dose-dependent vasodilatation. The A_2 adenosine receptor agonist, 5'-N-ethylcarboxamidoadenosine (NECA) was active at lower doses than adenosine, while the A_{2a}-selective agonist, CGS 21680 and the selective A_1 agonist, N^6-cyclopentyladenosine (CPA) failed to induce vasodilatation. ATP and its analogue, 2-methylthio ATP, elicited dose-dependent vasodilatation at doses 400 fold lower than adenosine.

3 Vasodilator responses to adenosine and 2-CADO were sensitive to antagonism by 1 μM 8-sulphophenyltheophylline (8-SPT) and were unaffected by inhibition of nitric oxide synthase by N^{ω}-nitro-L-arginine methyl ester (L-NAME). In contrast, vasodilator responses to ATP were not sensitive to antagonism by 8-SPT and were almost abolished by L-NAME treatment.

4 These results indicate that in the rat mesenteric arterial bed, while both adenosine and ATP participate in the purinergic control of vascular tone, adenosine appears to be a weaker vasodilator than ATP and lacks vasoconstrictor action. A_{2b} adenosine receptors account for the adenosine-induced vasodilatation which is independent of the production of nitric oxide.

Keywords: Adenosine; ATP; mesenteric vascular bed; A_{2b} receptor

Introduction

Since the original observation by Drury & Szent-Gyorgyi (1929) on the cardiovascular effects of adenosine and adenine nucleotides a growing body of evidence has indicated that adenosine and ATP play different roles in the physiological control of cardiovascular function, acting via distinct receptor systems and mechanisms of transduction (see Burnstock, 1990). The characterization of distinct P_1- and P_2-purinoceptors which mediate the effects of adenosine and ATP respectively, was based on the rank order of potency of the agonists, sensitivity to methylxanthines and coupling to adenylate cyclase activity (Burnstock, 1978). Further subdivision of P_1-purinoceptors into A_1, A_2 (A_{2a} and A_{2b}) and A_3 subtypes is now established, the pharmacology of the A_4 subtype still being undefined (Collis & Hourani, 1993; Fredholm *et al.*, 1994; Linden, 1994). For ATP and other adenine nucleotide receptors the existence of two subtypes (P_{2x}- and P_{2y}-purinoceptors) was proposed by Burnstock & Kennedy (1985), based on the rank order of potency of several ATP analogues and selective antagonists and subsequently P_{2t}-, P_{2z}- and P_{2u}-purinoceptors were also identified (Fredholm *et al.*, 1994). Most recently, the voluminous literature describing the investigations of the pharmacological actions of purines in several tissues and the cloning of several subclasses of P_2-purinoceptors (Barnard *et al.*, 1994), has suggested the subdivision of P_2-purinoceptors into families of P_{2X} and P_{2Y}-purinoceptors to embrace all subdivisions of P_2-purinoceptors (Abbracchio & Burnstock, 1994).

Purinergic control of vascular function is achieved as a result of direct actions of adenosine and ATP on vascular smooth muscle or by actions via the endothelium and indirectly, via modulation of perivascular neurotransmission. In most organs the primary role of adenosine is to couple blood perfusion of tissues to cellular energy state and available evidence suggests that adenosine plays an important role as a powerful direct muscle vasodilator in several vascular beds, such as the coronary and cerebral vasculature (Kalaria & Harik, 1988; Olsson & Pearson, 1990; Vials & Burnstock, 1993). Vasoconstrictor responses to adenosine have been shown in the pulmonary, placental and renal circulation (Lippton *et al.*, 1989; Olsson & Pearson, 1990). Vascular responses to ATP may also consist of vasodilatation or vasoconstriction, depending on the vascular tone and on the purinoceptor subtype activation (Ralevic & Burnstock, 1992). The endothelium via production of endothelium-derived relaxing factors, such as nitric oxide (NO), is involved in the vasodilator responses to several agents and the vasodilator activity of purines has been shown to be either endothelium-dependent or independent (Furchgott, 1984; Ralevic & Burnstock, 1992). In the rat isolated mesenteric vascular bed both P_{2x}- and P_{2y}-purinoceptor subtypes have been characterized, mediating vasoconstrictor and endothelium-dependent vasodilator responses to ATP, respectively (Ralevic & Burnstock, 1988). Furthermore the inhibitory modulation of adenosine on sympathetic and sensory-motor neurotransmission has been shown in the same vascular model (Jackson, 1987; Rubino *et al.*, 1993). However, no information is available on the direct effects of adenosine in the resistance vessels of the mesenteric vascular bed and on the receptor subtype(s) mediating such actions.

The present investigation was therefore designed to examine the direct actions of adenosine on the rat mesenteric arterial bed and to compare them with the effects of ATP. In addition we have investigated the vasodilator potency of analogues of adenosine and ATP. The vascular effect of the purines was further characterized by using the P_1-purinoceptor antagonist 8-(*p*-sulphophenyl)theophylline (8-SPT) and an inhibitor of endothelial NO formation, N^{ω}-nitro-L-arginine methyl ester (L-NAME).

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Methods

Experiments were carried out in the mesenteric arterial bed, prepared for perfusion as previously described (Rubino *et al.*, 1993). Briefly, male Wistar rats (300–350 g) were killed by asphyxiation with carbon dioxide, the abdomen was opened and the superior mesenteric artery exposed and cannulated. The superior mesenteric vein was severed, the gut dissected away and perfusion (5 ml min^{-1}) started with Krebs solution containing (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8, gassed with 95% O_2, 5% CO_2 and maintained at 37°C. The preparations was allowed to equilibrate for 30 min before experimentation. Because flow through the mesenteric arterial bed was kept constant (5 ml min^{-1}) throughout the experiment, changes in perfusion pressure (mmHg) were indicative of changes in mesenteric vascular resistance. These were measured with a pressure transducer (model P23XL, Viggo Spectramed, Oxnard, CA, U.S.A.) on a side arm of the perfusion cannula and were recorded on a polygraph (model 79D, Grass Instrument Co., Quincy, Mass, U.S.A.). Concentration-response curves to adenosine and ATP were constructed by perfusing the preparations with increasing concentrations of the two agonists. However, due to the high cost of the experiments, dose-response curves to their analogues were obtained by applying the drugs as bolus injections of 50 μl via an injection port proximal to the preparation. When the effects of 8-SPT and L-NAME were tested, injections of adenosine, 2-chloroadenosine (2-CADO) and ATP were repeated on the same preparation in the absence and in the presence of the drugs.

Statistical analysis

Results are presented in the text and figures as means ± standard error of the mean (s.e.mean), followed by number of observations in parentheses (n). Student's t test for paired data was used to test for statistical significance and a value of $P < 0.05$ was considered statistically significant.

Drugs

Adenosine (hemisulphate), 5'-N-ethylcarboxamidoadenosine (NECA), 2-chloroadenosine (2-CADO), adenosine 5'-triphosphate (ATP), methoxamine hydrochloride and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma. 8-(*p*-Sulphophenyl)theophylline (8-SPT), CGS 21680, cyclopentyladenosine (CPA) and 2-methylthio ATP (2-meSATP) were from RBI. Stock solutions (10^{-2} M) of all drugs except CGS 21680 which was dissolved in 50% dimethylsulphoxide (DMSO), were in distilled water.

Results

Dose-response to adenosine, ATP and their analogues

Basal perfusion pressure in the isolated mesenteric vascular bed was 40.6 ± 2.9 (n = 12) mmHg. In preparations at basal tone, adenosine used at doses of up to 10^{-4} mol failed to induce vasoconstriction (see Figure 1). Similarly, the adenosine analogues, 2-CADO, NECA, CPA and CGS 21680 lacked vasoconstrictor activity. In contrast, ATP (3×10^{-7}–10^{-5} mol) elicited dose-dependent vasoconstrictor responses (Figure 1) achieving 42.2 ± 6.0 (n = 4) mmHg constriction at the highest dose tested.

The addition of methoxamine (3–10 μM) to the perfusate raised the vascular tone by 80.2 ± 3.7 (n = 12) mmHg and this tone was maintained throughout the experiment. In preparations in which the tone had been raised by methoxamine, continuous perfusion with ATP (10^{-8}–10^{-5} M) elicited concentration-dependent vasodilatation (Figure 2). At higher concentrations (10^{-6}–3×10^{-4} M) adenosine mimicked the vasodilator action of ATP. Similarly, bolus injection of ATP and its analogue 2-meSATP elicited dose-dependent vasodilatation at doses ranging from 10^{-11} to 3×10^{-8} mol, while adenosine was active at higher doses (Figures 1 and 2). At the highest dose tested (10^{-6} mol), adenosine relaxed by 49.5 ± 5.1 (n = 7) % the methoxamine-sustained tone of the preparations. The vasodilator responses to 2-CADO were superimposable on those to adenosine, the maximal vasodilatation achieved being 52.6 ± 4.2% (n = 4) of the raised tone. The A_2-selective agonist, NECA, appeared to be more potent, inducing a vasodilator response of 44.0 ± 3.2% (n = 4) when injected as a bolus of 10^{-6} mol (Figure 2). No responses were detected following injection of CGS 21680 and CPA up to 10^{-6} mol (Figure 2).

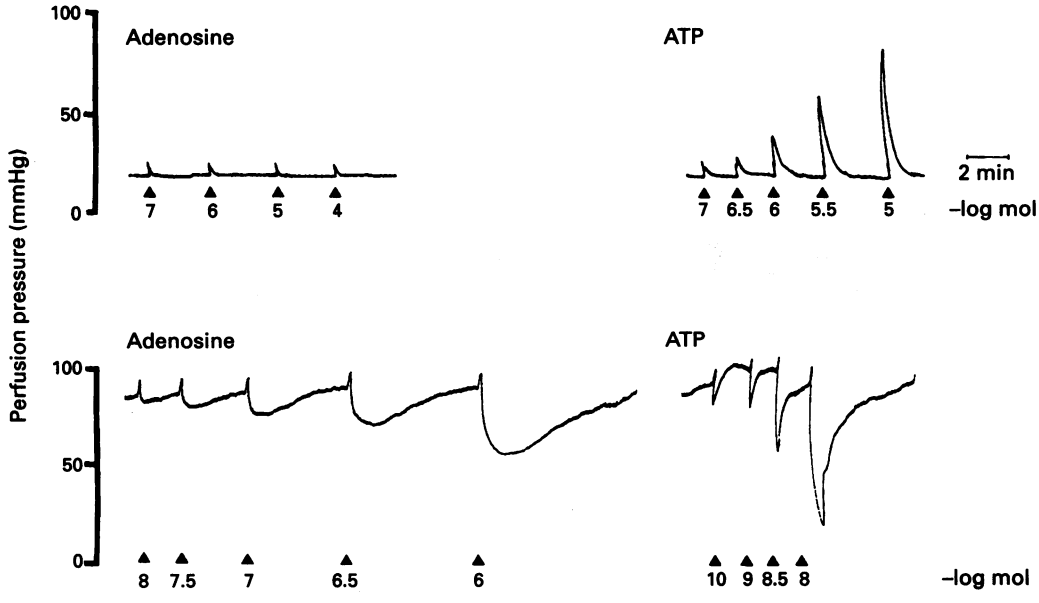

Figure 1 Representative tracings showing vasoconstrictor (upper tracings, preparation at basal tone) and vasodilator (lower tracings, preparation with tone raised by 10 μM methoxamine) responses of the rat mesenteric vascular bed to increasing doses of adenosine and ATP. Vascular responses are shown as increase in the perfusion pressure (mmHg). Doses were applied as 50 μl bolus injection and are labelled in the figure as (−log mol).

Effect of 8-sulphophenyltheophylline

In the presence of 8-SPT (1 μM) the tone of the preparations was not significantly different from controls, being 73.9 ± 5.2 mmHg (n = 8) above the basal value in the presence of methoxamine. 8-SPT attenuated the vasodilator responses to adenosine and its stable analogue 2-CADO, as indicated by the shift to the right in their dose-response curves (Figure 3). The vasodilatation induced by these agents was significantly reduced at all the doses, including the highest (Figure 3). In contrast, the dose-effect curve for ATP in the presence of 8-SPT was superimposable on that obtained in the absence of the antagonist (Figure 3).

Effect of L-NAME

Perfusion with L-NAME resulted in an increased sensitivity of the preparations to the vasoconstrictor action of methoxamine. Vasodilator responses were therefore evaluated in the presence of a reduced concentration of methoxamine (7 μM) which allowed the tone to be raised by 80.2 ± 4.0 mmHg (n = 4), as in control conditions (see above). L-NAME (30 μM) only partially but not significantly reduced the vasodilatation elicited by adenosine and 2-CADO (Figure 4). In contrast, L-NAME (30 μM) produced a drastic reduction of vasodilator responses to all doses of ATP tested (Figure 4).

Discussion

These data indicate that in the rat mesenteric arterial bed both P_1-and P_2-purinoceptors contribute to the regulation of vascular tone. Adenosine elicits vasodilatation via the A_{2b} receptor subtype and lacks vasoconstrictor effects, in contrast to ATP which is a powerful vasoconstrictor and a more potent vasodilator than adenosine.

When the preparations were perfused with increasing concentrations of adenosine and ATP, ATP was shown to be a more potent vasodilator than adenosine and this finding was confirmed when vascular responses to bolus injections of the two agonists and their analogues were evaluated. The vasodilator activity of ATP was mimicked by its analogue 2-meSATP used at the same doses, while the effect of adenosine was mimicked by 2-CADO and NECA. Dose-response curves for adenosine and 2-CADO were superimposable, while NECA elicited vasodilatation at lower doses. CPA, a selective agonist of A_1 adenosine receptors (Williams *et al.*, 1986), failed to in-

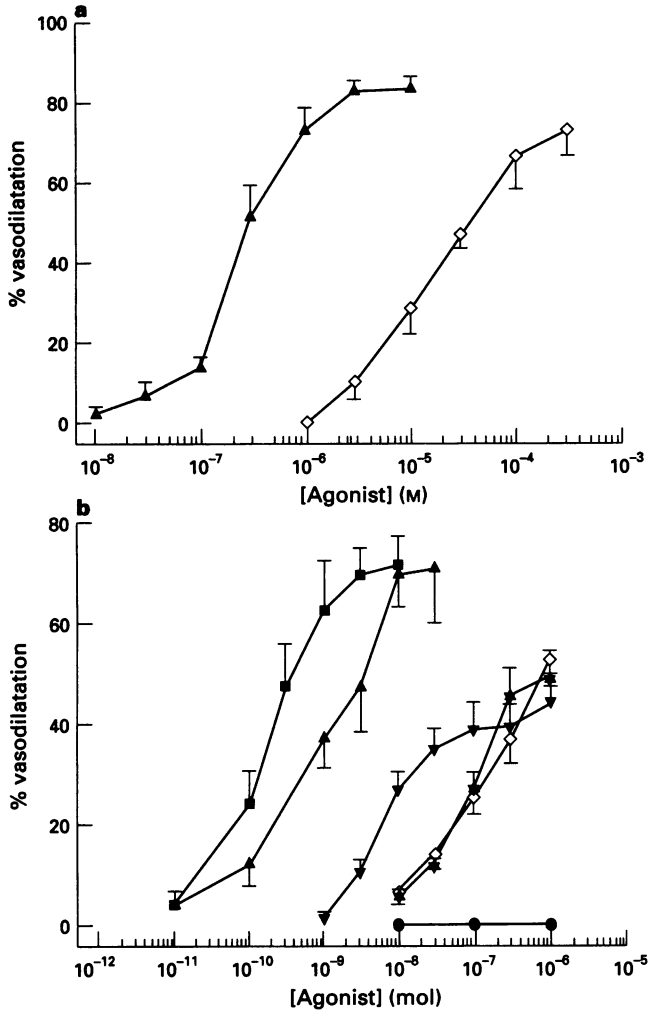

Figure 2 Dose-responses curves showing vasodilator responses of the rat mesenteric vascular bed to ATP (▲), 2-meSATP (■), adenosine (◇), 2-CADO (★), NECA (▼), CPA (○) and CGS 21680 (●). Vasodilator responses are shown as percent vasodilatation of the methoxamine sustained tone taken as 100% and are the mean of 4–7 preparations. (a) Shows vascular responses following perfusion of the preparations with increasing concentrations of ATP and adenosine; (b) shows vascular responses following bolus injection of increasing doses of agonists. Symbols show means ± s.e.mean. For abbreviations, see text.

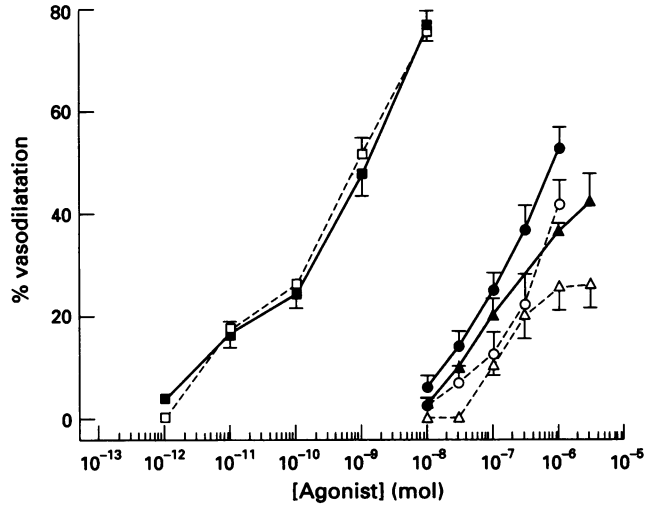

Figure 3 Dose-responses curves showing vasodilator responses of the rat mesenteric vascular bed to ATP (□, ■), adenosine (△, ▲) and 2-CADO (○, ●) in the absence (solid symbols) and in the presence (open symbols) of 1 μM 8-sulphophenyltheophylline. Symbols indicate means of 4 preparations ± s.e.mean.

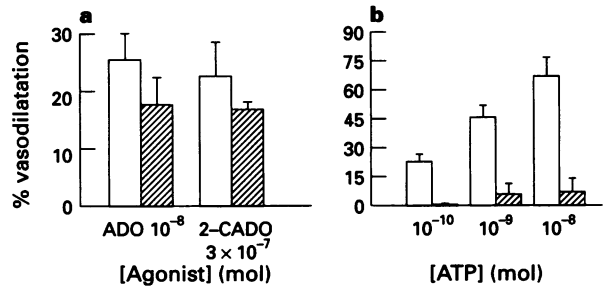

Figure 4 Vascular responses to adenosine (ADO) and 2-chloroadenosine (2-CADO) (a) and to ATP (b) in the absence (open columns) and in the presence (hatched columns) of N^{ω}-nitro-L-arginine methyl ester 30 μM. Columns indicate means ± s.e.mean of 4–6 preparations.

duce vasodilatation. Similarly, CGS 21680, which has been suggested as an A_{2a} receptor-selective agonist (Jarvis *et al.*, 1989; Lupica *et al.*, 1990) was devoid of vasodilator activity.

The vasodilator activity of adenosine and 2-CADO was sensitive to the antagonistic action of methylxanthine as indicated by the shift to the right of their respective dose-response curves in the presence of 8-SPT. In contrast, the vasodilator activity of ATP was unaffected by 8-SPT. The different sensitivity of adenosine- and ATP-mediated vasodilatation to antagonism by methylxanthine is in favour of the hypothesis that distinct receptor populations, namely P_1- and P_2-purinoceptors, mediate the vasodilator action of adenosine and ATP, respectively. Furthermore, the order of potency of the adenosine analogues and the lack of activity of CGS 21680 suggest that in the rat mesenteric arterial bed, P_1-purinoceptors of the A_{2b} subtype are responsible for the adenosine-mediated vasodilatation. The A_{2b} adenosine receptor has been characterized in other vascular preparations, such as guinea-pig aorta, where NECA was 400 fold more potent than CGS 21680 (Gurden *et al.*, 1993). The order of potency for NECA and CGS 21680 was reversed in dog and guinea-pig coronary artery, where the A_{2a} receptor subtype has been described (Gurden *et al.*, 1993; Vials & Burnstock, 1993). In the rabbit mesenteric artery the adenosine analogues, CGS 21680 and NECA, were equieffective as vasodilator agonists (Balwierczak *et al.*, 1991). It is noteworthy that the cloned A_{2b} receptor has been shown to be highly expressed in the rat intestine and bladder (Collis & Hourani, 1993), consistent with the functional data of this study.

In most vascular preparations vasodilator responses to adenosine appear to be endothelium-independent and the production of NO following adenosine receptor activation seems to play a minor role (Furchgott, 1984). However, A_2 adenosine receptors have been localized on endothelial cells in culture (Des Rosiers & Nees, 1987) and NO production has been suggested in the guinea-pig coronary circulation following activation of A_{2a} receptors (Vials & Burnstock, 1993). In this study the vasodilator effects of maximal doses of adenosine and 2-CADO were not significantly affected by a concentration of L-NAME that fully inhibited ATP-mediated vasodilatation thus suggesting that in the rat mesenteric circulation, adenosine-induced vasodilatation can be achieved independently from the production of NO. However, the contribution of NO to vasodilatation via A_{2b} receptors can be suggested in the light of the fact that L-NAME could slightly reduce, though not significantly, the vasodilator effect of adenosine and CADO. In contrast, ATP-mediated vasodilatation was largely dependent on NO formation, as shown previously in this preparation (Ralevic & Burnstock, 1988; 1992). These observations are in line with previous reports showing that in the rat mesenteric artery vasodilatation to ATP and adenosine is endothelium-dependent and -independent, respectively (Vourinen *et al.*, 1992).

In several vessels ATP has effects that are more potent than those of adenosine, for example in the constriction of the pulmonary circulation (Lippton *et al.*, 1989). Moreover, in the coronary circulation of every mammalian species ATP is a powerful vasodilator, often more potent than adenosine (Olsson & Pearson, 1990; Corr & Burnstock, 1991). In this study ATP elicited vasoconstrictor responses while adenosine and its stable analogues failed to induce vasoconstriction. When vasodilator responses were evaluated, ATP was able to elicit vasodilatation at concentrations 400 fold lower than adenosine and its analogues, thus confirming data obtained in the isolated mesenteric artery where ATP is a more potent vasodilator than adenosine (Vourinen *et al.*, 1992). However, as first described by McGregor (1965) and confirmed by more recent investigations (Longhurst *et al.*, 1986), the isolated mesenteric arterial bed rather than vascular rings isolated from the main mesenteric artery, is representative of vascular responsiveness at the level of resistance vessels and the whole-bed preparation can therefore be considered as a valuable *in vitro* model of splanchnic circulation. In the light of these considerations, this study rather than previous observations (Vourinen *et al.*, 1992) suggests that ATP is a more powerful vasoactive agent than adenosine in the intestinal vasculature.

Adenosine and ATP may change vascular tone in either of two ways, directly through receptors on the vascular wall or indirectly as modulators of perivascular innervation. The inhibitory effect of adenosine on sympathetic neurotransmission has been well documented *in vitro* and *in vivo* in the rat mesenteric circulation (Lukacsko & Blumberg, 1982; Jackson, 1987). Moreover, previous observations in this laboratory demonstrated that in the rat mesenteric arterial bed, adenosine and its analogues modulate sensory-motor neurotransmission via prejunctional A_1 receptors at concentrations devoid of any direct action on vascular tone (Rubino *et al.*, 1993). From the observations of this study and from data described in the literature it can be speculated that adenosine and ATP both participate in the regulation of the mesenteric circulation via activation of distinct receptor systems and via different mechanisms which are independent or dependent on NO production for adenosine and ATP, respectively. However, ATP appears to be more powerful than adenosine in causing direct vasoactive actions, whilst adenosine exerts indirect effects as a modulator of perivascular sympathetic and sensory-motor neurotransmission. These observations are in contrast with the hypothesis of Berne (1963; 1980) in favour of adenosine, a breakdown product of ATP, as the main endogenous vasodilator and further support the view that ATP itself is a powerful regulator of vascular tone, producing both vasodilatation and vasoconstriction (Burnstock, 1993).

In conclusion, this study shows ATP as a more powerful vasoactive agent than adenosine in the regulation of the mesenteric circulation via direct vascular effects and demonstrates for the first time that in the rat mesenteric arterial bed, adenosine elicits vasodilatation via A_{2b} receptors, independently of NO formation.

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Actions of endothelins and sarafotoxin 6c in the rat isolated perfused lung

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1 Endothelin (ET) receptors within the vasculature and airways were studied in a rat perfused lung model in which pulmonary perfusion pressure (PPP), pulmonary inflation pressure (PIP) and lung weight were continuously monitored.

2 The vascular potencies of ETs (ET-1 > ET-2 > ET-3) suggest an action via ET_A receptors. This was confirmed by use of the antagonist, BQ123 (2 μM). The vasoconstrictor effects of sarafotoxin 6c (SX6C) also indicated the presence of ET_B receptors.

3 Lung weight increases induced by ETs appeared to be a consequence of their vasoconstrictor potencies. The mixed ET receptor antagonist, bosentan (5 μM), markedly attenuated the responses of ET-1 and SX6C on PPP and lung weight, further implicating activation of both ET_A and ET_B receptors in these responses.

4 Endothelin-1 (ET-1) induced an accumulation of albumin-bound Evans blue dye in orthogradely perfused lungs. Retrograde perfusion attenuated the extravasation and increase in lung weight due to ET-1 but significantly augmented those induced by SX6C.

5 The bronchoconstrictor actions of ETs (ET-1 = ET-2 = ET-3) and SX6C suggest this is an ET_B-mediated response. However SX6C was more potent than ETs and the dose-response curve was significantly steeper and achieved a higher maximum.

6 Indomethacin did not affect the vascular or bronchial responses to ET-1 or SX6C.

7 These findings indicate that rat pulmonary vasculature contains both ET_A and ET_B receptors. Retrograde perfusion suggests that ET_B receptors are located arterially whereas ET_A receptors are predominantly venous in distribution. Differences in the bronchoconstrictor potency of SX6C (compared to ETs) and the antagonism by bosentan may indicate ET_B receptor heterogeneity in the airways.

Keywords: Endothelins; sarafotoxin 6c; BQ123; bosentan; rat lung; pulmonary vasoconstriction; bronchoconstriction; lung weight

Introduction

Endothelin (ET) was discovered as a potent vasoactive peptide produced by cultured endothelial cells (Yanagisawa *et al.*, 1988). It is now apparent that there are four forms of endothelin [endothelin-1 (ET-1), ET-2 and ET-3] (Inoue *et al.*, 1989) including vasoactive intestinal contractor (VIC) (Saida *et al.*, 1989). Sarafotoxins (SX6A, -B, -C and -D) also possess structural features and pharmacological activities similar to those of ETs (Kloog *et al.*, 1988). Based on differential affinities for ETs and related peptides, four types of ET receptor have also been described namely ET_A, ET_B, ET_C and ET_{AX}. The ET_A receptor is characterized by the rank order of binding affinities ET-1 $\geq$ ET-2 > ET-3 (Arai *et al.*, 1990), while the ET_B receptor is non-isopeptide-selective (ET-1 = ET-2 = ET-3) (Sakurai *et al.*, 1990) and at the ET_C receptor ET-3 is more potent than ET-1 (Karne *et al.*, 1993). The ET_{AX} receptor cloned recently shares 74%, 60% and 51% identities with human ET_A, human ET_B and *Xenopus* ET_C receptors (Kumar *et al.*, 1994). SX6B has a higher affinity for ET_A receptors whereas SX6C selectively binds to ET_B receptors (Williams *et al.*, 1991).

The actions of ETs in the lung have attracted considerable interest. ETs are potent constrictors of pulmonary blood vessels (McKay *et al.*, 1991; White *et al.*, 1993; Cardell *et al.*, 1993; MacLean *et al.*, 1994). In a recent report it has been shown that the constrictor action of ETs in segments of rat large pulmonary artery are mediated by ET_A receptors whereas constriction in the small pulmonary arteries is mediated by the activation of ET_B receptors (MacLean *et al.*, 1994). ETs also constrict tracheal rings (Black *et al.*, 1989; Cardell *et al.*, 1993; Battisitini *et al.*, 1994) and lung parenchymal strips (Battistini *et al.*, 1994), an action primarily mediated via ET_B receptors (Battistini *et al.*, 1994).

In the present study we have utilised an isolated perfused lung model (Lal *et al.*, 1994a) to investigate the effects of ETs and SX6C with a view to identifying the subtypes of ET receptor present. As an adjunct we have also studied the effects of an ET_A receptor antagonist, BQ123 (Ihara *et al.*, 1992) and bosentan, the mixed ET_A/ET_B receptor antagonist (Clozel *et al.*, 1994), on the responses to ETs and SX6C. In some experiments retrograde perfusion of the lungs via the pulmonary vein was used to localize further the sites of action of ET-1 and SX6C. Preliminary findings from these studies have been presented to the British Pharmacological Society (Lal *et al.*, 1994b,c).

Methods

Isolated ventilated perfused lung preparation

Lungs were isolated and perfused as described previously (Lal *et al.*, 1994a). Male Wistar rats (300-350 g) were anaesthetized with Sagatal (60 mg kg^{-1} body weight, i.p.). Heparin (500 iu) was injected i.v. via the tail vein; 5 min later the chest was opened and the pulmonary artery cannulated with a stainless steel cannula (external diameter = 1.5 mm, internal diameter = 1 mm) via the right ventricle. The left atrium was cut and the major part of the ventricles removed to allow free efflux of the perfusate. The trachea was cannulated, the lungs were isolated and immediately transferred into a warming jacket maintained at 37°C. Lungs were perfused via the pulmonary artery at 5 ml min^{-1} with Krebs solution of the fol-

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lowing composition (mM): potassium chloride 4.7, potassium dihydrogen phosphate 1.2, calcium chloride 1.25, magnesium sulphate 1.2, sodium chloride 118, sodium bicarbonate 25, glucose 11.1, (gassed with 95% O_2 and 5% CO_2). Pulmonary perfusion pressure (PPP) was recorded via a pressure transducer connected to the pulmonary artery cannula. The tracheal cannula was attached to a ventilator and the lungs were ventilated with room air, stroke volume (1 ml) using 28 strokes min^{-1} (no positive end expiratory pressure). A pressure transducer was attached to the tracheal cannula for recording the pulmonary inflation pressure (PIP). Lungs were suspended from an isometric transducer for recording changes in lung weight. All parameters were recorded on a multichannel recorder (Grass model 7D polygraph). Lungs were allowed to stabilize for 30 min before drug administration. After the initial stabilization, basal values for recorded parameters were as follows: PPP, 6.7 ± 0.5 mmHg; PIP, 5.0 ± 0.01 mmHg and lung weight, 2.8 ± 0.06 g, $n = 28$. Drugs were injected as bolus doses (10–100 μl) via the pulmonary artery.

Effects of indomethacin and endothelin antagonists

In a series of experiments, BQ123 (2 μM) or bosentan (5 μM) was added to the perfusate 20 min or 30 min, respectively, before giving the bolus injections of drugs via the pulmonary artery cannula. These concentration ranges have previously been shown to produce inhibition of ET-1-induced effects (Hay *et al.*, 1993; Clozel *et al.*, 1994). Experiments were also performed with indomethacin (10 μM) to study the involvement of cyclo-oxygenase products. Indomethacin (10 μM) was added to the perfusate 30 min before agonist injection to inhibit prostanoid production (Palmer *et al.*, 1973).

Retrograde perfusion

In some experiments a cannula was introduced into the left atrium via the left ventricle and lungs were perfused via the pulmonary vein and pulmonary venous pressure monitored together with LW and PIP. Drugs were injected as bolus doses (10–100 μl) via the pulmonary vein cannula.

Determination of albumin-bound dye accumulation

Evans blue dye was added to a 10% (w/v) solution of bovine serum albumin (BSA) in saline to give a final concentration of 1 mg ml^{-1}. The mixture was dialysed overnight in Visking tubing (22/23) against an excess of 0.9% saline. Aliquots of the solution were infused into the pulmonary artery (0.1 ml min^{-1}) for 5 min, 1 min after ET-1 or SX6C injection. Lungs were perfused for a further 10 min to remove unbound dye from the vasculature, lungs were removed and after removal of cardiac tissue and trachea, the lungs were oven dried (50–60°C) overnight and dry weight noted. Lungs were then digested with formamide (2 ml) at 40°C in a water bath for 20 h and the contents were centrifuged (Eppendorf centrifuge 5414) at 9880 *g* for 30 min. The absorbance of standard concentrations of Evans blue (0.25–20 μg ml^{-1}) or lung extracts were read at 622 nm in a spectrophotometer (Pye Unicam). The concentration of dye in lung extracts was calculated by reference to the standard curve. From this value, fluid accumulation in the lung could be calculated by reference to the concentration of dye in the fluid perfusing the lung.

Drugs used

BQ123 cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]) was supplied by Dr. K. Clark, Glaxo Group Research (Ware, Herts) and bosentan (Ro47-0203; 4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulphonamide sodium salt) was provided by Hoffmann-La Roche Ltd (Basel, Switzerland). Endothelin-1, endothelin-2 and endothelin-3 were from Novabiochem (Nottingham), indomethacin was from Sigma (Poole, Dorset) and sarafotoxin 6c from Peninsula Laboratories (Merseyside). Stock solutions of drugs were prepared in 0.9% normal saline and stored at −20°C.

Data analyses

Increases in PPP, PIP and lung weight are expressed as relative to the basal values prior to the addition of each dose. When lung weight measurement was needed for comparative purposes, weight gain was calculated 7 min after the maximum dose of agonist. Data are expressed as mean ± standard error of the mean (s.e.mean). ANOVA, Dunnett's and Student's *t* test were used to test the levels of significance. Probability values of $P < 0.05$ were considered significant.

Results

Pulmonary vascular and bronchial actions of ETs and SX6C

Effects on pulmonary perfusion pressure (PPP) The accumulated results for ETs (ET-1, ET-2 and ET-3) and SX6C on PPP are shown in Figure 1. From Figure 1a, it can be seen that over the dose-range used the order of potency of the ETs was ET-1 > ET-2 > ET-3. SX6C was of similar potency to ET-1 with ED_{50} values of 165 ± 19 pmol, $n = 6$ and 130 ± 17 pmol respectively ($n = 10$).

Effects on lung weight and albumin accumulation Figure 1b shows the accumulated results for the effects of ETs and SX6C on lung weight. The order of potency of the ETs was ET-1 > ET-2 > ET-3. In contrast, SX6C which was equipotent with ET-1 on PPP caused no significant change in lung weight over the dose-range used.

In a separate set of experiments the increase in lung weight caused by ET-1 (800 pmol) was shown to be associated with a large accumulation of albumin bound dye (97.5 ± 22 ng mg^{-1} dry weight of tissue compared to 1.32 ± 0.54 ng mg^{-1} in control lungs without ET-1; $P < 0.01$, $n = 4$). Back-calculation of fluid retention from retained dye indicated that 800 pmol of ET-1 caused an accumulation of 1.62 ± 0.28 ml of fluid ($n = 4$). Assuming a specific gravity of 1 for this fluid a comparison of this calculated weight gain with weight gain recorded experimentally (4.25 ± 0.25 g, $n = 4$) illustrates that the values are significantly different ($P < 0.01$), i.e. fluid accumulation without albumin.

Effects on pulmonary inflation pressure (PIP) As shown in Figure 1c the three ETs were equipotent on PIP (ED_{50} values for ET-1, ET-2 and ET-3 on PIP were 216 ± 2 pmol, $n = 10$; 277 ± 7 pmol, $n = 4$ and 268 ± 4 pmol, $n = 8$ respectively). However SX6C was the most potent bronchoconstrictor agent (ED_{50} 80 ± 6 pmol, $n = 6$). In addition the slope of the PIP response to SX6C was significantly steeper than that for any of the ETs ($P < 0.001$).

Effects of indomethacin and endothelin antagonists

Indomethacin (10 μM) had no effect on basal PPP, PIP or lung weight ($n = 14$), and did not affect the actions of ET-1 on PPP (ED_{50} value of 132 ± 14 pmol was not different from control ED_{50} of 130 ± 17 pmol, $n = 10$), PIP (ED_{50} of 200 ± 2 pmol compared to control ED_{50} of 216 ± 2 pmol, $n = 10$) or lung weight (maximum increase in lung weight produced by 400 pmol ET-1 in the presence and absence of indomethacin was 4 ± 0.9 g and 4.5 ± 0.8 g $n = 10$ respectively).

SX6C-mediated responses were also unaffected by indomethacin (10 μM). PPP ED_{50} values for SX6C in the presence and absence of indomethacin were 142 ± 11 pmol ($n = 4$) and 165 ± 19 pmol ($n = 6$) respectively, while the corresponding PIP ED_{50} values in the presence and absence of indomethacin were 81 ± 8 pmol ($n = 4$) and 80 ± 6 pmol ($n = 6$). SX6C had no significant effect on lung weight with or without indomethacin.

BQ123 (2 μM), a selective ET_A receptor antagonist, had no effect on basal PPP, PIP and lung weight ($n=4$). However, perfusion of BQ123 significantly attenuated the effects of lower doses of ET-1 on PPP (Figure 2a). The ED_{50} value for ET-1 was increased from 130±17 pmol ($n=10$) to 311±3 pmol ($n=4$; $P<0.01$) in the presence of BQ123. However, BQ123 had no effect on the maximal increase in PPP induced by ET-1. The maximum increase in lung weight produced with 400 pmol ET-1 in control lungs (4.5±0.8 g, $n=10$) was significantly reduced in the presence of BQ123 (0.75±0.1 g, $n=4$, $P<0.01$) as shown in Figure 2b. Interestingly, BQ123 significantly augmented the effects of ET-1 (200 pmol) on PIP ($P<0.05$) but it had no effect on responses to other doses of ET-1 (Figure 2c).

BQ123 (2 μM) did not affect the PPP response to SX6C; control ED_{50} was 165±19 pmol ($n=6$) and in the presence of BQ123 was 184±20 pmol ($n=3$). Similarly, SX6C-induced changes in PIP were not altered by BQ123; control ED_{50} was 80±6 pmol ($n=6$) and in presence of BQ123 was 68±2 pmol ($n=3$).

Bosentan (5 μM), a mixed endothelin receptor antagonist, had no effect on the basal PPP, PIP and lung weight ($n=4$). However, it significantly ($P<0.001$, $n=4$) reduced the ET-1-induced PPP and lung weight responses as shown in Figure 3a and 3b, respectively. In contrast, bosentan did not affect ET-1-induced PIP responses (Figure 3c). Responses to SX6C on PPP, PIP and lung weight were significantly ($P<0.001$, $n=4$) attenuated by bosentan (Figure 3).

Effects of ET-1 and SX6C in retrogradely perfused lungs

Figure 4 illustrates the actions of ET-1 and SX6C on recorded parameters in orthogradely and retrogradely perfused lungs. ET-1 (50–400 pmol) and SX6C (50–800 pmol) produced dose-dependent increases in PPP, PIP and lung weight. However, a comparison of the upper panels shows that the actions

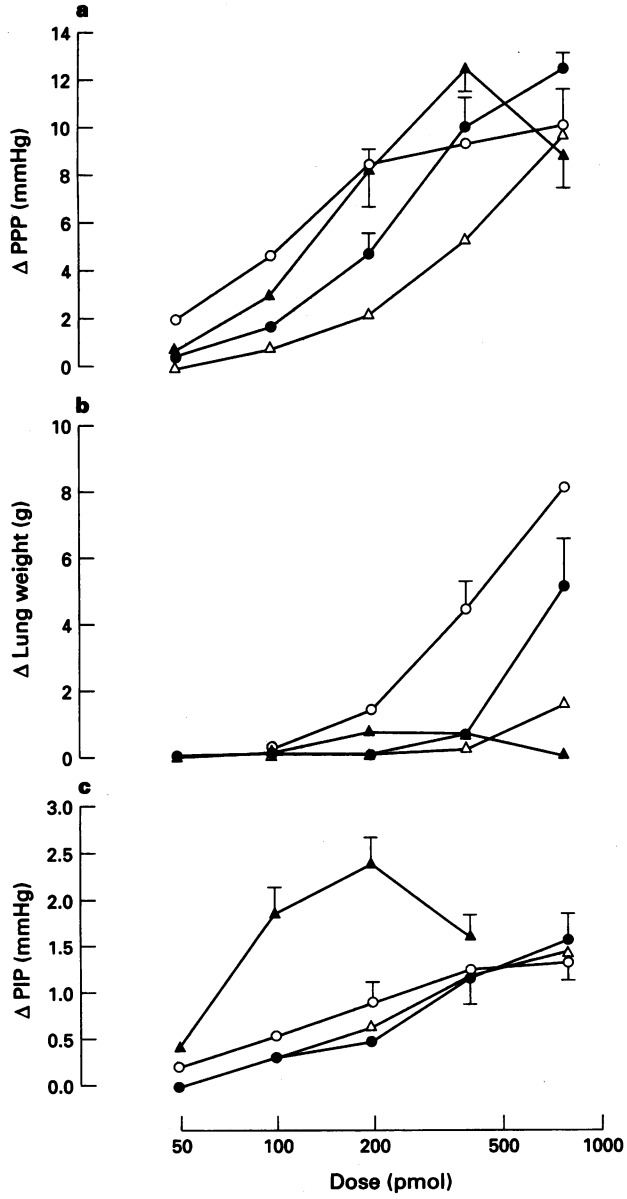

Figure 1 Actions of endothelin-1 (ET-1, ○), ET-2 (●), ET-3 (△) and sarafotoxin 6c (SX6C, ▲) on (a) pulmonary perfusion pressure (PPP), (b) lung weight and (c) pulmonary inflation pressure (PIP). Each point represents mean±s.e.mean, $n=4-10$ experiments.

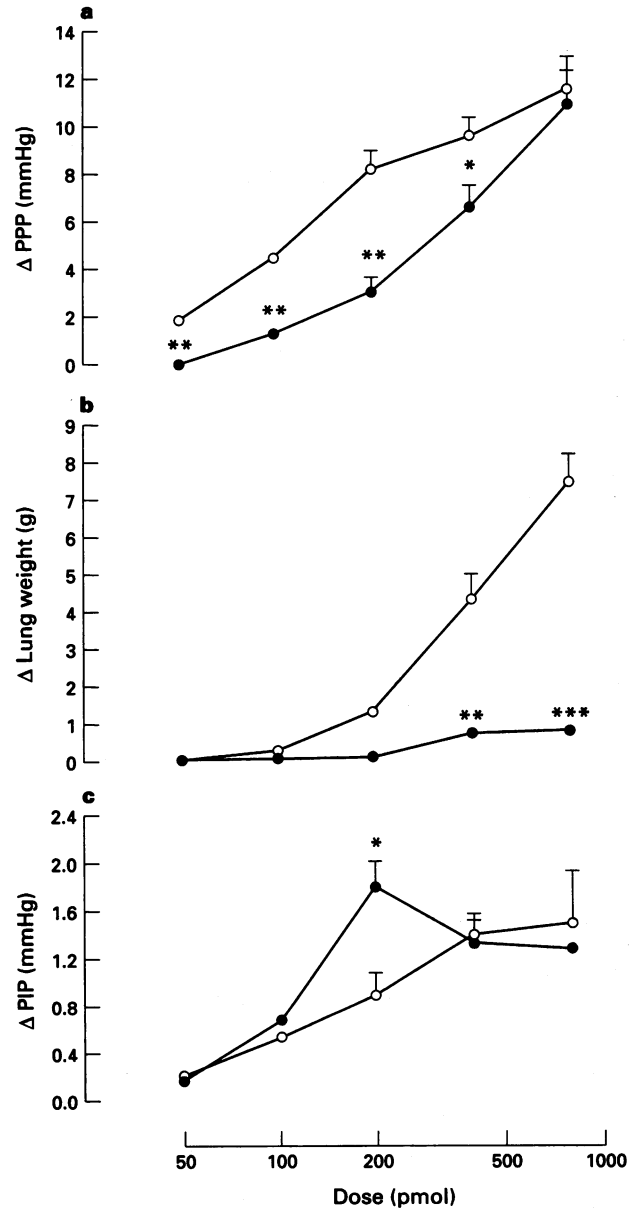

Figure 2 Endothelin-1 (ET-1)-induced increases in (a) pulmonary perfusion pressure (PPP), (b) lung weight and (c) pulmonary inflation pressure (PIP) in the absence (○, control data as in Figure 1) and presence of BQ123 (2 μM) (●). Each point represents mean±s.e.mean, $n=4-10$ experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$: significantly different from control ET-1.

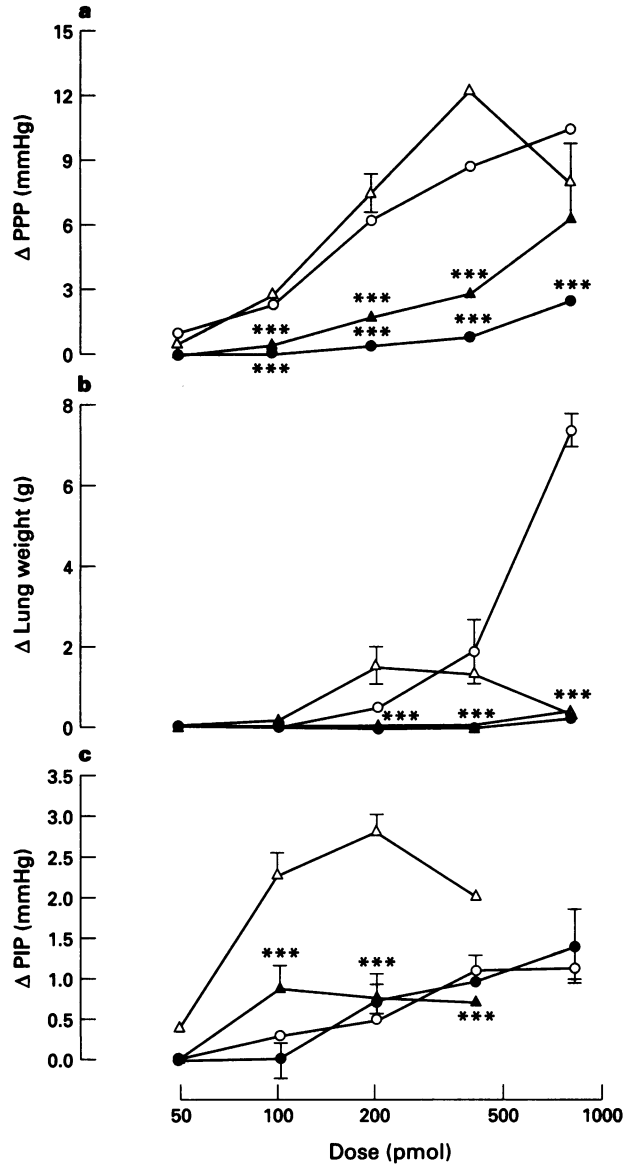

Figure 3 Effects of bosentan (5 μM) on endothelin-1 (ET-1, ○, ●) and sarafotoxin 6c (SX6C, △, ▲) on (a) pulmonary perfusion pressure (PPP) (b) lung weight and (c) pulmonary inflation pressure (PIP) in absence (open symbols) and presence of bosentan (closed symbols). Each point represents mean ± s.e.mean, $n = 4-13$ experiments. ***$P < 0.001$: significantly different from control ET-1 or SX6C.

of ET-1 on lung weight were markedly reduced by retrograde perfusion. Using orthograde perfusion, 400 pmol of ET-1 produced an increase in lung weight of 6.9 ± 0.21 g ($n = 4$) whereas 400 pmol of ET-1 in retrograde perfusion produced only a 1.8 ± 0.36 g ($n = 4$, $P < 0.01$) increase. Furthermore, albumin bound dye accumulation (1.48 ± 0.85 ng mg^{-1} of dry wt, $n = 4$) produced by a higher dose of ET-1 (800 pmol) with retrograde perfusion was still significantly lower ($P < 0.01$) than the accumulation seen with 400 pmol ET-1 (25 ± 9.5 ng mg^{-1} of dry wt., $n = 4$) given via orthograde perfusion.

Comparison of the lower panels in Figure 4 shows that the actions of SX6C on lung weight were markedly increased by retrograde perfusion. Using orthograde perfusion, 800 pmol of SX6C produced a 0.11 ± 0.06 g ($n = 4$) increase in lung weight whereas the same dose given via retrograde perfusion produced a 7.5 ± 0.29 g ($n = 4$, $P > 0.001$) increase in lung weight. Furthermore, in a separate set of experiments accumulation of albumin bound dye (216 ± 2.6 ng mg^{-1} dry tissue, $n = 4$) caused by SX6C (800 pmol) in retrograde perfusion was significantly greater ($P < 0.01$) than albumin bound dye accumulation (70 ± 4 ng mg^{-1} dry wt, $n = 4$) caused by SX6C in orthograde perfusion. Back calculation of fluid retention from retained dye indicated that 800 pmol of SX6C caused an accumulation of 1.44 ± 0.06 ml and 6.6 ± 0.39 ml fluid in orthogradely and retrogradely perfused lungs, respectively ($n = 4$). Assuming a specific gravity of 1 for this fluid a comparison of this calculated weight gain with weight gain recorded experimentally (1.46 ± 0.24 g and 7.3 ± 0.15 g, $n = 4$, in orthogradely and retrogradely perfused lung, respectively) illustrates that the values are in good agreement.

Discussion

We have studied the effects of ETs and SX6C in a rat perfused lung model which allows simultaneous measurement of PPP, PIP and lung weight. The pulmonary vasoconstrictor potencies of ETs (ET-1 > ET-2 > ET-3) suggest an action mediated via ET_A receptors (Webb, 1991). This was supported by the finding that a low concentration of BQ123, a selective ET_A receptor antagonist (Ihara *et al.*, 1992), markedly attenuated the pulmonary vasoconstrictor actions of ET-1. A similar finding has been reported by Bonvallet *et al.* (1993). However SX6C, a highly selective ET_B agonist (Williams *et al.*, 1991) was also found to be a pulmonary vasoconstrictor with a potency similar to ET-1. This shows that stimulation of ET_B receptors can also produce vasoconstriction in the lung; a similar finding has been reported in guinea-pig lung (Noguchi *et al.*, 1993). In addition ET_B receptors have been shown to be present on isolated rings of rat small pulmonary artery (MacLean *et al.*, 1994). In the present experiments, perfusion of bosentan a mixed ET_A/ET_B receptor antagonist (Clozel *et al.*, 1994) blocked the responses of ET-1 and SX6C on PPP confirming the findings that pulmonary vasoconstriction is mediated via both ET_A and ET_B receptors.

Our data indicate that ETs are also very potent in producing increases in lung weight, which supports the findings of Ercan *et al.* (1993). The potency profile of ETs in causing increases in lung weight parallelled their vasoconstrictor potencies which indicates that changes in lung weight are secondary to their vasoconstrictor actions. Interestingly SX6C produced similar increases in PPP to the ETs but caused very little increase in lung weight. We have previously reported that phenylephrine selectively increases PPP whereas bradykinin increases both PPP and lung weight. These different effects were explained by phenylephrine producing arteriolar vasoconstriction whereas bradykinin caused venoconstriction (Lal *et al.*, 1994a). Thus SX6C, like phenylephrine is probably acting on the arteriolar side of the pulmonary circulation whereas ETs, acting like bradykinin, cause venoconstriction, an action ETs are known to exert (Horgan *et al.*, 1991; Rodman *et al.*, 1992) and oedema formation. In order to investigate this possibility retrograde perfusion was used. Under these conditions, arterial vasoconstriction will now cause an increase in hydrostatic pressure within the microcirculation and increase lung weight via hydrostatic means whereas venous constriction would be expected to cause little effect. This is exactly what was found, SX6C now produced significant increases in lung weight, whilst ET-1 had a reduced effect on lung weight, suggesting that ET_B receptors stimulated by SX6C are predominantly located on the arterial side of the pulmonary circulation whereas the majority of ET_A receptors appear to be located in the pulmonary venous vessels. These results agree with findings in other vascular beds (D'Orleans-Juste *et al.*, 1993; White *et al.*, 1994; MacLean *et al.*, 1994).

Increases in lung weight produced by ET-1 were associated with large accumulations of albumin bound dye. We have previously reported that increases in venous outflow pressure in this lung model can cause significant retention of albumin bound dye (Lal *et al.*, 1994a). In the present experiments ET-1

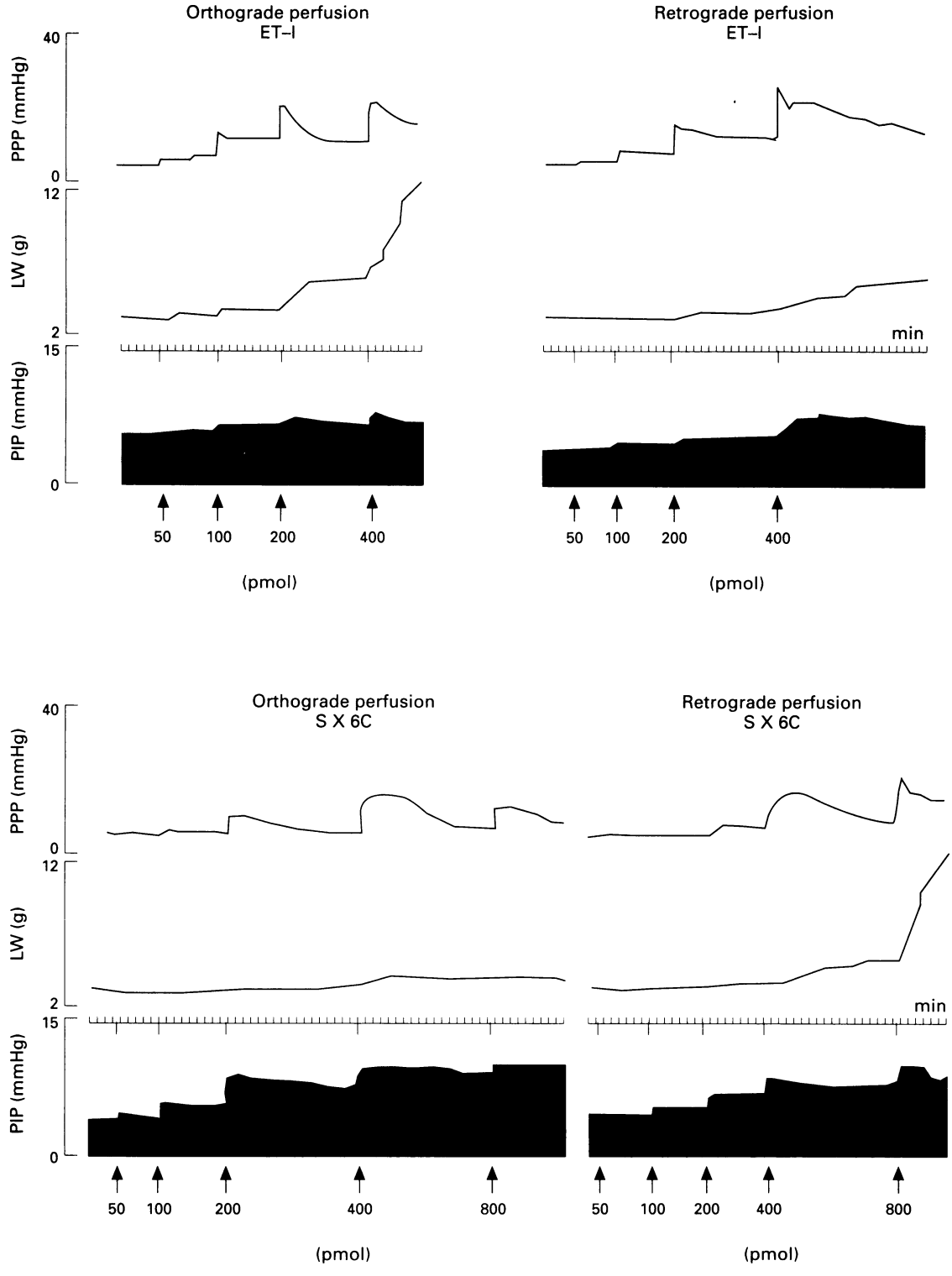

Figure 4 Experimental traces comparing the effects of endothelin-1 (ET-1, upper section) and sarafotoxin 6c (SX6C, lower section) on pulmonary perfusion pressure (PPP), lung weight (LW) and pulmonary inflation pressure (PIP) in rat lung perfused orthogradely (right hand panels) and retrogradely (left hand panels). This figure represents one of 4 similar experiments.

when given in the orthograde mode of perfusion produced marked accumulation of albumin bound dye, whereas when ET-1 was given via the retrograde mode of perfusion significantly less dye retention was seen. These findings further support the suggestion that increases in lung weight and retention of dye produced with ET-1 are due to potent venoconstriction. Furthermore increases in lung weight resulting from fluid accumulation were significantly higher than the expected lung weight increases calculated from the retention of dye. This suggests that accumulation of fluid in the lung is primarily due to hydrostatic oedema rather than to a direct increase in vascular permeability to albumin.

When SX6C was given via orthograde perfusion it also caused retention of albumin bound dye which indicates the presence of ET_B receptors mediating constriction of pulmonary venous smooth muscle which would contribute to the pulmonary oedema via a hydrostatic mechanism. However when SX6C was given via retrograde mode of perfusion it

produced a significantly higher retention of albumin bound dye compared to orthograde perfusion. This provides further evidence that the majority of ET_B receptors are located at the arteriolar level.

BQ123 significantly attenuated the changes in lung weight produced by ET-1 suggesting the involvement of ET_A receptors in this action. Filep *et al.* (1992, 1993) have also recently concluded that ET-1 increases vascular permeability via an action on ET_A receptors. However, these findings are not in agreement with those of Bonvallet *et al.* (1993), who showed that in the rat isolated perfused lung, ET-1-associated hydrostatic oedema was unaffected by BQ123. Bosentan, a mixed ET_A/ET_B receptor antagonist, was shown to block the increases in PPP and lung weight produced by ET-1 or SX6C. This again indicates that stimulation of both ET_A and ET_B receptors can produce pulmonary oedema.

The bronchoconstrictor profile of ETs (ET-1 ≈ ET-2 ≈ ET-3) and SX6C suggests the presence of ET_B receptors in the bronchial smooth muscle. The fact that BQ123 at a concentration which reduced ET-1 induced increases in PPP and oedema, did not produce any reduction in the bronchoconstrictor effects of ET-1 or SX6C shows that bronchoconstriction was not mediated by ET_A receptors. Similar findings have been reported in guinea-pig (Hay *et al.*, 1993; Battistini *et al.*, 1994) and human bronchus (Hay *et al.*, 1993). The finding that SX6C was more potent than the ETs in causing bronchoconstriction and that the slope of the dose-response curve was significantly steeper and achieved a higher maximum could be due to a number of factors. Firstly ETs are known to be more susceptible to breakdown by neutral endopeptidase present in the lung, this could reduce the effects of ETs compared with SX6C (Sokolovsky *et al.*, 1990). However, we feel this is unlikely as we have shown that the bronchoconstrictor actions of ETs were not influenced by the addition of the neutral endopeptidase inhibitor phosphoramidon (unpublished observation). A recent report by Hisaki *et al.* (1994) has also shown that in rat isolated lung, pressor responses to ET-1 were not influenced by phosphoramidon. Secondly, ETs could be releasing some bronchodilator substance resulting in a physiological antagonism, as reported by Uchida *et al.* (1991). In the present study use of the selective ET_A receptor antagonist, BQ123, significantly augumented the bronchoconstrictor response of ET-1 at 200 pmol. This could indicate that activation of ET_A receptors may release a bronchodilator substance which antagonizes the bronchoconstrictor responses of ET-1. Interestingly, Battistini *et al.* (1994) also reported that BQ123 potentiated the ET-1-induced contractions of guinea-pig isolated trachea.

Perfusion with the mixed ET_A/ET_B receptor antagonist, bosentan, produced a significant reduction in the bronchoconstrictor responses of SX6C without having any affect on the bronchoconstrictor responses of ET-1. This could indicate that in bronchial smooth muscles, ET_B receptors activated by ET-1 are different from the ET_B receptors activated by SX6C. The existence of different ET_B receptor subtypes in the guinea-pig isolated trachea has also been suggested by Battistini *et al.* (1993). Alternatively the strong binding affinity of ET-1 for ET_B receptors may explain this discrepancy (Takasuka *et al.*, 1992; Wu-Wong *et al.*, 1994). Indomethacin did not inhibit the vascular and bronchial actions of ET-1 or SX6C suggesting that the effects of ETs or SX6C were not mediated by eicosanoids. Similar findings have been reported by several other workers (O'Donnell *et al.*, 1990; Raffestin *et al.*, 1991; Rodman *et al.*, 1992).

In summary, our experiments show that activation of both ET_A and ET_B receptors can lead to constriction in the pulmonary vascular bed. In addition we have provided indirect evidence that ET_A receptors are predominantly present on the venous side of the pulmonary circulation whereas ET_B receptors are located arterially. Changes in lung weight probably reflect hydrostatic oedema in the pulmonary microvasculature resulting from intense venoconstriction mediated by ET_A receptors. Results also suggest the possibility of two ET_B receptor subtypes in mediating bronchial smooth muscle contraction.

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Characterization of the receptors and mechanisms involved in the cardiovascular actions of sCCK-8 in the pithed rat

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1 The cardiovascular actions of cholecystokinin and related peptides were investigated in the pithed rat. The receptors and the mechanisms involved in these experiments were characterized.

2 Sulphated cholecystokinin octapeptide (sCCK-8, 0.1-100 nmol kg^{-1}, i.v.) elicited a dose-dependent bradycardia and increase in mean arterial blood pressure. Neither gastrin-17 nor pentagastrin had any effect at concentrations up to 100 nmol kg^{-1}.

3 Both the pressor response and bradycardia elicited by sCCK-8 were reduced by the selective CCK_A receptor antagonists, devazepide (0.5-50 nmol kg^{-1}) and lorglumide (1-7 μmol kg^{-1}). The selective CCK_B receptor antagonists, CI-988 (1 μmol kg^{-1}) and L-365,260 (15 μmol kg^{-1}) did not inhibit the effects of sCCK-8.

4 The pressor response induced with sCCK-8 was reduced by treatment with either phentolamine (3 μmol kg^{-1}) or guanethidine (2 μmol kg^{-1}) and was unaffected by treatment with propranolol, atropine or hexamethonium. The pressor response also persisted following bilateral adrenalectomy.

5 The bradycardia induced with sCCK-8 was unaffected by treatment with phentolamine, propranolol, guanethidine, atropine, hexamethonium or bilateral adrenalectomy.

6 The tetrapeptide of cholecystokinin (CCK-4) elicited a dose-dependent pressor response but did not induce bradycardia. The pressor response was unaffected by devazepide (50 nmol kg^{-1}), L-365260 (15 μmol kg^{-1}) or phentolamine (3 μmol kg^{-1}).

7 In the pithed rat, sCCK-8 acted via CCK_A receptors to increase arterial blood pressure indirectly, at least in part, through activation of α-adrenoceptors. The observed bradycardia was also mediated by CCK_A receptors but possibly through a direct action on the heart.

Keywords: cholecystokinin; CCK_A receptor; CCK_B receptor; cardiovascular actions

Introduction

Gastrin and cholecystokinin (CCK) are related peptides with an identical pentapeptide sequence at the biologically relevant carboxy terminal (Rehfeld, 1981). These peptides demonstrate different physiological actions which have been attributed to actions at more than one receptor. At least two receptor subtypes have been identified and classified as CCK_A and CCK_B receptors (Moran *et al.*, 1986). The CCK_A receptor is distributed in the pancreas (Innis & Snyder, 1980), gastrointestinal tissue (Patel & Spraggs, 1992) and in discrete regions of the central nervous system (Hill *et al.*, 1987). The CCK_B receptor is more widely distributed throughout the central nervous system (Hill *et al.*, 1987) and in the gastrointestinal tract (Bock *et al.*, 1989; Woodruff & Hughes, 1991; Patel & Spraggs, 1992). These receptors can be functionally distinguished by the use of selective receptor blockers and by comparing the rank order of potency of selective agonists (Jensen *et al.*, 1982).

Cholecystokinin has been demonstrated to have cardiovascular actions in both conscious and anaesthetized animals (Marker & Roberts, 1988; Janssen *et al.*, 1991). Intravenous (i.v.) administration of sulphated cholecystokinin octapeptide (sCCK-8) produced a complex series of blood pressure changes and dose-dependent bradycardia in anaesthetized rats (Marker & Roberts, 1988). Intravenous administration of sCCK-8 to conscious Long Evans rats produced dose-dependent increases in arterial blood pressure and a variation in heart rate responses, i.e. low doses of sCCK-8, (0.5 μg kg^{-1}) caused tachycardia and higher doses of sCCK-8 (5 μg kg^{-1}) caused bradycardia (Janssen *et al.*, 1991). The changes in arterial blood pressure and heart rate were both inhibited by devazepide implying an involvement of CCK_A receptors (Janssen *et al.*, 1991). The tachycardia was thought to be due to a CCK_A receptor-mediated inhibition of vagal tone whereas the bradycardia was attributed to a non-adrenergic, non-cholinergic action of sCCK-8.

As a consequence of these observations a full characterization of the receptors responsible for the cardiovascular effects of sCCK-8 has been carried out in the pithed rat. A rank order of agonist potency was obtained for a range of cholecystokinin related peptides and used to characterize the receptors involved (Jensen *et al.*, 1982; Woodruff & Hughes, 1991; Patel & Spraggs, 1992). Receptor blockers selective for CCK_A receptors, such as devazepide and lorglumide (Chang & Lotti, 1986; Rovati *et al.*, 1987), and gastrin/CCK_B receptors such as L-365,260 and CI-988 (Lotti & Chang, 1989; Hayward *et al.*, 1991; Woodruff & Hughes, 1991), were investigated against sCCK-8-induced responses. The mechanisms of action involved in the responses elicited with sCCK-8 were also characterized. Preliminary accounts of this study have been presented to the British Pharmacological Society. (Gaw *et al.*, 1992; Hill *et al.*, 1992).

Methods

Male rats (Glaxo AHA, 350-450 g) were anaesthetized with a gaseous mixture of N_2O (2 l min^{-1}), O_2 (1 l min^{-1}) and isoflurane (5%). A midline incision was made in the neck and the trachea intubated. Animals were pithed by insertion of a thin metal rod, via the right eye socket, to destroy the brain and spinal cord and vagotomized by ligation. Animals were connected to a respiratory pump and artificially respired with room air. The right carotid artery was exposed and cannulated (Portex Ltd i.d. = 0.5 mm). The cannula was connected to a pressure transducer (Bell and Howell) for measurement of arterial blood pressure. Heart rate was obtained by processing

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the signal through a Devices integrator. Blood loss from the brain was reduced by ligation of the left carotid artery. The right jugular vein was cannulated (Portex Ltd. i.d. = 0.5 mm) to allow intravenous administration of various agents. Following a 15 min stabilization period, sCCK-8 was administered i.v. as a bolus dose. In preliminary studies a six point dose-response curve to sCCK-8 was obtained (0.1–30 nmol kg^{-1}, i.v.) in each of four animals. To assess the effects of potential inhibitor agents, a three point dose-response curve to sCCK-8 was constructed, before and after administration of inhibitor, which consisted of a low (0.1 nmol kg^{-1}), medium (1 nmol kg^{-1}) and maximal (10 nmol kg^{-1}) dose. Three consecutive curves were obtained with 5 min intervals between doses and 10 min intervals between each dose-response curve. There was considerable variation between first and second curves with respect to arterial blood pressure. However, little variation was observed between the second and third curves. The effects of potential inhibitor agents were observed by administration of these agents between the second and third dose-response curves to sCCK8. To determine the order of agonist potency the peptides pentagastrin (PTG), gastrin (G-17) and CCK-4 were used in place of sCCK-8.

Some studies were undertaken to investigate the contribution of the adrenal glands in the sCCK-8 responses. For these studies a separate group of animals was prepared in which after pithing and cannulation, each animal received a bilateral adrenalectomy. Another group of animals was treated similarly but did not have the adrenal glands removed. Three consecutive dose-response curves to sCCK-8 were obtained in the adrenalectomized and sham-operated animals.

In all experiments saline and potential inhibitor agent vehicles were administered as bolus doses of no more than 0.05 ml and flushed through the jugular cannula with 0.1 ml of saline.

Statistics

All data points are expressed as arithmetic means ± s.e.mean. ED_{50} values are expressed as the dose of agonist required to produce 50% of the maximal response to sCCK-8. DR_{10} values are expressed as the dose of antagonist required to produce a tenfold, rightward shift in the dose-response curve to sCCK-8. Data were analysed by use of Student's *t* test for unpaired data. Differences were considered to be significant at *P* values <0.05.

Materials

The following peptides and drugs were used during the experiments: sulphated-CCK-8, CCK-4, G-17, pentagastrin (Bachem), devazepide and L-365,260 (Department of Medicinal Chemistry, Glaxo Research and Development), CI-988 (Department of Medicinal Chemistry, Glaxo SpA, Verona, Italy), phentolamine hydrochloride, propranolol hydrochloride, atropine sulphate, hexamethonium iodide, guanethidine (Sigma Chemical Company). Sulphated CCK-8 was dissolved in 0.05 M NH_4HCO_3 in saline. Devazepide, lorglumide, CI-988 and L-365,260 were dissolved in 20% cremophor in saline. Phentolamine, propranolol, atropine, hexamethonium, and guanethidine were dissolved in saline.

Results

Cardiovascular effects of sCCK-8, gastrin-17 and pentagastrin

Resting systolic and diastolic pressures in the pithed rat were found to be 72.4 ± 4.0 mmHg and 42.2 ± 1.7 mmHg respectively (mean arterial pressure (MAP) of 52.3 ± 2.9 mmHg) and basal heart rate was 314.2 ± 11.7 beats min^{-1}.

Administration of sCCK-8 (0.1, 1.0 and 10 nmol kg^{-1}) caused an immediate, dose-dependent bradycardia and increase in MAP with ED_{50} values of 1.3 (9.6–18.4) nmol kg^{-1} and 6.2 (3.6–10.7) nmol kg^{-1} respectively (Figure 1). Addition of the vehicle for sCCK-8 (saline) did not affect resting MAP or heart rate. Neither gastrin-17 nor pentagastrin (1–100 nmol kg^{-1}) elicited any changes in heart rate or MAP.

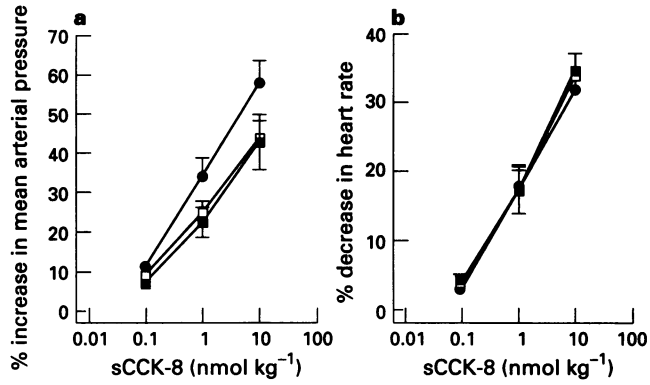

Figure 1 Dose-response curves to sCCK-8 showing increases in mean arterial pressure (a) and heart rate (b) in the pithed rat. The graphs show the first (●), second (□) and third (■) consecutive curves. Each point indicates the mean of six experiments with s.e. mean shown.

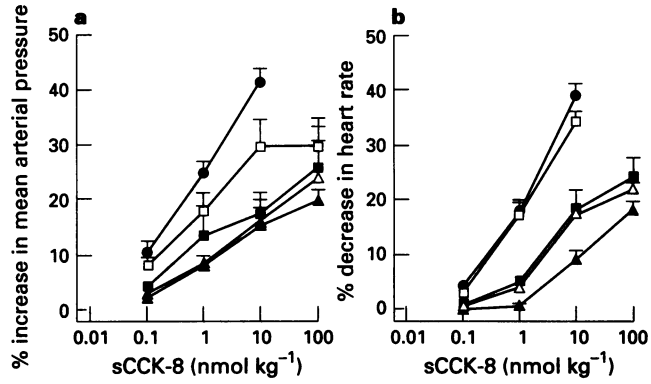

Figure 2 Effect of devazepide on sCCK-8-induced increases in mean arterial pressure (a) and heart rate (b) in the pithed rat. The curves were obtained in the presence of vehicle (●), and devazepide at 1.5 nmol kg^{-1} (□), 0.5 nmol kg^{-1} (■), 15 nmol kg^{-1} (Δ) and 50 nmol kg^{-1} (▲). Each point indicates the mean of 6–8 experiments with s.e. mean shown.

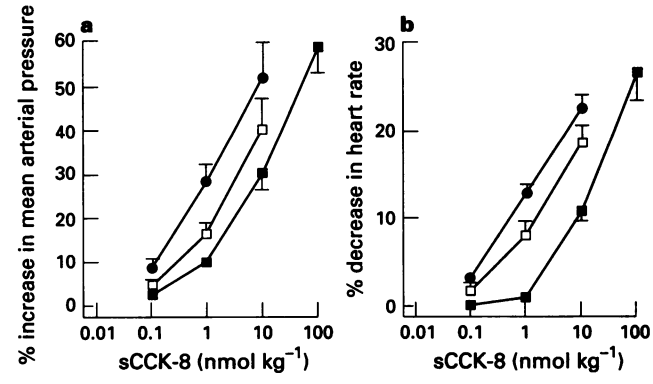

Figure 3 Effect of lorglumide on sCCK-8 induced increases in mean arterial pressure (a) and heart rate (b) in the pithed rat. The curves were obtained in the presence of vehicle (●), lorglumide at 1 μmol kg^{-1} (□) and 7 μmol kg^{-1} (■). Each point indicates the mean of 5 experiments with s.e. mean shown.

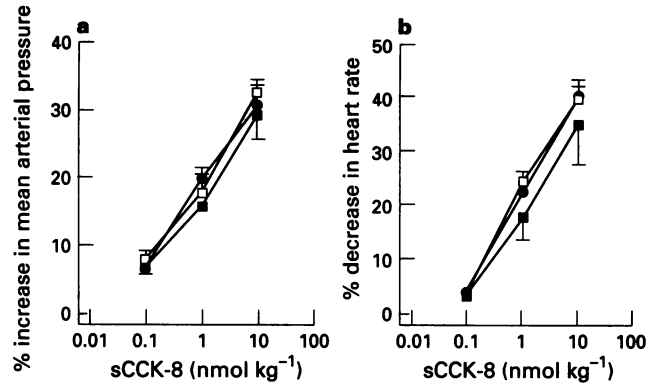

Figure 4 Effects of CCK_B antagonists on sCCK-8-induced increases in mean arterial pressure (a) and heart rate (b) in the pithed rat. The curves were obtained in the presence of vehicle (●), in the presence of L-365,260 at 15 μmol kg^{-1} (□) and CI-988 at 1 μmol kg^{-1} (■). Each point indicates the mean of 6 experiments with s.e. mean shown.

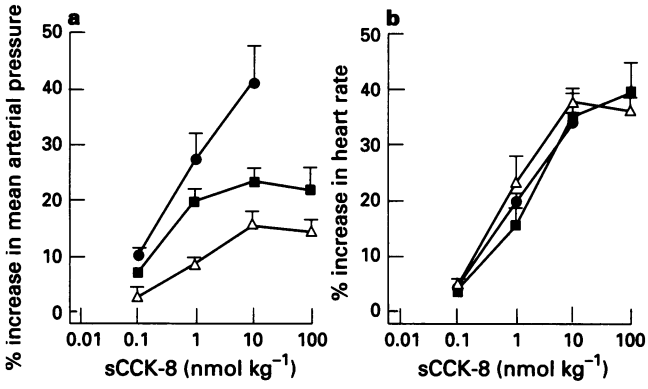

Figure 5 Effects of adrenoceptor blocking agents on sCCK-8-induced increases in mean arterial pressure (a) and heart rate (b) in the pithed rat. The curves were obtained in the presence of vehicle (●), phentolamine at 3 μmol kg^{-1} (■) and guanethidine at 2 μmol kg^{-1} (Δ). Each point indicates the mean of 6 experiments with s.e. mean shown.

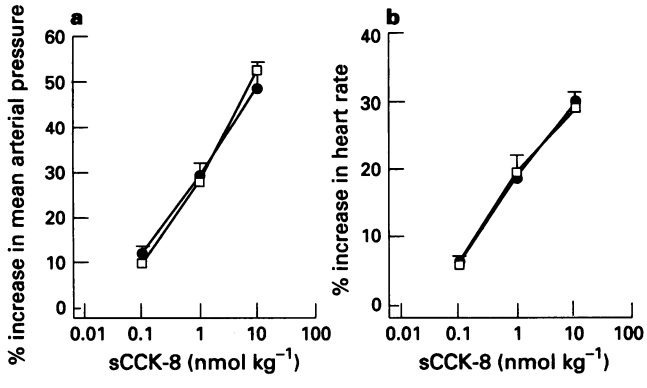

Figure 6 Effect of bilateral adrenalectomy on sCCK-8-induced increases in mean arterial pressure (a) and heart rate (b) in the pithed rat. The curves were obtained in sham-operated (●) and in bilaterally adrenalectomized rats (□). Each point indicates the mean of 4 experiments with s.e. mean shown.

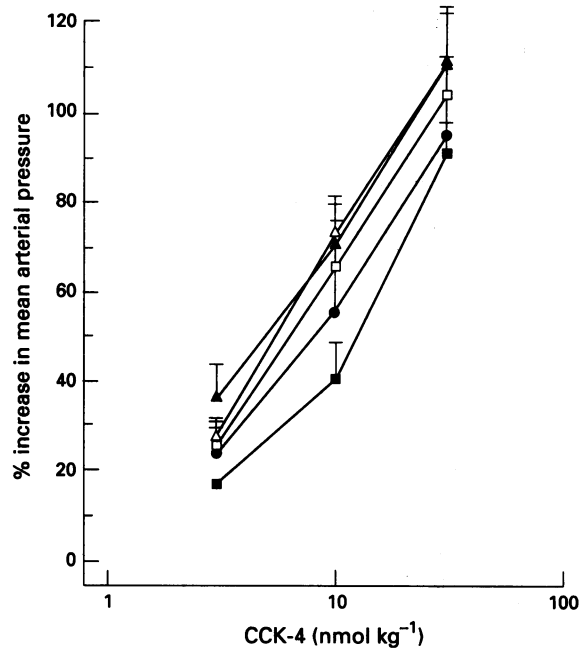

Figure 7 Dose-response curves to CCK-4 showing increases in mean arterial pressure in the pithed rat. The curves were obtained in the presence of vehicle (●), devazepide (50 nmol kg^{-1}, □), L-365,260 (15 μmol kg^{-1}, ■), phentolamine (3 μmol kg^{-1}, Δ) and guanethidine (2 μmol kg^{-1}, ▲). Each point indicates the mean of 5 experiments with s.e. mean shown.

Inhibition of sCCK-8 effects with CCK receptor selective antagonists

Both the increase in MAP and the fall in heart rate elicited by sCCK-8 were dose-dependently inhibited by the CCK_A receptor antagonist, devazepide, at doses up to 50 nmol kg^{-1} (Figure 2). Devazepide produced an insurmountable antagonism and so quantitative assessments of antagonist activity could not be determined. In comparison, the structurally dissimilar CCK_A receptor antagonist, lorglumide produced a rightward, parallel, dose-dependent displacement of the sCCK-8 elicited effects on blood pressure and heart rate with DR_{10} values of 5.3 and 3.4 μmol kg^{-1} respectively (Figure 3). The CCK_B receptor antagonist, L-365,260 and CI-988, at concentrations of 15 μmol kg^{-1} and 1 μmol kg^{-1} respectively, doses known to be antisecretory in rats *in vivo* (Hayward *et al.*, 1991), had no effect on the sCCK-8 elicited pressor response or bradycardia (Figure 4).

Mechanisms of sCCK-8 induced pressor responses and bradycardia

Phentolamine (3 μmol kg^{-1}) and guanethidine (2 μmol kg^{-1}) both reduced the increases in MAP (Figure 5) but not the bradycardia, produced by sCCK-8 (Figure 5). Propranolol (3 μmol kg^{-1}, $n=5$), atropine (7 μmol kg^{-1}, $n=5$) or hexamethonium (2 μmol kg^{-1}, $n=5$), all failed to reduce the sCCK-8 induced pressor response or bradycardia.

In animals subjected to a bilateral adrenalectomy, administration of sCCK-8 produced dose-dependent increases in MAP and dose-dependent bradycardia, similar to those observed in sham-operated animals ($n=4$, Figure 6).

CCK-4 induced responses in the pithed rat

Cholecystokinin-4 produced a dose-dependent increase in MAP (Figure 7) but had no effect on heart rate (data not shown). The pressor response was not affected by devazepide

(50 nmol kg^{-1}, $n=4$), L-365,260 (15 μmol kg^{-1}, $n=4$), CI-988 (1 μmol kg^{-1}, $n=3$), phentolamine (3 μmol kg^{-1}, $n=4$) or guanethidine (2 μmol kg^{-1}, $n=4$) suggesting a different mechanism of action from sCCK-8.

Discussion

It is clear from previous work that i.v. administration of sCCK-8 can produce complex cardiovascular changes in conscious and anaesthetized rats. The complexity of these actions may be a consequence of both central and peripheral actions of sCCK-8. By using the pithed rat as a model it was possible to clarify the mechanism of action of sCCK-8 without involving central neural reflexes. The aim of this study was therefore to characterize the cholecystokinin receptors which influence the cardiovascular system of the pithed rat.

The dose-dependent inhibition of the sCCK-8-induced increases in MAP by devazepide suggested that sCCK-8 produced these cardiovascular changes through an activation of CCK_A receptors. This supports earlier work indicating the involvement of CCK receptors in the sCCK-8 induced responses in conscious rats (Janssen *et al.*, 1991). The potency of devazepide in this preparation was similar to that reported for the *in vivo* inhibition of pancreatic secretion in the rat (Louie *et al.*, 1988). Although a competitive antagonist *in vitro* (Chang & Lotti, 1986), in the pithed rat devazepide demonstrated a non-competitive profile against sCCK-8-induced increases in both MAP and bradycardia. There are several explanations for this: (1) *In vivo* devazepide may dissociate from the receptor slowly. (2) Devazepide, once administered to the rat, may be subject to degradation to an active metabolite with a non-competitive profile. (3) Since devazepide inhibits both the pressor response and bradycardia, the observed responses may be a consequence of interactions within the cardiovascular system. Devazepide did not alter CCK-4-elicited increases in MAP, thus eliminating any non-specific effects of the antagonist. The inhibitory activity of lorglumide (Rovati *et al.*, 1987) supported the involvement of CCK_A receptor activation in this response. The lack of activity of the CCK_B-selective agonists, G-17 and pentagastrin, also support the hypothesis that sCCK-8 acted through CCK_A receptors. There is no apparent involvement of gastrin/CCK_B receptors in these responses since neither of the antagonists for these receptors, CI-988 and L-365,260, had any effect at doses which inhibit pentagastrin-stimulated acid secretion in anaesthetized rats (Hayward *et al.*, 1991).

The increases in MAP induced by sCCK-8 were attenuated in the presence of phentolamine suggesting that activation of peripheral α-adrenoceptors was involved. Guanethidine had no effect on CCK-4-induced increases in MAP but did reduce the sCCK-8-induced increase in MAP, implying that guanethidine was not acting nonselectively. This would support a hypothesis that sCCK-8 stimulated the release of noradrenaline from sympathetic neurones which then acted at α-adrenoceptors on vascular smooth muscle to produce a vasoconstriction and subsequent rise in recorded MAP. The pressor response of sCCK-8 was not through stimulation of the adrenal glands since a pressor response was still present in animals subjected to bilateral adrenalectomy. The lack of effect of hexamethonium would suggest that sCCK-8 stimulated the postganglionic sympathetic neurones. It has recently been shown that CCK_A receptors are present in the sympathetic ganglia of the rat and rabbit (Mantyh *et al.*, 1992), thus it is possible that sCCK-8 could act at this site to stimulate sympathetic nerves and cause the release of noradrenaline, resulting in a vasopressor response. This mechanism would also explain the depression of the maximum pressor response by phentolamine if the maximal release of noradrenaline by sCCK-8 was not of sufficient magnitude to displace phentolamine.

The increases in MAP produced by sCCK-8 were insensitive to propranolol, atropine and hexamethonium pretreatment indicating the lack of β-adrenoceptor, muscarinic receptor or nicotinic receptor involvement. Previous work investigating the cardiovascular effects of sCCK-8 in conscious rats showed a secondary depressor response to sCCK-8 in the presence of phentolamine which was sensitive to propranolol pretreatment (Janssen *et al.*, 1991), i.e. β-adrenoceptor mediated vasodilatation by noradrenaline unmasked by α-adrenoceptor blockade. In this pithed rat model, phentolamine pretreatment failed to unmask any vasodilatation in response to sCCK-8. Therefore, some centrally-mediated mechanism is likely to be involved in the activation of β-adrenoceptors, possibly a reflex depression in MAP, and not simply noradrenaline released in response to sCCK-8.

Of the test agents used in this study, only devazepide and lorglumide reduced CCK-8-induced bradycardia in the pithed rat, suggesting that the bradycardia was not a result of baroreceptor-mediated mechanisms, the implication being that CCK_A receptors directly control cardiac function or activate a nonadrenergic, noncholinergic pathway.

Cholecystokinin tetrapeptide (CCK-4) caused an increase in blood pressure with no effect on heart rate. The increase in MAP by CCK-4 was far greater than that observed with sCCK-8 but the mechanism involved is unclear. Since neither G-17 nor pentagastrin had an effect on MAP and none of the selective CCK receptor antagonists blocked the pressor response to CCK-4, it suggests that the action was not mediated by CCK_A or CCK_B receptors. The mechanism of the pressor response was also unlike that of sCCK-8 since phentolamine had no effect. These results suggest that CCK-4 can stimulate an increase in MAP independently of activation of any known cholecystokinin receptor. This effect may be worthy of further elucidation since, if the characteristics of the receptor involved can be identified, it may be of use in subclassification of that receptor family.

In conclusion we have demonstrated that i.v. administration of sCCK-8 into the pithed rat produced both an increase in MAP and a bradycardia. Both of these actions were dose-dependent. The increase in MAP probably involved an activation of CCK_A receptors situated on postganglionic sympathetic neurones and resulted in a subsequent release of noradrenaline which produced vasoconstriction via α-adrenoceptor activation. The bradycardia involved a nonadrenergic, noncholinergic mechanism which was most likely mediated by direct activation of CCK_A receptors on the cardiac tissue. CCK-4 was also shown to increase MAP but via a mechanism apparently unrelated to CCK_A and CCK_B receptors.

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No evidence for a role of muscarinic M_2 receptors in functional antagonism in bovine trachea

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1 The functional antagonism between methacholine- or histamine-induced contraction and β-adrenoceptor-mediated relaxation was evaluated in bovine tracheal smooth muscle *in vitro*. In addition, the putative contribution of muscarinic M_2 receptors mediating inhibition of β-adrenoceptor-induced biochemical responses to this functional antagonism was investigated with the selective muscarinic antagonists, pirenzepine (M_1 over M_2), AF-DX 116 and gallamine (M_2 over M_3), and hexahydrosiladiphenidol (M_3 over M_2).

2 By use of isotonic tension measurement, contractions were induced with various concentrations of methacholine or histamine, and isoprenaline concentration-relaxation curves were obtained in the absence or presence of the muscarinic antagonists. Antagonist concentrations were chosen so as to produce selective blockade of M_2 receptors (AF-DX 116 0.1 μM, gallamine 30 μM), or half-maximal blockade of M_3 receptors (pirenzepine 0.1 μM, AF-DX 116 0.5 μM, hexahydrosiladiphenidol 0.03 μM). Since these latter antagonist concentrations mimicked K_B values towards bovine tracheal smooth muscle M_3 receptors, antagonist-induced decreases in contractile tone were compensated for by doubling the agonist concentration.

3 It was found that isoprenaline-induced relaxation of bovine tracheal smooth muscle preparations was dependent on the nature and the concentration of the contractile agonist used. Thus, isoprenaline pD_2 ($-\log EC_{50}$) values were decreased 3.7 log units as a result of increasing cholinergic tone from 22 to 106%, and 2.4 log units by increasing histamine tone over a similar range. Furthermore, maximal relaxability of cholinergic tone decreased gradually from 100% at low to only 1.3% at supramaximal contraction levels, whereas with histamine almost complete relaxation was maintained at all concentrations applied. As a result, isoprenaline relaxation was clearly hampered with methacholine compared to histamine at equal levels of contractile tone.

4 In the presence of gallamine, isoprenaline relaxation was facilitated for most concentrations of methacholine, and for all concentrations of histamine. These changes could be explained by the decreased contraction levels for both contractile agonists in the presence of gallamine.

5 Isoprenaline-induced relaxation of cholinergic contraction was also facilitated by AF-DX 116 as well as by pirenzepine and hexahydrosiladiphenidol, and these (small) changes were again related to the (small) decreases in cholinergic contraction levels that were present in these experiments despite the additional administration of the agonist to readjust contractile tone. Similarly, changes in isoprenaline relaxation of histamine-induced tone could be explained by different contraction levels.

6 These results can be explained by the sole involvement of muscarinic M_3 receptors, and provide no evidence for a role of muscarinic M_2 receptors in functional antagonism in bovine trachea. Furthermore, they stress the importance of taking into account non-cholinergic controls as well as contraction levels in these experiments.

Keywords: Muscarinic M_2 receptors; functional antagonism; β-adrenoceptor-mediated relaxation; smooth muscle tone; bovine tracheal smooth muscle

Introduction

Muscarinic cholinoceptor binding sites in airways smooth muscle of mammalian species, including guinea-pig, rat, cow, dog and rabbit, have been shown to consist of both M_2 and M_3 receptor subtypes, and recent biochemical evidence suggests that this is also true for man (Widdop *et al.*, 1993). The M_2 type binding sites represent the major population (75-90%) of muscarinic receptors (Roffel *et al.*, 1988; Fryer *et al.*, 1990; Lucchesi *et al.*, 1990; Schaefer *et al.*, 1992; Fernandes *et al.*, 1992; Mahesh *et al.*, 1992), except in guinea-pig trachea where both populations are approximately equal (Haddad *et al.*, 1991). Among these receptor subtypes, muscarinic M_3 receptors are unanimously found to mediate contraction under normal conditions *in vitro* (Mutschler *et al.*, 1988; Roffel *et al.*, 1988; 1990a; Yang *et al.*, 1991; Gardier *et al.*, 1991; Mahesh *et al.*, 1992), presumably via activation of phospholipase C (Roffel *et al.*, 1990b; Yang *et al.*, 1991). The M_2 receptors have been shown to inhibit β-agonist- or forskolin-stimulated cyclic AMP production in intact canine, bovine and human airways smooth muscle cells (Sankary *et al.*, 1988; Yang *et al.*, 1991; Schaefer *et al.*, 1992; Challiss *et al.*, 1993; Widdop *et al.*, 1993), as well as in membrane preparations of canine, bovine and guinea-pig trachealis (Jones *et al.*, 1987; Meurs *et al.*, 1992; Pyne *et al.*, 1992), the latter demonstrating a direct inhibitory effect on adenylyl cyclase, presumably via the inhibitory G-protein, G_i (Sankary *et al.*, 1988; Pyne *et al.*, 1992; Widdop *et al.*, 1993). As an additional biochemical response, G_i-mediated inhibition of calcium-dependent potassium channel activity has been put forward (Kotlikoff *et al.*, 1994).

Concerning a functional role for this major population of muscarinic M_2 receptors in airways smooth muscle, it has been demonstrated recently that they may mediate contraction of guinea-pig longitudinal ileum smooth muscle under conditions of elevated cyclic AMP concentrations and in the presence of a non-cholinergic contractile agonist (i.e. histamine) *in vitro*

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(Thomas *et al.*, 1993). Furthermore, these M_2 receptors have been implicated in the functional antagonism between cholinergic airways smooth muscle contraction and β-adrenoceptor-mediated relaxation in canine (Fernandes *et al.*, 1992; Mitchell *et al.*, 1993), rabbit (Arjona *et al.*, 1993) and guinea-pig (Watson & Eglen, 1994) trachea. Thus, muscarinic M_2 receptor-mediated inhibition of adenylyl cyclase activity (or potassium channel closure) may contribute to the phenomenon that isoprenaline-mediated relaxation of cholinergic tone is hampered compared to similar contraction levels induced by leukotriene D_4 (Torphy, 1984) or histamine (Russell, 1984; Van Amsterdam *et al.*, 1989), and that such relaxation is dependent on the level of cholinergic tone *per se* (Torphy *et al.*, 1983; Russell, 1984; Van Amsterdam *et al.*, 1989). However, the increases in isoprenaline relaxation potency after muscarinic M_2 receptor blockade or inactivation of G_i, using pertussis toxin (Mitchell *et al.* 1993), i.e. 2 to 6 fold shift in isoprenaline pD_2 values, were rather small compared to the maximal changes in isoprenaline relaxant potency that can be obtained as a result of varying cholinergic contraction levels (85 and 490 fold shift for guinea-pig and canine trachea, respectively, cf. Torphy *et al.*, 1983; Roffel *et al.*, 1993). Moreover, in guinea-pig trachea such a role for muscarinic M_2 receptors was found to be completely absent when gallamine was used as the M_2-selective antagonist (Roffel *et al.*, 1993).

Transductional cross-talk between receptor-mediated phospholipase C activation and β-adrenoceptor function has also been proposed as a mechanism involved in functional antagonism between (cholinergic) airways contraction and β-adrenoceptor-mediated relaxation, showing a correlation between the production of inositol phosphates and the shift in isoprenaline potencies (Van Amsterdam *et al.*, 1989; 1990). Such cross-talk may involve protein kinase C-mediated uncoupling of β-adrenoceptors (as observed in bovine trachea, Grandordy *et al.*, 1993) through functional inactivation of G_s, (as reported for guinea-pig trachea, Pyne *et al.*, 1992), as has also been suggested for blood lymphocytes of asthmatic patients after allergen provocation (Meurs *et al.*, 1987), and may explain why contractions induced by partial muscarinic receptor agonists are more easily relaxed by isoprenaline than those induced by full muscarinic agonists (Gunst *et al.*, 1989; Van Amsterdam *et al.*, 1989; Al-Hassani *et al.*, 1993).

The present study was undertaken to investigate in detail functional antagonism between methacholine- or histamine-induced contraction and isoprenaline-mediated relaxation in bovine trachea. Furthermore, the putative role of muscarinic M_2 receptors in this process was investigated. This may be especially interesting since bovine tracheal smooth muscle has been reported to comprise a higher proportion of muscarinic M_2 binding sites (Roffel *et al.*, 1988; Lucchesi *et al.*, 1990; Schaefer *et al.*, 1992) than guinea-pig trachea (Haddad *et al.*, 1991). This latter question was addressed by comparing the influence of the selective muscarinic receptor antagonists, AF-DX 116 and gallamine (M_2/M_3-selective, cf. Fernandes *et al.*, 1992; Arjona *et al.*, 1993), pirenzepine (M_1/M_2-selective, cf. Mitchell *et al.*, 1993), and hexahydrosiladiphenidol (M_3/M_2-selective, cf. Fernandes *et al.*, 1992), on isoprenaline-induced relaxation of methacholine- and histamine-induced contractile tone, the latter serving as a control. Part of this work was presented to the British Pharmacological Society (Meurs *et al.*, 1993).

Methods

General

Fresh bovine tracheae were obtained from the local slaughterhouse and transported to the laboratory in room temperature-Krebs-Henseleit buffer solution that had been pre-gassed with 95% O_2/5% CO_2; pH 7.4. The tracheal smooth muscle was carefully dissected and smooth muscle strips (10 × 1 mm) were prepared free of mucosa and connective tissue in Krebs-Henseleit solution gassed with 95% O_2/5% CO_2 at room temperature. These strips were mounted in 20 ml organ baths (Krebs-Henseleit, 37°C) under isotonic recording with a preload of 0.5 g. After a 90 min equilibration period the strips were precontracted twice by cumulative administration of methacholine (0.1, 1, 10 and 0.1, 1, 10, 100 μM, respectively) with washing periods of 60 min, between which maximal relaxation was established with isoprenaline (0.1 μM) immediately followed by a 30 min washing period.

Gallamine experiments

The preparations were subsequently contracted with different concentrations of methacholine (0.01 – 1000 μM) or histamine (0.1 – 1000 μM), building up smooth muscle tone in 2 – 4 concentration steps, and cumulative concentration-relaxation curves were obtained with isoprenaline. When the maximal response to isoprenaline had been obtained, the preparations were washed twice and maximal relaxation was re-established with 10 and 100 μM isoprenaline. The effect of muscarinic M_2 receptor blockade on isoprenaline-induced relaxation was determined with the M_2/M_3-selective muscarinic receptor antagonist, gallamine (30 μM), administered 30 min before building up tone with methacholine or histamine. This concentration was chosen so as to block selectively muscarinic M_2 receptors (pK_i on bovine heart 6.5; fractional receptor occupancy 99%) compared to muscarinic M_3 receptors (apparent pK_B on bovine trachea 4.1; fractional receptor occupancy 28%) (Roffel *et al.*, 1988).

Stability of the contractions for the duration of the isoprenaline relaxation curve was not monitored in paired controls. However, from independent experiments it was inferred that the methacholine- and histamine-induced contractions are stable for at least 45 and 25 min, respectively i.e. for the largest part of the relaxation curve.

Readjusted tone experiments

In another type of experiment, contractile tone was first induced with methacholine (0.1, 0.3 or 1.0 μM) or histamine (10 μM) and then the M_2/M_3-selective muscarinic receptor antagonist, AF-DX 116 (0.1 or 0.5 μM), the M_1/M_2-selective antagonist, pirenzepine (0.1 μM), or the M_3/M_2-selective antagonist, hexahydrosiladiphenidol (0.03 μM) was added. After 30 min of antagonist incubation, the original agonist concentration was added once again to compensate for antagonist-induced decrease of contractile tone, if any (cf. Fernandes *et al.*, 1992), and isoprenaline relaxation curves were obtained as described above. The antagonist concentrations were chosen so as to produce selective blockade of M_2 receptors (AF-DX 116 0.1 μM; fractional receptor occupancy 50% for M_2 and 17% for M_3), or half-maximal blockade of M_3 receptors (AF-DX 116 0.5 μM, pirenzepine 0.1 μM, hexahydrosiladiphenidol 0.03 μM; fractional M_2 receptor occupancy 83%, 14%, and 7%, respectively), based on pK_B and pK_i values for muscarinic M_3 and M_2 receptors in bovine trachea and heart, respectively (Roffel *et al.*, 1988). Readjustment of tone was achieved by a second dose of the original agonist concentration because antagonist concentrations (except AF-DX 116 0.1 μM) had been chosen to reflect K_B values towards bovine tracheal smooth muscle M_3 receptors (Roffel *et al.*, 1988), thereby also mimicking the pirenzepine concentration applied in the previous study (Mitchell *et al.*, 1993).

Data analysis

Contractile responses were expressed as percentages of the response to 100 μM methacholine in the second precontraction in each experiment. Maximal relaxant effects of isoprenaline (E_{max}) were expressed as percentages of the full relaxation obtained with isoprenaline at the end of each experiment. Isoprenaline pD_2 ($-\log EC_{50}$) and E_{max} values in the absence and presence of selective muscarinic antagonists were com-

pared by Student's paired t test. Statistical significance was assumed at the 5% level.

Drugs

Methacholine chloride, histamine dihydrochloride, (−)-isoprenaline hydrochloride and gallamine triethiodide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pirenzepine dihydrochloride and AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5, 11-dihydro-6H-pyrido-[2,3-b][1,4]benzodiazepin-6-one) were kind gifts from Thomae GmbH (Biberach an der Riss, Germany) and hexahydrosiladiphenidol hydrochloride was a gift from Dr G Lambrecht (Frankfurt am Main, Germany). All other chemicals were of reagent grade. Fresh solutions of isoprenaline were made up every day in Krebs-Henseleit buffer solution containing 0.5 mM ascorbic acid as the anti-oxidant. Fresh solutions of the other drugs were made up every day in Krebs-Henseleit buffer solution (methacholine, histamine) or ultrapure water (muscarinic antagonists).

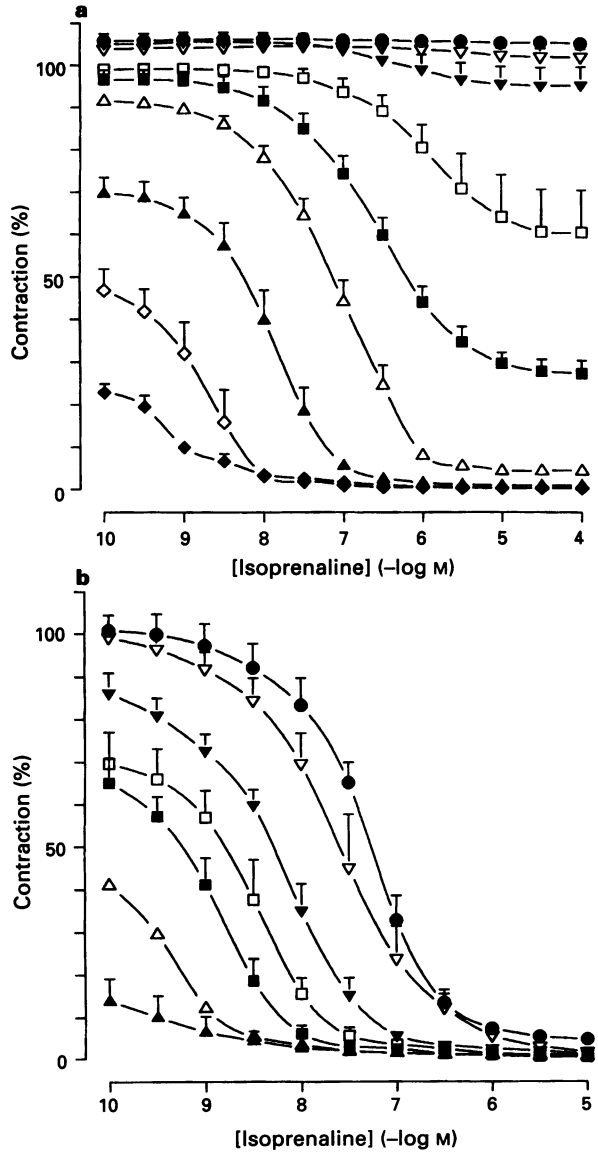

Figure 1 Relaxation of bovine tracheal smooth muscle preparations by isoprenaline following contraction by increasing concentrations of methacholine (a) and histamine (b) in the absence of gallamine; methacholine 0.01 μM (◆), 0.03 μM (◇), 0.1 μM (▲), 0.3 μM (△), 1.0 μM (■), 3.0 μM (□), 10 μM (▼), 100 μM (▽), 1000 μM (●); histamine 0.1 μM (▲), 0.3 μM (△), 1.0 μM (■), 3.0 μM (□), 10 μM (▼), 100 μM (▽), 1000 μM (●) Contraction levels are expressed as percentage of the response to 100 μM methacholine in the second precontraction (see Methods). Data represent means ± s.e.mean of 4 or 5 experiments.

Results

Functional antagonism in bovine trachea and the influence of gallamine

Isoprenaline-induced relaxation of bovine tracheal smooth muscle preparations was dependent on the nature and the concentration of the contractile agonist used (Figure 1). Both with methacholine and histamine, isoprenaline pD_2 values decreased with increasing contractile tone. However, the effect was more pronounced when methacholine was used. i.e. a maximum shift of 3.7 log units compared to 2.4 log units with histamine (Table 1). Maximum relaxation was also decreased with increasing contractile tone in the case of methacholine, but hardly in the case of histamine. Thus, methacholine-induced contractions were almost fully (99%) relaxed up to 70% contractile tone (0.1 μM methacholine) but E_{max} gradually decreased to only 1.3% at higher contraction levels. With histamine, only the highest contraction level (103%, 1 mM) showed a small (4%) decrease in isoprenaline E_{max} values. As a result, isoprenaline pD_2 and E_{max} values were higher with histamine compared to methacholine at equal levels of contractile tone (Table 1, Figure 2a and c). In the presence of the M_2/M_3-selective muscarinic receptor antagonist, gallamine (30 μM), isoprenaline relaxation curves were shifted to the left (by 0.5 to 1.1 log units) for the lower concentrations of methacholine (0.03–3.0 μM) and by 0.1 to 0.4 log units for all concentrations of histamine (Table 1). Maximal relaxation was increased for all concentrations of methacholine. Furthermore, the contraction levels induced by a given concentration of either agonist were decreased in the presence of gallamine, in such a way that a 3 fold higher concentration of agonist induced approximately the same level of contraction in the presence of the antagonist (Table 1). No contraction was obtained with methacholine 0.01 μM and histamine 0.1 μM in the presence of gallamine, whereas contraction levels with histamine 0.3 μM in the presence of gallamine were variable. As

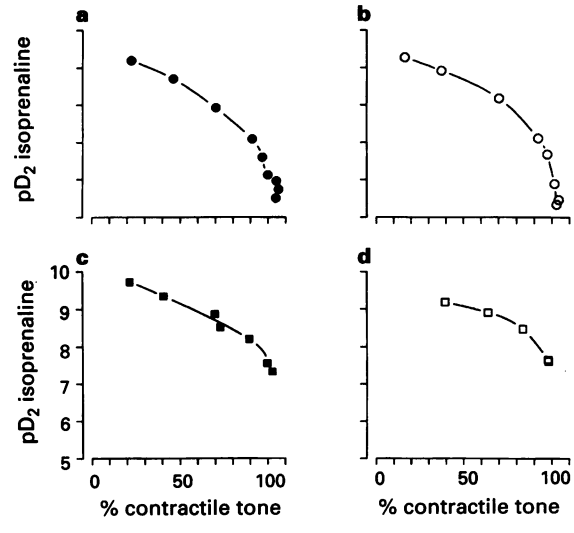

Figure 2 Relationships between contractile tone induced by various concentrations of methacholine (a and b) or histamine (c and d), in the absence (a and c) and presence (b and d) of the M_2-selective muscarinic antagonist, gallamine (30 μM), and isoprenaline relaxation potencies (pD_2) in bovine trachea. Each data point represents one agonist concentration (methacholine or histamine), according to Table 1. Contractile tone expressed as in Figure 1. Error bars (Table 1) have been omitted for reasons of clarity.

shown in Table 1 and seen in Figure 2, the increase in isoprenaline pD_2 values at a given concentration of both methacholine and histamine in the presence of gallamine parallelled the decrease in contractile tone under these conditions to an important extent, and this was similarly true for isoprenaline E_{max} values (not shown).

The influence of selective muscarinic antagonists on functional antagonism in bovine trachea

Isoprenaline-induced relaxation of bovine tracheal smooth muscle strips in the absence of muscarinic receptor antagonists in this independent series of experiments was in good agreement with the controls of the gallamine experiments (cf. Tables 1 and 2). Isoprenaline pD_2 values were increased in the presence of *all* types of selective muscarinic antagonist, i.e. pirenzepine 0.1 μM, AF-DX 116 0.1 and 0.5 μM and hexahydrosiladiphenidol 0.03 μM, for all concentrations of methacholine investigated (0.1, 0.3 and 1.0 μM). With histamine 10 μM, isoprenaline pD_2 values decreased in the presence of the muscarinic antagonists, except for pirenzepine (Table 2). Amongst these changes in isoprenaline pD_2 values in the presence of muscarinic antagonists, only the increase with hexahydrosiladiphenidol at 1.0 μM methacholine and the decrease at 10 μM histamine were statistically significant ($P<0.05$ and 0.02, respectively). Maximum relaxation of 1.0 μM methacholine-induced contractile tone by isoprenaline was also increased by all types of selective muscarinic antagonist (Figure 3), from 66.9±4.4% in controls (mean±s.e.mean, $n=12$) to 76.8±4.1% in the presence of 0.1 μM AF-DX 116 ($n=8$; not significantly different from control), 87.2±3.3% with 0.5 μM AF-DX 116 ($n=8$; $P<0.005$ compared to control), 89.8±4.2% with 0.03 μM hexahydrosiladiphenidol ($n=8$; $P<0.005$), and 93.4±2.2% in the presence of 0.1 μM pir-

Table 1 Isoprenaline pD_2 and E_{max} values obtained after contraction of bovine tracheal smooth muscle preparations with different concentrations of methacholine and histamine in the absence or presence of an M_2-selective concentration of the muscarinic receptor antagonist, gallamine (30 μM)

[Methacholine] (μM)		*Contraction level (%)*		*pD_2 (-log M)*		*E_{max} (%)*	
Control	*Gallamine*	*Control*	*Gallamine*	*Control*	*Gallamine*	*Control*	*Gallamine*
0.01	0.03	22.4±3.9	16.6±8.3	9.19±0.03	9.27±0.24	98.8±0.8	100.0±0.0
0.03	0.1	46.2±4.5	37.6±3.6	8.70±0.13	8.92±0.19	99.4±0.05	98.8±0.2
0.1	0.3	70.2±4.0	70.2±2.5	7.92±0.10	8.18±0.15	98.8±0.6	97.8±1.1
0.3	1.0	90.9±1.6	92.3±1.2	7.09±0.10	7.11±0.12	95.2±0.9	84.3±4.7
1.0	3.0	96.7±2.1	97.6±1.8	6.60±0.12	6.68±0.21	71.6±3.6	65.2±9.6
3.0	10	99.8±0.9	101.6±1.1	6.12±0.09	5.88±0.20	39.4±9.6	25.3±4.7
10	100	104.8±2.1	102.8±0.9	5.96±0.18	5.33±0.12	10.0±2.7	8.4±2.4
100	1000	104.4±1.4	103.9±1.4	5.50±0.23	5.46±0.21	3.0±0.8	3.0±0.1
1000		106.1±1.2		5.74±0.31		1.3±0.3	
[Histamine]							
0.1	0.3	21.5±3.9	[a]	9.73±0.27	[a]	99.1±0.9	[a]
0.3	1.0	40.6±6.9	39.4±5.1	9.35±0.03	9.19±0.18	99.3±0.7	100.0±0.0
1.0	3.0	69.6±6.5	63.8±4.6	8.87±0.07	8.91±0.16	99.6±0.4	99.8±0.1
3.0		72.8±6.1		8.51±0.10		99.2±0.5	
10	10	89.2±4.9	83.5±3.4	8.21±0.08	8.47±0.14	98.4±0.7	99.4±0.3
100	100	99.6±1.9	97.7±1.5	7.57±0.16	7.67±0.26	98.5±1.0	96.7±2.0
1000	1000	102.6±2.6	98.0±3.5	7.33±0.05	7.63±0.24	95.3±1.5	92.4±6.1

Results are means±s.e.mean of 4–5 (control) or 7 (gallamine) experiments, except methacholine 0.03 mM in the presence of gallamine (3 experiments). Contraction levels are expressed as percentage of the response to 100 μM methacholine in the second precontraction (see Methods). Note that methacholine and histamine concentrations in the absence and presence of gallamine have been arranged so as to compare equal contraction levels.

[a] Data highly variable (see text).

Table 2 Isoprenaline pD_2 values for relaxation of bovine tracheal smooth muscle, contracted by selected concentrations of methacholine and histamine, in the absence or presence of selective muscarinic receptor antagonists

	Methacholine 0.1	*Methacholine* 0.3	*Methacholine* 1.0	*Histamine* 10 μM
Control	8.11±0.10 (8) (73.7±2.2)	7.33±0.11 (12) (89.8±1.4)	6.59±0.12 (12) (97.8±1.0)	8.72±0.13 (8) (71.9±4.8)
Pirenzepine 0.1 μM	8.13±0.17 (4) (76.2±5.0)	7.75±0.21 (4) (84.0±2.0)	7.06±0.19 (4) (92.9±2.3)	8.77±0.17 (4) (74.8±2.0)
AF-DX 116 0.1 μM	8.20±0.09 (4) (66.6±6.6)	7.63±0.12 (8) (84.0±2.6)*	6.88±0.15 (8) (95.7±2.2)	8.59±0.26 (4) (74.0±11.9)
AF-DX 116 0.5 μM	8.44±0.07 (4) (61.6±6.1)*	7.70±0.12 (8) (85.0±3.8)	6.84±0.12 (8) (92.1±1.6)***	8.60±0.24 (4) (73.7±9.6)
Hexahydrosiladiphenidol 0.03 μM	8.22±0.16 (4) (64.7±5.2)	7.68±0.10 (8) (82.9±2.5)***	6.95±0.11 (8)* (93.2±1.8)***	8.23±0.13 (4)** (81.7±4.0)

Results are expressed as means±s.e.mean of (n) experiments. Values in parentheses represent contraction levels after 30 min of antagonist incubation and readjustment of tone, if necessary (see Methods), and are expressed as in Table 1. Significantly different from control: *$P<0.05$, **$P<0.02$, ***$P<0.01$.

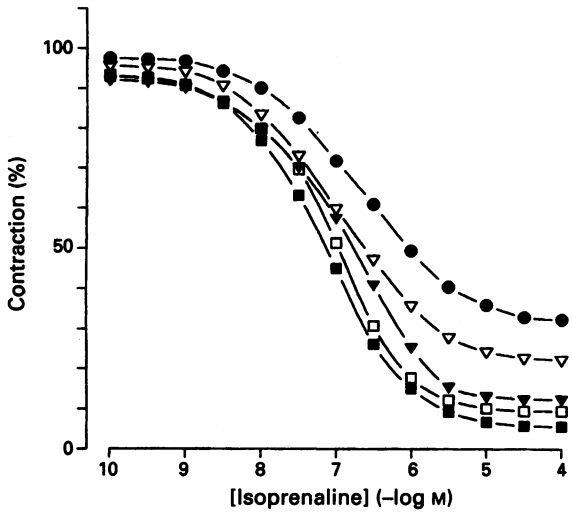

Figure 3 Relaxation of bovine tracheal smooth muscle preparations by isoprenaline after contraction by 1.0 μM methacholine in the absence (●) and presence of muscarinic antagonists (AF-DX 116 0.1 μM, ▽; AF-DX 116 0.5 μM, ▼; hexahydrosiladiphenidol 0.03 μM, □; pirenzepine 0.1 μM, ■), with readjustment of tone if necessary (see Methods). Contraction levels (means of 4–12 experiments as indicated in Table 2) expressed as in Figure 1; error bars did not exceed 7% and have been omitted for reasons of clarity.

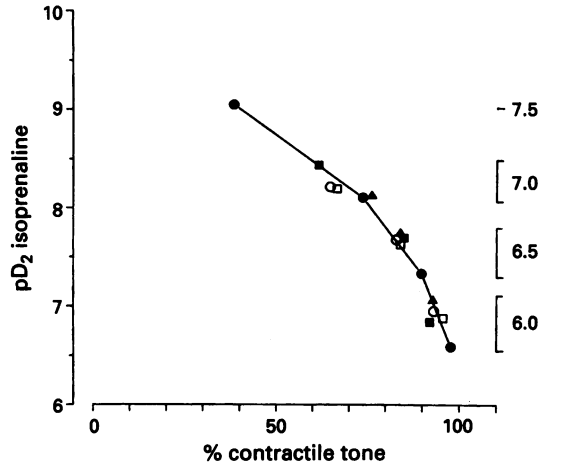

Figure 4 Relationship between contractile tone induced by various concentrations of methacholine (indicated as the antilog of the molar concentration on the right hand ordinate scale) in the absence (●) and presence of the selective muscarinic antagonists, AF-DX 116 (M_2, 0.1 μM, □; 0.5 μM, ■), pirenzepine (M_1, 0.1 μM, ▲), and hexahydrosiladiphenidol (M_3, 0.03 μM, ○), and isoprenaline relaxation potencies (pD_2) in bovine trachea. Contractile tone (expressed as in Figure 1) was readjusted after 30 min of antagonist incubation, if necessary. Control data for 0.03 μM methacholine were taken from pirenzepine experiments (not shown) and gallamine experiments (Table 1).

enzepine ($n=4$; $P<0.02$). Furthermore, the contraction levels induced by either agonist were decreased by the addition of muscarinic receptor antagonists in almost half of the experiments, dependent on both the agonist and the antagonist concentration involved, i.e. more frequently with methacholine 0.1 μM and AF-DX 116 0.3 μM or hexahydrosiladiphenidol 0.03 μM. The decrease in tone was largely but not fully corrected by doubling (after 30 min of antagonist incubation, see Methods) the original agonist concentration. Agonist concentrations were not increased further to exclude the risk of introducing additional inhibition of the β-adrenergic signalling system through M_3 receptor-mediated cross-talk (see below), which would easily obscure the putative effects of M_2 receptor blockade on isoprenaline relaxation potencies. As shown in Figure 4, the increase in isoprenaline pD_2 values at a given concentration of methacholine in the presence of selective muscarinic antagonists parallelled the decrease in contractile tone under these conditions to an important extent, and this was similarly true for isoprenaline E_{max} values (not shown).

Discussion

The aim of the present study was to establish the nature and the extent of the functional antagonism between contractile agonists, i.e. methacholine and histamine, and isoprenaline in bovine tracheal smooth muscle *in vitro*, and the putative role that muscarinic M_2 receptors inhibiting β-adrenoceptor-stimulated adenylyl cyclase (or potassium channel) activity play in this process. It was found that gradually increasing cholinergic tone from low to supramaximal levels resulted in a pronounced, methacholine concentration-dependent, decrease in the ability of isoprenaline to relax the smooth muscle, both regarding the concentration giving half-maximal effect (4,900 fold shift) as well as the magnitude of the maximal effect (from 100 to 1%). Interestingly, a clear decrease in E_{max} only started to emerge when contractile tone rose over 90%, when the shift in pD_2 values was already more than 2.0 log units. Increasing histamine-induced tone in bovine trachea also resulted in a marked decrease in the ability of isoprenaline to relax the tissue, but in contrast to methacholine-induced tone this hardly affected E_{max} values, and the shift in pD_2 values was markedly smaller than in the former case (250 fold). These observations may be related to quantitative differences in receptor reserve between muscarinic and histamine receptors, as have indeed been demonstrated in bovine trachea (Grandordy & Barnes, 1987). Such difference in receptor reserve may also explain the differential ability of isoprenaline to relax identical levels of contractile tone induced by methacholine and histamine, as signified in Figure 2a and c.

Comparing bovine trachea to guinea-pig (Torphy, 1984; Van Amsterdam *et al.*, 1989; Roffel *et al.*, 1993; Watson & Eglen, 1994) and dog trachea (Torphy *et al.*, 1983; Russell, 1984; Gunst *et al.*, 1987; 1989; Mitchell *et al.*, 1993), and to human bronchial smooth muscle (Van Amsterdam *et al.*, 1990), it appears that functional antagonism by isoprenaline can be quite different quantitatively, both regarding the difference between histamine and cholinoceptor agonists, and the influence of raising cholinergic tone on isoprenaline pD_2 and E_{max} values. These differences may be related to receptor reserve (as discussed above) but also to receptor cross-talk between contractile and relaxant agonists, and/or to a differential importance of muscarinic M_2 receptors inhibiting isoprenaline-induced biochemical responses as discussed below.

Gallamine experiments

The question whether muscarinic M_2 receptors inhibiting β-adrenoceptor-mediated biochemical responses contribute to the functional antagonism between methacholine (but not histamine) and isoprenaline in bovine tracheal smooth muscle *in vitro* was first addressed by use of the muscarinic M_2/M_3-selective receptor antagonist, gallamine. This compound was chosen because it is more selective for M_2 over M_3 receptors than AF-DX 116 and methoctramine (Roffel *et al.*, 1988; Lee & El-Fakahany, 1991), which is of major importance when studying mixed M_2/M_3 receptor systems. As shown in Table 1, it was observed that isoprenaline relaxation in the presence of gallamine was facilitated both in terms of pD_2 and E_{max} values. This would indeed suggest that muscarinic M_2 receptors play a role in this functional antagonism, as has recently also been reported using gallamine in rabbit trachea (Arjona *et al.*, 1993). However, isoprenaline relaxation of histamine-induced tone, which should not involve muscarinic M_2 receptors in any fashion, was also facilitated in the presence of gallamine, al-

though to a smaller extent (average pD_2 shift for isoprenaline 0.3 versus 0.7 log units in case of methacholine). Moreover, gallamine did influence contraction levels obtained with all methacholine and histamine concentrations, the effect being rather marked at lower levels, and it was concluded that the increase in isoprenaline relaxant action in the presence of gallamine could be fully attributed to these decreases in contractile tone for *both* agonists (Figure 2). As a result, these experiments do not provide any evidence for a role of muscarinic M_2 receptors in the functional antagonism between methacholine and isoprenaline in bovine trachea. Furthermore, they stress the importance of performing non-cholinoceptor controls (as also incorporated in the study of canine trachea by Fernandes *et al.* (1992)), and taking into account differences in contraction levels.

It should of course be questioned why gallamine in apparent M_2-selective concentration influenced cholinoceptor as well as histamine-induced contractile tone, especially since this concentration of gallamine hardly shifted methacholine concentration-response curves in previous experiments (Roffel *et al.*, 1988), which was confirmed in the course of the present study (not shown). In fact, we do not have a satisfactory explanation for this, although the observation that gallamine affected both methacholine- and histamine-induced contractions suggests that some unspecific property of this compound is involved.

Readjusted tone experiments

The putative role of muscarinic M_2 receptors in functional antagonism in bovine trachea was next investigated by adopting the experimental paradigm applied successfully in canine trachea (Fernandes *et al.*, 1992). Thus, the M_2/M_3-selective muscarinic receptor antagonist AF-DX 116, in concentrations giving selective (half-maximal) M_2 receptor blockade (0.1 μM) or half-maximal M_3 receptor blockade (0.5 μM), the M_3/M_2-selective antagonist hexahydrosiladiphenidol, in a concentration giving half-maximal M_3 receptor blockade (0.03 μM, concentrations based on pK_B and pK_i values taken from Roffel *et al.*, 1988), or vehicle were added after contractile tone had been induced, and contractile tone was readjusted after 30 min of antagonist incubation to compensate for antagonist-induced inhibition of tone, if any (see Methods and Results). Pirenzepine was also subjected to this protocol because this compound had also been reported to facilitate isoprenaline relaxation in canine trachea (Mitchell *et al.*, 1993), which was explained by functional uncoupling of M_3 receptors from adenylyl cyclase. Finally, in contrast to previous studies, three different methacholine concentrations (and thus contraction levels) in addition to one histamine concentration (serving as a control) were investigated, in order to increase the possibility of detecting a specific role for M_2 receptors.

It was found that isoprenaline relaxation potencies were increased, up to 3 fold and in large part not significantly, by *all* types of selective muscarinic receptor antagonist and for all concentrations of methacholine, whereas for histamine-induced contractions isoprenaline relaxation was somewhat decreased, except with pirenzepine. Maximum relaxation to the highest concentration of methacholine was also increased by all antagonists (Figure 3). Although the results with AF-DX 116 and pirenzepine are in apparent agreement with those obtained in canine trachea (Fernandes *et al.*, 1992; Mitchell *et al.*, 1993), they do not appear to suggest a *specific* role for muscarinic M_2 receptors in the functional antagonism between methacholine and isoprenaline in bovine trachea. This conclusion is based on the observation that, in contrast to the dog (Fernandes *et al.*, 1992), the M_3/M_2-selective muscarinic receptor antagonist hexahydrosiladiphenidol also produced increased relaxant action by isoprenaline in bovine trachea, and on the fact that the effect of the M_1/M_2-selective antagonist pirenzepine cannot be attributed to selective blockade of M_2 muscarinic receptors, the affinity towards this receptor subtype being (somewhat) lower than for M_3 and of course M_1 receptors (Roffel & Zaagsma, 1995). By contrast, the (small) effects of the selective muscarinic receptor antagonists on isoprenaline relaxation in bovine trachea can be fully explained (similar to the gallamine experiments discussed above) by the (small) differences in contractile tone that were encountered in these experiments. Thus, despite the readjustment of contractile tone following antagonist incubation, contractile levels were generally slightly, although only occasionally significantly, lower in the preparations incubated with antagonist, and it can be observed from Figure 4 that the increase in isoprenaline pD_2 values was related to these changes in contractile tone (cf. gallamine experiments); a similar explanation applies to the increases in maximum relaxability observed in case of 1.0 μM methacholine (relationship not shown). Similarly, the significant change in isoprenaline pD_2 values in the presence of hexahydrosiladiphenidol with histamine as the contractile agonist appears to be related to the higher contraction level reached in those incubations (Figure 3, Table 2).

A role for muscarinic M_2 receptors in functional antagonism?

Summarizing the experiments with the selective muscarinic antagonists, pirenzepine, AF-DX 116, gallamine, and hexahydrosiladiphenidol, it must be concluded that the data can be explained without introducing a specific role for muscarinic M_2 receptors in the functional antagonism between methacholine and isoprenaline in bovine trachea. Furthermore, these experiments clearly showed the importance of taking into account the starting level of contraction, since rather small variation in this level, i.e. only a few percent, can critically determine isoprenaline relaxation, both regarding pD_2 and E_{max} values. The question remains why, in contrast to bovine trachea, muscarinic M_2 receptors inhibiting β-adrenoceptor-stimulated adenylyl cyclase or potassium channel activity appear to contribute, although only moderately, to functional antagonism in canine, rabbit and guinea-pig trachea, provided that these studies have indeed taken into account any small differences in starting contractions. The first and rather interesting possibility is that a role for these M_2 receptors can only be observed using isometric tension measurement, as was applied in all studies concerned (Fernandes *et al.*, 1992; Arjona *et al.*, 1993; Mitchell *et al.*, 1993; Watson & Eglen, 1994). This is indirectly supported by our own observation, using isotonic measurement, that such a role is absent in guinea-pig trachea (Roffel *et al.*, 1993). Alternatively, the relative population of tracheal smooth muscle M_2 receptors in these species and/or their ability to inhibit adenylyl cyclase may be involved. The first of these possibilities does not seem to be valid, since this proportion has been reported to be smaller in guinea-pig than in the other species (Haddad *et al.*, 1991), and rather similar in dog, cow, and rabbit (Roffel *et al.*, 1988; Lucchesi *et al.*, 1990; Schaefer *et al.*, 1992; Fernandes *et al.*, 1992; Mahesh *et al.*, 1992). Concerning the inhibition of adenylyl cyclase by these M_2 receptors, it appears that this response is less efficacious in guinea-pig compared to canine and bovine trachea (cf. Jones *et al.*, 1987; Meurs *et al.*, 1992; Pyne *et al.*, 1992; Challiss *et al.*, 1993; Ethier *et al.*, 1993; see Roffel & Zaagsma, 1995 for review), which therefore does not readily explain the reported differences in contraction/relaxation experiments. As a final explanation, it may be suggested that the other mechanism that has been implicated in functional antagonism, i.e. cross-talk between receptor-mediated phospholipase C activation and β-adrenoceptor function through protein kinase C (see Introduction), is more pronounced in bovine trachea compared to the other species. However, information on this subject is not available, and further experiments should resolve this intriguing possibility.

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Elevation of plasma noradrenaline levels in urethane-anaesthetized rats by activation of central prostanoid EP_3 receptors

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1 We studied the effects of intracerebroventricular (i.c.v.) administration of prostaglandin E_2 (PGE_2) and its receptor subtype ligands on plasma levels of catecholamines in urethane-anaesthetized rats.

2 Administration of PGE_2 (0.15, 0.3 and 1.5 nmol per animal, i.c.v.) dose-dependently elevated plasma levels of noradrenaline (NA), while the levels of adrenaline were not affected.

3 Administration of sulprostone (EP_3/EP_1 agonist) and misoprostol (EP_3/EP_2 agonist) effectively elevated plasma NA levels in a dose-dependent manner (0.1, 0.3, and 1.0 nmol per animal). Butaprost (EP_2 agonist) (0.3, 1.0 and 3.0 nmol per animal) was without effect. 17-Phenyl-ω-trinor PGE_2 (EP_1/EP_3 agonist) effectively elevated plasma NA levels only at its highest dose (1.0 nmol per animal), but this elevation was not attenuated by pretreatment with SC-19220 (selective EP_1 antagonist) (20 nmol per animal, i.c.v.).

4 The potency of these test agents in elevating plasma levels of NA was as follows; misoprostol > sulprostone > PGE_2 > > 17-phenyl-ω-trinor PGE_2 > > > butaprost. These results suggest that activation of central prostanoid EP_3-receptors induces central sympathetic outflow in rats.

Keywords: Central nervous system; EP_3 receptor, plasma noradrenaline

Introduction

Prostaglandins are synthesized within the central nervous system of mammals (Wolfe, 1982). Increasing evidence has demonstrated that prostaglandins are implicated in central regulation of a variety of functions, including body temperature (Coceani *et al.*, 1988; Milton, 1989), cardiovascular function (Hoffman & Schmid, 1979; Chiu & Richardson, 1983), hormone secretion (Behrman, 1979; Heaulme & Dray, 1984; Brooks *et al.*, 1986) and some behavioural activities (Johnson *et al.*, 1993). With regard to central regulatory roles of prostaglandins in the sympathetic nervous system, prostaglandin E_2 (PGE_2) administered into the brain has been shown to increase both blood pressure and plasma levels of catecholamines (Okuno *et al.*, 1982; Feuerstein *et al.*, 1982). We also demonstrated that intracerebroventricularly (i.c.v.) applied PGE_2 activates central sympathetic outflow, thereby inhibiting vagally-mediated gastric acid secretion (Yokotani *et al.*, 1988). Receptors coupled to PGE_2 are pharmacologically divided into at least three subtypes, EP_1, EP_2 and EP_3 (Coleman *et al.*, 1994) and activation of these EP receptor subtypes induces Ca^{2+} mobilization, stimulation and inhibition of adenylate cyclase, respectively (Sonnenburg & Smith, 1988; Smith, 1992). However, the EP receptor subtypes related to centrally-mediated activation of sympathetic nervous system have not yet been characterized. In the present study, therefore, we examined central effects of PGE_2 and various EP receptor agonists on plasma levels of catecholamines in urethane-anaesthetized rats.

Methods

Procedures

Male Wistar rats weighing 350 to 400 g were maintained in a room at 22-24°C under a constant day-night rhythm for more than 2 weeks, and given food (laboratory chow, CE-2, Clea Japan, Inc., Japan) and water *ad libitum*. Under urethane anaesthesia (1.2 g kg^{-1}, i.p.), the femoral vein was cannulated for infusion of saline (1.6 ml h^{-1}), and the femoral artery was cannulated for collecting blood samples. After these procedures, the animal was placed in a stereotaxic apparatus, as described by Okuma *et al.* (1991).

I.c.v. administration of prostaglandins

Three hours after the animal was placed in a stereotaxic apparatus, a stainless-steel cannula (0.35 mm outer diameter) was inserted into the right lateral ventricle at co-ordinates AP -0.8 mm from the bregma, L 1.5 mm from the midline, H 4.0 mm below the surface of the brain according to the rat brain atlas of Paxinos & Watson (1986). PGE_2, prostaglandin-related ligands or vehicle were slowly injected into the right lateral ventricle in a volume of 10 μl using 50 μl Hamilton syringe.

PGE_2 and related ligands (sulprostone, misoprostol, butaprost and 17-phenyl-ω-trinor PGE_2) were dissolved in 99% ethanol and stored at -20°C. These stock solutions were diluted with saline whenever we used them and the final concentration of ethanol was adjusted to 0.5% (saline containing 0.5% of ethanol was used as vehicle for control experiments). SC-19220 dissolved in saline containing 5% dimethylsulphoxide (DMSO) was administered i.c.v. 15 min before application of prostaglandin-related ligands.

Measurement of plasma catecholamines

Blood samples (400 μl) were collected through an arterial catheter. Catecholamines in the plasma were extracted by the method of Anton & Sayre (1962) with slight modifications, and were assayed electrochemically by high performance liquid chromatography (h.p.l.c.). The modifications were as follows: plasma (180 μl) was transferred to a centrifuge tube containing 30 mg of activated alumina, 2 ml of twice deionized water and 1 ml of 1.5 M Tris buffer (pH 8.6) containing 0.1 M disodium

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EDTA, after which the preparation was shaken for 5 min. After several washes with 4 ml aliquots of ice-cold twice deionized water, catecholamines adsorbed onto alumina were eluted with 300 μl of 4% acetic acid containing 0.1 mM disodium EDTA. A pump (880 PU; Japan Spectroscopic Co., Ltd., Japan) and an electrochemical detector (ECD-100, Eicom Co., Ltd., Japan) equipped with a graphite electrode were used with h.p.l.c. Analytical conditions were as follows: detector, +700 mV potential; column, Cosmosil-packed column (ODS) 4.6 × 150 mm (Nacalai Tesque, Inc., Japan); mobile phase, 0.1 M phosphate buffer pH 3.5, 20 mM EDTA, 4 mM 1-octane sulphate sodium (Nacalai Tesque, Inc.) containing 16% methanol, as shown in our previous paper (Yokotani & Osumi, 1993). The amount of catecholamines in each sample was calculated by using the peak height ratio relative to 3,4-dihydroxybenzylamine, an internal standard. By this assay, 5 pg of noradrenaline (NA) and adrenaline (Ad) could be determined accurately.

Compounds

The following drugs were used: prostaglandin E_2 was purchased from Sigma Chemical Co., U.S.A.; 17-phenyl-ω-trinor PGE_2 was purchased from Cayman Chemicals, U.S.A. The following compounds were gifts which we gratefully acknowledge: butaprost from Bayer, U.K.; sulprostone from Schering AG, Germany; misoprostol and SC-19220 (1-acetyl-2-(8-chloro-10,11-dihydrodibenz(b,f)(1,4) oxazepine-10-carbonyl)-hydrazine) from G.D. Searle, U.S.A.

Statistical analysis

Results are expressed as the mean ± s.e.mean. Statistical analysis was carried out using Student's unpaired t test in Figure 4 or the Bonferroni method for comparing a control to all other means after one-way analysis of variance (ANOVA) in Figures 1,2 and 3. P values of less than 0.05 were taken to indicate significant differences.

Results

Effects of PGE_2 on plasma levels of catecholamine

Basal plasma levels of NA and Ad were 338 ± 25 pg ml^{-1} and 226 ± 20 pg ml^{-1} (n = 16), respectively. After i.c.v. administration of vehicle (10 μl saline containing 0.5% ethanol), blood sampling at 0, 5, 10, 30 and 60 min failed to detect any alteration in the plasma levels of either NA or Ad (Figure 1). However, administration of PGE_2 (0.15, 0.3 and 1.5 nmol per animal, i.c.v.) caused a rapidly developing and dose-dependent elevation of plasma levels of NA, while those of Ad were not affected (Figure 1). The plasma NA level reached maximum 10 min after the administration of PGE_2, and then rapidly declined toward the basal level.

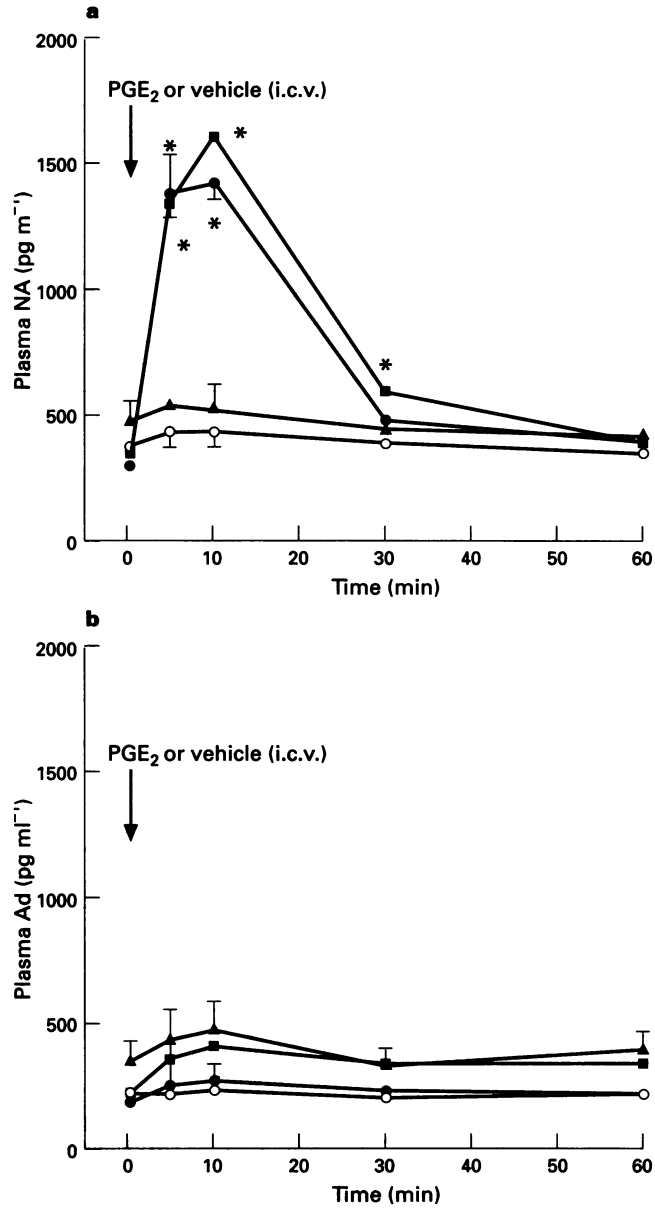

Figure 1 Effects of i.c.v. administered prostaglandin E_2 (PGE_2) on plasma levels of catecholamines. Arrow indicates administration of test substances. NA, noradrenaline; Ad, adrenaline. Vehicle (saline 10 μl per animal) (○), n = 4; PGE_2; 0.15 nmol per animal (▲), n = 4; 0.3 nmol per animal (●), n = 4; 1.5 nmol per animal (■), n = 4. Each point represents the mean ± s.e.mean. *Significantly different (P < 0.05) from vehicle-treated control. The actual values of basal plasma NA and Ad were 291 ± 67 pg ml^{-1} and 222 ± 46 pg ml^{-1} for vehicle-treated control group, 473 ± 66 pg ml^{-1} and 351 ± 83 pg ml^{-1} for PGE_2 (0.15 nmol per animal)-treated group, 268 ± 14 pg ml^{-1} and 181 ± 54 pg ml^{-1} for PGE_2 (0.3 nmol per animal)-treated group, 333 ± 41 pg ml^{-1} and 183 ± 16 pg ml^{-1} for PGE_2 (1.5 nmol per animal)-treated group, respectively.

Effects of sulprostone (EP_3/EP_1 agonist) and misoprostol (EP_3/EP_2 agonist) on plasma levels of NA

Basal plasma levels of NA and Ad were 502 ± 23 pg ml^{-1} and 280 ± 37 pg ml^{-1} (n = 30), respectively. After i.c.v. administration of vehicle (10 μl saline containing 0.5% ethanol), blood sampling at 0, 5, 10, 20, 30 and 60 min did not show any alteration in the plasma levels of NA and Ad (Figure 2). The administration of sulprostone (0.1, 0.3 and 1.0 nmol per animal, i.c.v.) rapidly and dose-dependently elevated plasma NA levels; maximal responses being obtained 5–10 min after its administration, followed by a rapid decline toward the basal level (Figure 2a). Misoprostol (0.1, 0.3 and 1.0 nmol per animal, i.c.v.) also rapidly elevated plasma NA concentrations (Figure 2b). The maximal response evoked by misoprostol (1.0 nmol per animal, i.c.v.) was almost the same as that evoked by the smaller dose of this agent (0.3 nmol per animal, i.c.v.). However, the duration of the response to misoprostol, 1.0 nmol per animal, was more prolonged than that to the smaller dose (0.3 nmol per animal).

Plasma levels of Ad were not affected by either sulprostone or misoprostol (i.c.v.) (data not shown).

Effects of butaprost (EP_2 agonist) and 17-phenyl-ω-trinor PGE_2 (EP_1/EP_3 agonist) on plasma NA level

Basal plasma levels of NA and Ad were 550 ± 26 pg ml^{-1} and 209 ± 24 pg ml^{-1} (n = 26), respectively. Blood sampling at 0, 5,

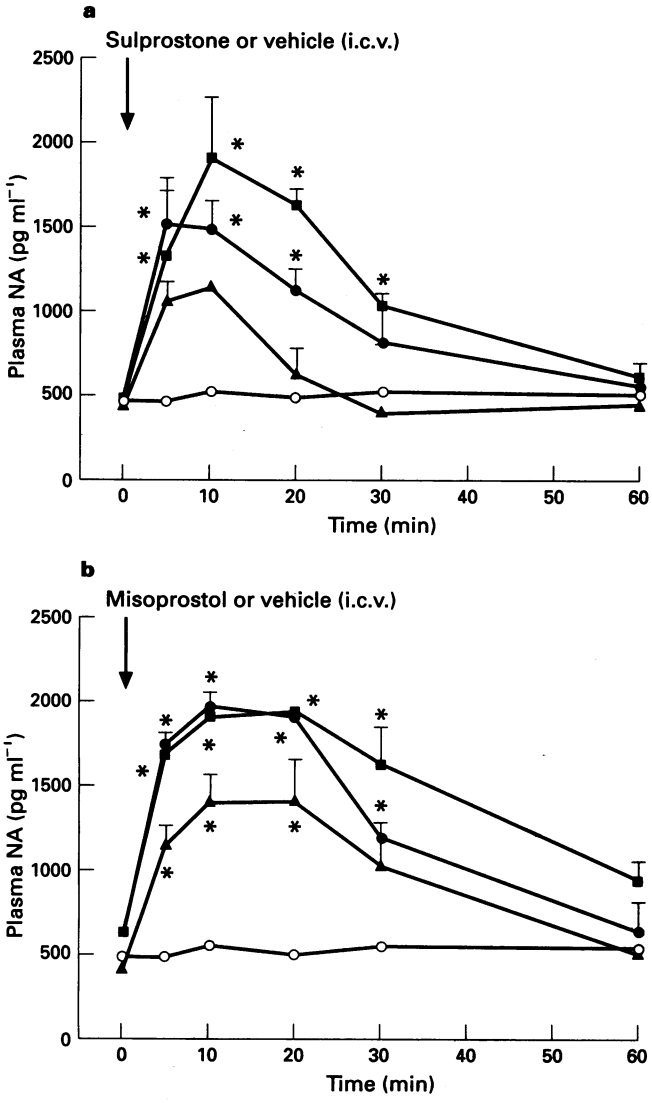

Figure 2 Effects of i.c.v. administered sulprostone and misoprostol on plasma noradrenaline (NA) concentrations. Arrow indicates administration of test substances. In (a), vehicle (○), $n=6$; sulprostone: 0.1 nmol per animal (▲), $n=4$; 0.3 nmol per animal (●), $n=4$; 1.0 nmol per animal (■), $n=4$. In (b), vehicle (○) (as in a), $n=6$; misoprostol: 0.1 nmol per animal (▲), $n=4$; misoprostol 0.3 nmol per animal (●), $n=4$; misoprostol 1.0 nmol per animal (■), $n=4$. *Significantly different ($P<0.05$) from vehicle-treated control. Other conditions were the same as those in Figure 1. The actual values of basal plasma NA were 480 ± 23 pg ml^{-1} for vehicle-treated control group, 439 ± 86 pg ml^{-1} for sulprostone (0.1 nmol per animal)-treated group, 439 ± 64 pg ml^{-1} for sulprostone (0.3 nmol per animal)-treated group, 494 ± 19 pg ml^{-1} for sulprostone (1.0 nmol per animal)-treated group, 416 ± 30 pg ml^{-1} for misoprostol (0.1 nmol per animal)-treated group, 622 ± 87 pg ml^{-1} for misoprostol (0.3 nmol per animal)-treated group, and 633 ± 35 pg ml^{-1} for misoprostol (1.0 nmol per animal)-treated group.

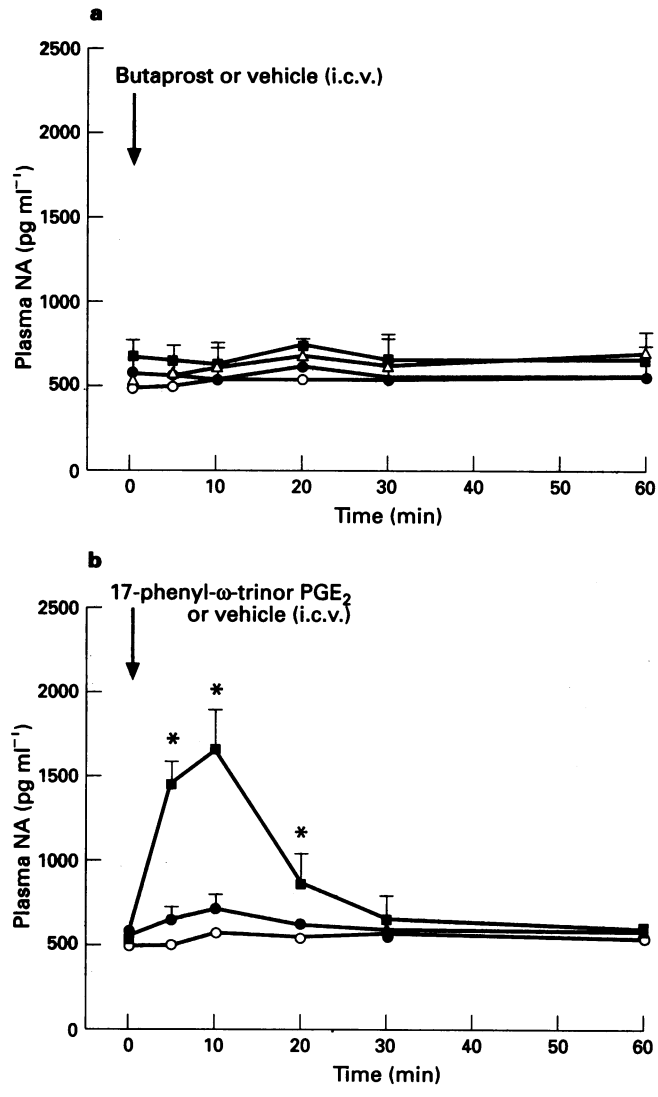

Figure 3 Effects of i.c.v. administered butaprost and 17-phenyl-ω-trinor PGE_2 on plasma noradrenaline (NA) concentrations. Arrow indicates administration of test substances. In (a), vehicle (○), $n=6$ (as in Figure 2); butaprost: 0.3 nmol per animal (●), $n=4$; 1.0 nmol per animal (■), $n=4$; 3.0 nmol per animal (△), $n=4$. In (b), vehicle (○) (as in Figure 2); 17-phenyl-ω-trinor PGE_2: 0.3 nmol per animal (●), $n=4$; 1.0 nmol per animal (■), $n=4$. *Significantly different ($P<0.05$) from vehicle-treated control. Other conditions were the same as those in Figures 1 and 2. The actual values of basal plasma NA were 480 ± 23 pg ml^{-1} for vehicle-treated control group, 578 ± 51 pg ml^{-1} for butaprost (0.3 nmol per animal)-treated group, 672 ± 105 pg ml^{-1} for butaprost (1.0 nmol per animal)-treated group, 558 ± 93 pg ml^{-1} for butaprost (3.0 nmol per animal)-treated group, 545 ± 59 pg ml^{-1} for 17-phenyl-ω-trinor PGE_2 (0.3 nmol per animal)-treated group, and 502 ± 51 pg ml^{-1} for 17-phenyl-ω-trinor PGE_2 (1.0 nmol per animal)-treated group.

10, 20, 30 and 60 min did not show any alteration in the plasma levels of NA in vehicle-treated control animals, as shown as Figure 2. Butaprost (0.3, 1.0 and 3.0 nmol per animal, i.c.v.) did not affect the plasma NA at any time point (Figure 3a). In contrast, 17-phenyl-ω-trinor PGE_2 (1.0 nmol per animal, i.c.v.) rapidly elevated plasma NA levels (Figure 3b). The maximal response was obtained 10 min after its administration, after which the level rapidly declined toward basal by 30 min. A lower dose of this agent (0.3 nmol per animal, i.c.v.) was without effect.

Plasma Ad was not affected by either butaprost, or 17-phenyl-ω-trinor PGE_2 (i.c.v.) (data not shown).

Effects of SC-19220 (selective EP_1 antagonist) on 17-phenyl-ω-trinor PGE_2-induced elevation of plasma NA levels

In the animals pretreated with SC-19220 (20 nmol per animal, i.c.v.), basal plasma levels of NA were 420 ± 35 pg ml^{-1} ($n=8$). Fifteen min after administration of SC-19220, 17-phenyl-ω-trinor PGE_2 (1.0 nmol per animal) or vehicle was administered i.c.v. Administration of SC-19220 had no direct effect on the basal plasma level of NA (at 0, 5, 10, 20, 30 and 60 min) and pretreatment with SC-19220 did not affect the 17-phenyl-ω-trinor PGE_2 (1.0 nmol per animal, i.c.v.)-induced elevation of plasma NA (Figure 4).

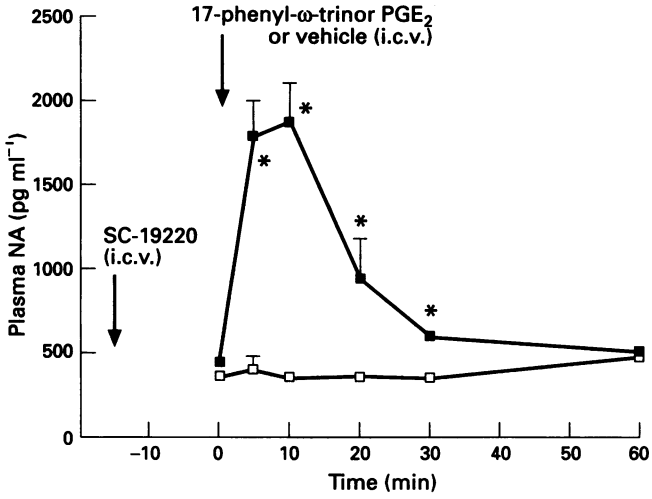

Figure 4 Effects of SC-19220 on the 17-phenyl-ω-trinor PGE_2-induced elevation of plasma noradrenaline (NA) concentrations. The first arrow (at −15 min) indicates the administration of SC-19220 (20 nmol per animal, i.c.v.). The second arrow (at 0 min) indicates the administration of 17-phenyl-ω-trinor PGE_2 (1.0 nmol per animal, i.c.v.) or vehicle alone (i.c.v.). Vehicle (□), $n=4$; 17-phenyl-ω-trinor PGE_2 1.0 nmol per animal (■), $n=4$. *Significantly different ($P<0.05$) from SC-19220 plus vehicle-treated control. Other conditions were the same as in Figures 1,2 and 3. The actual values of basal plasma NA were 381 ± 68 pg ml^{-1} for SC-19220 and vehicle-treated group and 459 ± 10 pg ml^{-1} for SC-19220 and 17-phenyl-ω-trinor PGE_2-treated group.

Discussion

Feuerstein *et al.* (1982) have already shown that PGE_2 (0.5–5 nmol kg^{-1}) injected into the lateral cerebral ventricle of the rat increased plasma levels of catecholamines, especially NA. In the present study, we first confirmed that administration of PGE_2 (0.15–1.5 nmol per animal, i.c.v.) elevated plasma NA concentrations in a dose-dependent manner, without affecting the plasma level of Ad.

In the next series of experiments, we used various agonists selective for EP receptor subtypes to characterize central EP receptor which mediates elevation of plasma NA levels. There are however no prostanoid analogues which show absolute selectivity for each of the EP-receptor subtypes, EP_1, EP_2 and EP_3. Sulprostone was first identified as a potent EP_1 receptor agonist, but it is more potent at EP_3 receptors (agonist potencies: $EP_3 > EP_1 > > EP_2$) (Coleman *et al.*, 1987a,b; Bunce *et al.*, 1990). Misoprostol is a potent EP_3 receptor agonist and also has moderate EP_2 and weak EP_1 receptor agonist activities (agonist potencies: $EP_3 > EP_2 > > > EP_1$) (Mantelli *et al.*, 1991; Lawrence *et al.*, 1992). In the present experiments, sulprostone and misoprostol (0.1, 0.3 and 1.0 nmol per animal, i.c.v.) both effectively elevated plasma NA concentrations in a dose-dependent manner. This evidence suggests that EP_3 receptors in the brain are probably involved in the PGE_2-induced elevation of plasma NA. However, there was a possibility that central EP_1 and/or EP_2 receptors are also involved in this elevation.

To clarify whether or nor central EP_2 receptors are involved, butaprost was then tested. Although butaprost behaves as a selective EP_2 receptor agonist (agonist potencies: $EP_2 > > > EP_1$), it is of relatively low potency (Gardiner, 1986). However, even in a large dose of 3.0 nmol i.c.v., butaprost was ineffective on plasma NA levels. It is therefore likely that central EP_2 receptors are not involved in the PGE_2-induced elevation of plasma NA.

We finally examined the roles of EP_1 receptors in the PGE_2-induced elevation of plasma NA. 17-Phenyl-ω-trinor PGE_2 is an EP_1 receptor agonist, with some weaker EP_3 agonist activity (agonist potencies: $EP_1 > EP_3 > > EP_2$) (Dong *et al.*, 1986; Lawrence *et al.*, 1989; 1992). In the present study, a relatively large dose of 17-phenyl-ω-trinor PGE_2 (1.0 nmol per animal, i.c.v.) was necessary to elevate plasma NA. Furthermore, SC-19220, which has been shown to be a highly selective and competitive albeit rather weak EP_1 receptor antagonist, (Sanner, 1969; Kennedy *et al.*, 1982; Coleman *et al.*, 1985), failed to attenuate 17-phenyl-ω-trinor PGE_2-induced elevation of plasma NA even at a dose of 20 nmol per animal (i.c.v.). This dose of SC-19220 is probably sufficient to block central EP_1 receptors (Ferreira *et al.*, 1978; Kandasamy & Williams, 1982). The present results therefore seem to indicate that the elevation of plasma levels of NA by a large dose of 17-phenyl-ω-trinor PGE_2 (1.0 nmol per animal, i.c.v.) involves the activation of central EP_3-receptors.

In the central nervous system, EP_3 receptor mRNA is highly expressed in brain regions such as hippocampus, preoptic area, hypothalamus, mammillary body, locus coeruleus and raphe nuclei (Sugimoto *et al.*, 1994). The paraventricular nucleus of the hypothalamus is a major site sending signals to the spinal sympathetic preganglionic neurones (Swanson & Sawchenko, 1983). It is therefore likely that EP_3 receptors in the hypothalamus are involved in the PGE_2-induced central sympathetic outflow.

In summary, we demonstrate here that EP_3-receptors in the rat brain are involved in the PGE_2-induced elevation of plasma NA.

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5-Hydroxytryptamine receptors that facilitate excitatory neuromuscular transmission in the guinea-pig isolated detrusor muscle

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1 In isolated detrusor strips from the guinea-pig urinary bladder, contractile responses to electrical field stimulation were mostly mediated by neurally released acetylcholine (ACh) and adenosine 5'-triphosphate (ATP).

2 5-Hydroxytryptamine (5-HT) produced a concentration-dependent increase in the amplitude of stimulated detrusor strip contractions. The 5-HT concentration-response curve showed a biphasic profile: the high potency phase was obtained at sub-micromolar concentrations (10-300 nM), while the low potency phase in the range 1-30 μM. The maximum response of the first phase was 30% of the total 5-HT response.

3 Like 5-HT, the 5-HT_3 receptor agonist, 2-methyl-5-hydroxytryptamine (2-methyl-5-HT: 0.3-100 μM), the 5-HT_2 receptor agonist, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI: 30 nM-3 μM) and the 5-HT_4 receptor agonist, 5-methoxytryptamine (5-MeOT: 0.1-30 μM) potentiated, though with lower potency, detrusor contractions. The resulting concentration-response curves were monophasic in nature. 2-Methyl-5-HT had a maximum effect comparable to that of 5-HT. By contrast, the maximal effects of DOI and 5-MeOT were only 20% and 30% of that elicited by 30 μM 5-HT, respectively.

4 The 5-HT_3 receptor antagonist, granisetron (0.3 μM) had no effect on the high potency phase, but caused a rightward parallel shift of the low potency phase of the 5-HT curve ($pK_B = 7.3$). Granisetron (0.3 μM) antagonized with comparable affinity ($pK_B = 7.1$) 5-HT-induced responses after pharmacological isolation of 5-HT_3 receptors with the 5-HT_1/5-HT_2 receptor antagonist, methiothepin (0.3 μM) and the 5-HT_4 receptor antagonist, GR 125487 (30 nM). Granisetron (0.1, 0.3 and 1 μM) competitively antagonized the potentiating effect of 2-methyl-5-HT with an estimated pA_2 of 7.3.

5 Methiothepin (0.3 μM) and the 5-HT_{2A} receptor antagonist, ketanserin (0.3 μM) produced a slight inhibition of the first phase of the 5-HT curve. In the presence of ketanserin, an equimolar concentration of methiothepin was ineffective in further reducing the effect of 5-HT. Similarly, the 5-HT_4 receptor antagonist, GR 125487 (30 nM) slightly inhibited the first phase of the 5-HT curve. Conversely, this phase was suppressed when detrusor strips were coincubated with ketanserin (or methiothepin) and GR 125487.

6 In a separate set of experiments, the interactions of 5-HT with either the purinergic or cholinergic components of excitatory neuromuscular transmission were investigated. In the presence of hyoscine (1 μM), 5-HT was mostly effective at sub-micromolar concentrations, while in the presence of the P_2-purinoceptor antagonist, suramin (300 μM), 5-HT-induced potentiation was mainly obtained with micromolar concentrations.

7 Thus, in electrically stimulated detrusor strips from guinea-pig, 5-HT potentiated excitatory neuromuscular transmission by activating at least three separate neural 5-HT receptors. These include the 5-HT_{2A} and 5-HT_4 receptors, which mediate the 5-HT high potency phase mainly by activation of purinergic transmission. On the other hand, the potentiating effect caused by micromolar concentrations of 5-HT mostly involves cholinergic transmission and is mediated by the 5-HT_3 receptors.

Keywords: 5-HT; 5-HT_{2A}, 5-HT_3 and 5-HT_4 receptors; 5-HT receptor agonists and antagonists; hyoscine; suramin; excitatory neuromuscular transmission; urinary bladder

Introduction

It is well established that in the isolated detrusor muscle of several mammals, including man, the excitatory neuromuscular transmission evoked by electrical stimulation is mainly mediated by the neuronal release of acetylcholine (ACh) and adenosine 5'-triphosphate (ATP), acting at post-junctional muscarinic receptors and P_2-purinoceptors, respectively (see Anderson, 1993 for review). However, other endogenous substances may directly and/or indirectly affect bladder contractility. For example, 5-hydroxytryptamine (5-HT) enhances motor activity of resting detrusor through a mechanism which involves, at least in part, ketanserin-sensitive muscular 5-HT_{2A} receptor subtypes, as observed in man (Klarskov & Hørby-Petersen, 1986) and various animal species (Saxena *et al.*, 1985; Cohen, 1990), as well as neural 5-HT_3 receptors (Saxena *et al.*, 1985; Chen, 1990). With regard to excitatory neurotransmission, 5-HT potentiates neurogenic contractile responses to electrical stimulation in the mouse (Holt *et al.*, 1986) and human urinary bladder (Hindmarsh *et al.*, 1977; Corsi *et al.*, 1991). In the mouse, the potentiating effect is probably mediated by the 5-HT_{1B} receptor subtype (Holt *et al.*, 1986), while

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in human detrusor strips the receptor involved, which is different from the classical 5-HT_1, 5-HT_2 or 5-HT_3 sites (Corsi *et al.*, 1991), has been recently characterized as the 5-HT_4 receptor (Tonini *et al.*, 1994).

In a preliminary report (Rizzi *et al.*, 1994), we first showed that 5-HT potentiates the electrically induced excitatory neuromuscular transmission in isolated detrusor strips from the guinea-pig. The present study extends our previous observation by characterizing the 5-HT receptors facilitating neuromuscular transmission in this species by means of 5-HT and 5-HT-related agonists (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), 2-methyl-5-HT, 5-methoxytryptamine (5-MeOT)) and antagonists (methiothepin, ketanserin, granisetron, GR 125487). Additionally, 5-HT was examined for interactions with both the cholinergic and purinergic components of excitatory neuromuscular transmission. These components were isolated pharmacologically by using suramin or hyoscine to block post-junctional P_2-purinoceptors and muscarinic receptors, respectively (Hoyle *et al.*, 1990; Creed *et al.*, 1991).

Methods

Electrically stimulated detrusor strips

Male guinea-pigs weighing 400–600 g were stunned and bled. Mucosa-free detrusor strips, approximately 2 by 15 mm in size, were prepared by dissecting the bladder along the minor axis. The strips were suspended isometrically (tension: 5 mN) in 5 ml organ baths containing Tyrode solution of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.04, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.5, and gassed with 95% O_2/5% CO_2 (pH 7.3–7.4). A minimum initial equilibration period of 45 min was allowed before the experiments were started, during which the solution was changed every 15 min. Electrical field stimulation was applied via two platinum electrodes placed at the top and the bottom of the chamber, and connected to a MARB ST 87 stimulator. Trains of electrical pulses at 10 Hz and 5 s in duration were delivered at 1 min intervals, at 0.1 ms pulse width and 45 V. This stimulation evoked reproducible submaximal contractile responses, the amplitude of which was approximately 40% of that obtained by 50 Hz stimulation.

Experimental design

Cumulative concentration-response curves to 5-HT and 5-HT-related agonists were obtained using 0.5 log unit increments until the maximum effect was reached. Two agonist concentration-response curves were constructed in each preparation. Preliminary experiments showed that the second curve was superimposable, provided that there were 60 min intervals between curves and frequent solution changes (every 10 min). In order to allow direct (between-)agonist comparisons, a series of concentration-response curves to 5-HT, DOI (a 5-HT_2 receptor agonist), 2-methyl-5-HT (a 5-HT_3 receptor agonist) and 5-MeOT (a 5-HT_4 receptor agonist) were constructed in single preparations. Responses to each agonist were expressed as a percentage of the maximal response to 5-HT. The potentiating effect of 5-HT was studied in the absence and in the presence of 0.3 μM methiothepin (a mixed 5-HT_1/5-HT_2 receptor antagonist), 0.3 μM ketanserin (a 5-HT_{2A} receptor antagonist), 0.3 μM granisetron (a 5-HT_3 receptor antagonist) and 30 nM GR 125487 (a potent and selective 5-HT_4 receptor antagonist: Gale *et al.*, 1993; Bunce *et al.*, 1994). The incubation time for each antagonist was 30 min. The potentiation induced by 2-methyl-5-HT was investigated in the absence and in the presence of granisetron (0.1, 0.3, and 1 μM).

In a separate set of experiments, the effects of 5-HT were examined on the cholinergic (in the presence of 300 μM suramin) and the purinergic (in the presence of 1 μM) hyoscine) components of excitatory neuromuscular transmission.

Data analysis

To evaluate whether curves to 5-HT and 5-HT-related agonists were mono- or biphasic in nature, they were analyzed by use of the following logistic equation: $\text{Effect} = E_{maximum}/1 + e^{\{-2.303 \times \text{slope} \times (\log[A] - \log [A_{50}])}$ where: $E_{maximum}$ = maximum response; [A] = molar agonist concentration; $[A_{50}]$ = molar agonist concentration inducing 50% of the maximum response. Data were fitted either to a single logistic expression or to the sum of two logistics. Goodness of fit of the data to a single or double logistic expression was evaluated on the basis of the residual variance by means of an appropriate *F*-test, using a significant criterion of $P < 0.05$.

Any antagonist-induced inhibition of agonist response was calculated as a percentage of the maximum effect of agonist obtained under control conditions. Antagonist pA_2 estimates were calculated following Schild regression analysis (Arunlakshana & Schild, 1959), using agonist concentration-ratios (CR) determined at the EC_{50} levels in control and test curves. Confidence limits (CL) at 95% probability for the slope of the regression were evaluated by use of a computer programme (PHARM/PCS, Version 4.1) based on a manual of pharmacological calculations (Tallarida & Murray, 1986). Apparent affinity estimates (pK_B) from single antagonist concentrations were calculated using the Gaddum (1957) equation.

All data are expressed as means ± s.e.mean of *n* experiments. Differences between means were analyzed by Student's two tailed *t* test. Values of $P < 0.05$ were considered as statistically significant.

Drugs

5-Hydroxytryptamine creatinine sulphate (5-HT), hyoscine hydrochloride, hexamethonium bromide and tetrodotoxin (TTX) were obtained from Sigma Chemical Co.; 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) maleate, (±)-DOI [(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride], 5-methoxytryptamine (5-MeOT) hydrochloride, ketanserin tartrate and methiothepin mesylate from RBI; granisetron and GR 125487 ([1-[2(methylsulphonylamino)ethyl]4-piperidinyl]methyl-5-fluoro-2-methoxy-1H-indole-3-carboxylate hydrochloride) were kindly donated by SmithKline Beecham and Glaxo, respectively; suramin was obtained as a generous gift from Bayer S.p.A., Milan, courtesy of Dr A. Faggiotto.

With the exception of ketanserin, all drugs were dissolved in distilled water and administered in volumes not exceeding 1% v/v of the bath volume. Stock solutions of ketanserin were prepared in 1 : 100 v/v ethanol/water. Further dilutions were in water.

Results

In isolated strips of guinea-pig detrusor muscle, electrical field stimulation (10 Hz for 5 s every 60 s, 0.1 ms pulse duration, 45V) evoked submaximal twitch contractions, which were 40.8 ± 7.5%, $n = 5$, of those obtained by 50 Hz stimulation. These contractions were suppressed by 0.6 μM TTX ($n = 5$), indicating their neurogenic origin.

Cumulative administration of 5-HT caused a concentration-dependent (range 0.03–10 μM) increase in the amplitude of twitch contractions ($n = 20$). A representative tracing of 5-HT-induced potentiating effect is shown in Figure 1, while Figure 2 illustrates a quantitation of the response. The concentration-response curve to 5-HT was biphasic in nature, since it was better fitted to a summation of two logistics than to a single logistic function. The first phase was obtained with concentrations of 5-HT in the range of 0.03–1 μM. The mean maximum response of this phase was 31.1 ± 2.0% ($n = 8$) of the total response. The mean potency values were 7.1 ± 0.08 ($n = 8$) for the first phase

($-\log EC_{50'}$) and 5.7 ± 0.04 ($n=8$) for the second phase ($-\log EC_{50''}$). None of these parameters was altered by hexamethonium (30 μM, $n=5$).

Contractile responses to field stimulation were also enhanced by the 5-HT-related agonists, 2-methyl-5-HT, DOI and 5-MeOT (Figure 2). The concentration-response curve to 2-methyl-5-HT was monophasic ($-\log EC_{50} = 5.3 \pm 0.03$, $n=12$) and the maximum effect was superimposable to that obtained with 30 μM 5-HT. Similarly, exposure to DOI and 5-MeOT produced monophasic responses, although maximal effects were only $20.0 \pm 4.6\%$ ($n=3$) and $30.5 \pm 9.8\%$ ($n=3$) of that elicited by 5-HT, respectively.

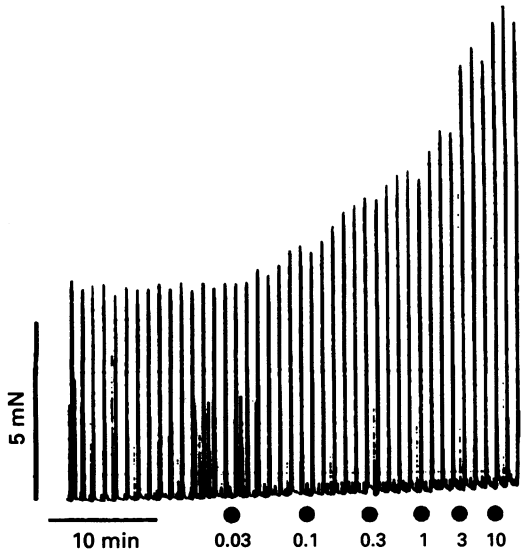

Figure 1 A representative tracing illustrating the potentiating effect of 5-HT (0.03–10 μM) on the excitatory neuromuscular transmission elicited by repetitive trains of electrical pulses in guinea-pig isolated detrusor strips.

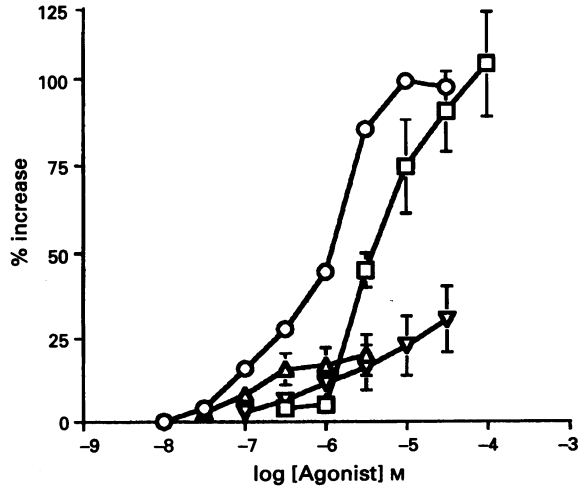

Figure 2 Concentration-response curves for 5-HT (○), 2-methyl-5-HT (□), DOI (△) and 5-MeOT (▽) in enhancing excitatory neuromuscular transmission in guinea-pig isolated detrusor strips. The curve to 5-HT is biphasic, while the curves to other 5-HT related agonists are monophasic. Values are expressed as means ± s.e.mean, $n=4-20$. For abbreviations, see text.

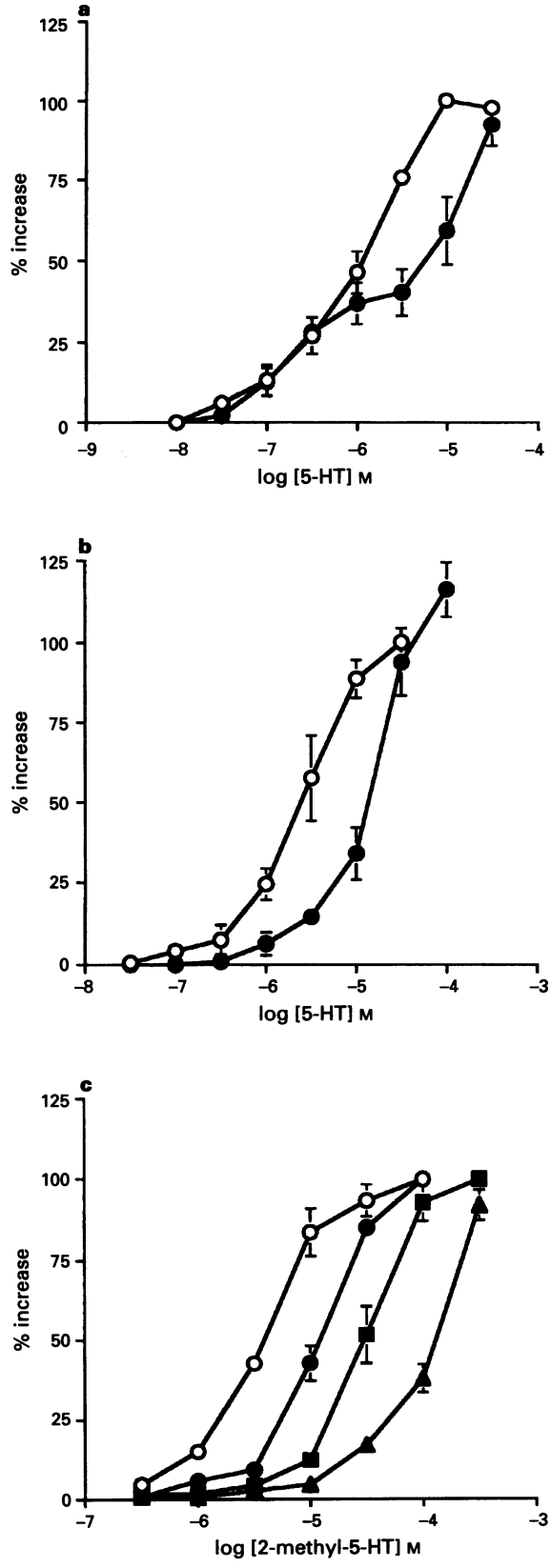

Figure 3 Effect of granisetron on responses to 5-HT (a,b) and 2-methyl-5-HT (c). In (a) control 5-HT responses (○); responses in the presence of 0.3 μM granisetron (●). Note that the effect of submicromolar concentrations of 5-HT is not antagonized by granisetron. In (b) responses to 5-HT in 0.3 μM methiothepin- and 30 nM GR 125487-pretreated preparations, both in the absence (○) and in the presence of 0.3 μM granisetron (●); In (c) control 2-methyl-5-HT responses (○); responses in the presence of 0.1 (●), 0.3 (■) and 1 μM (▲) granisetron. Values are expressed as means ± s.e.mean, $n=4-11$.

Since the 5-HT$_3$ receptor agonist, 2-methyl-5-HT behaved, like 5-HT, as a full agonist in our experimental model, the effect of the 5-HT$_3$ receptor antagonist, granisetron was investigated on the responses induced by both compounds. As illustrated in Figure 3a, 0.3 μM granisetron did not affect the first phase of the 5-HT curve, but shifted the second low potency phase to the right in a parallel fashion. Use of the Gaddum equation in the latter phase yielded a pK_B value of 7.3 ± 0.1 (n = 4). In a separate set of experiments, the effect of granisetron (0.3 μM) on the 5-HT response was also assessed after pharmacological isolation of 5-HT$_3$ receptors with methiothepin (0.3 μM) and GR 125487 (30 nM). Under these conditions, the concentration-response curve to 5-HT was monophasic with a mean potency value (−log EC$_{50}$) of 5.6 ± 0.1 (n = 4). Granisetron (0.3 μM) produced a parallel rightward shift of the 5-HT curve without depression of the agonist response maximum (Figure 3b). The apparent pK_B value was 7.1 ± 0.1 (n = 4). Granisetron (0.1, 0.3 and 1 μM) caused parallel rightward displacements of the concentration-response curve to 2-methyl-5-HT (Figure 3c). Schild analysis yielded a line with a slope of 1.09 (0.87 − 1.32), which was not different from unity, and a pA$_2$ estimate of 7.32 ± 0.04 (slope constrained to 1).

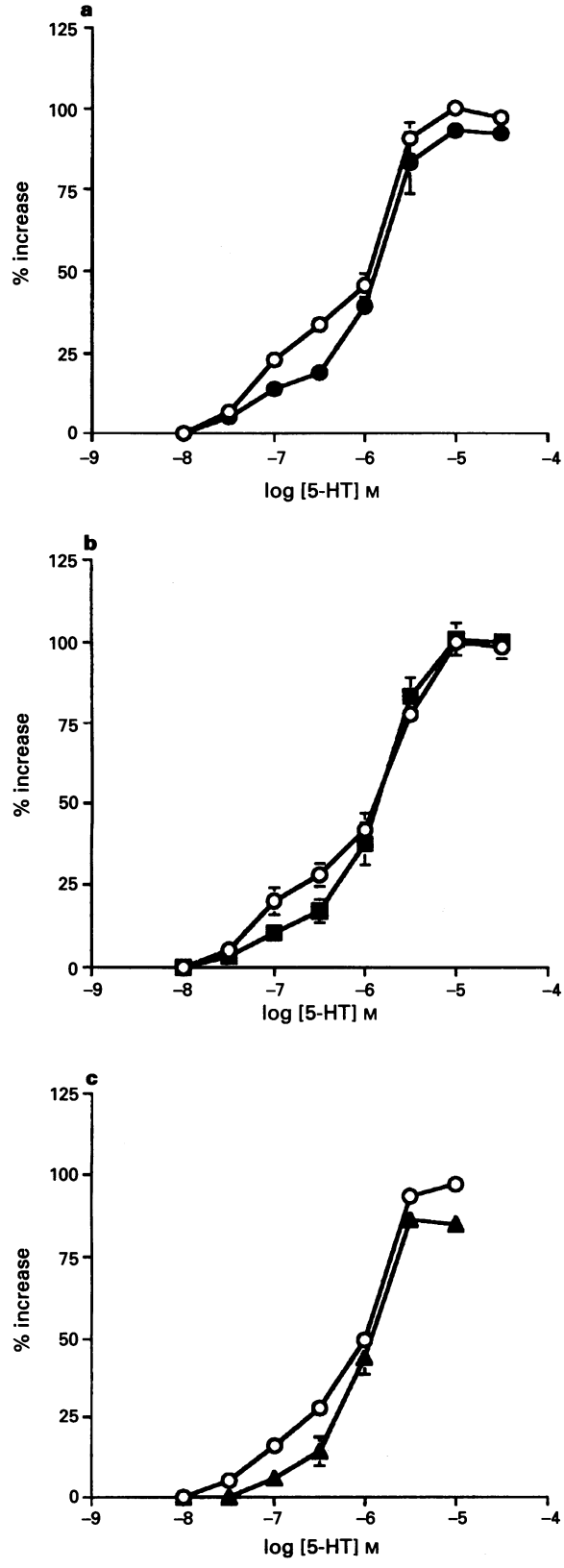

Figure 4 Effect of methiothepin (a), ketanserin (b) and GR 125487 (c) on responses to 5-HT. In (a, b and c) control 5-HT responses (○); responses in the presence of 0.3 μM (●) methiothepin (P < 0.05 versus control at 0.1 and 0.3 μM 5-HT), 0.3 μM (■) ketanserin (P < 0.05 versus control at 0.1 and 0.3 μM 5-HT) and 30 nM (▲) GR 125487 (P < 0.05 versus control at 0.1 μM 5-HT). Values are expressed as means ± s.e.mean, n = 4–6.

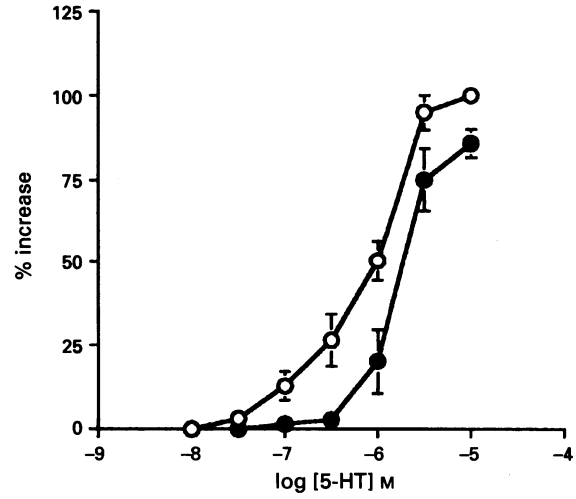

Figure 5 Effect of a combination of 0.3 μM ketanserin and 30 nM GR 125487 (●) on responses to 5-HT (control: ○). Note that in the presence of both antagonists the high potency phase is suppressed (P < 0.05 versus control at 0.1, 0.3 and 1 μM 5-HT), and the curve to 5-HT becomes monophasic. Values are expressed as means ± s.e.mean, n = 4.

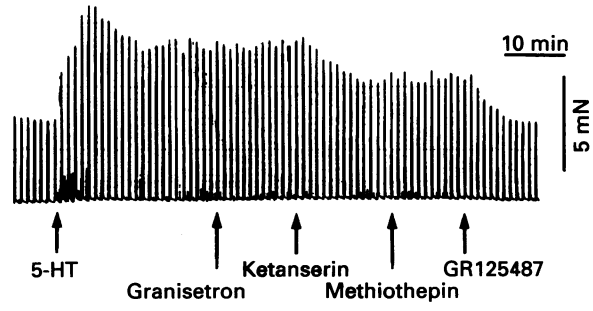

Figure 6 Effect of various 5-HT receptor antagonists on the potentiating effect caused by 0.3 μM 5-HT on excitatory neuromuscular transmission evoked by electrical field stimulation in guinea-pig isolated detrusor strips. 5-HT-induced potentiation peaked within 4 min, and peak response then decayed to a stable lower level. Under these conditions granisetron (0.3 μM) was ineffective, ketanserin (0.3 μM) inhibited the amplitude of contractions, and the subsequent addition of methiothepin (0.3 μM) had no effect. GR 125487 (30 nM) further antagonized contractile responses up to recovery of their control height.

Based on these findings, experiments with other 5-HT receptor antagonists were performed to assess the nature of receptors involved in the first phase of the 5-HT response. Equimolar concentrations of methiothepin and ketanserin (each at 0.3 μM) had a similar slight inhibitory effect on the first phase, without altering the second phase (Figure 4a,b). A slight inhibition of the first phase of the 5-HT curve was also caused by 30 nM GR 125487 (Figure 4c). Conversely, this phase was almost entirely suppressed (and the 5-HT curve changed into a monophasic curve) by coincubating the preparation with ketanserin (or methiothepin, see Figure 3b) and GR 125487 (Figure 5). Ketanserin (0.3 μM) and GR 125487 (30 nM) were also able to completely reverse the potentiating effect caused by a sub-micromolar concentration (0.3 μM) of 5-HT (Figure 6). By contrast, granisetron (1 μM) was ineffective. Methiothepin (0.3 μM) was also ineffective once a previous administration of ketanserin (0.3 μM) had partially inhibited the potentiating effect of 5-HT (Figure 6). None of the tested antagonists altered control contractions to field stimulation.

In a separate set of experiments, the effects of 5-HT were studied on the cholinergic and purinergic components of the excitatory neuromuscular transmission to the detrusor muscle (Burnstock *et al.*, 1978; Anderson, 1993). In agreement with previous findings (Hoyle *et al.*, 1990), the hyoscine (1 μM)-sensitive (cholinergic) component of stimulated twitch contractions was 43.6 ± 7.0%, $n = 5$, while the suramin (300 μM)-sensitive (purinergic) component was 44.4 ± 4.2%, $n = 6$, of the total response. 5-HT potentiated twitch contractions irrespective of antagonist pretreatment. However, in the presence of hyoscine, twitch contractions were mainly enhanced by sub-micromolar concentrations of 5-HT, while in the presence of suramin, 5-HT-induced potentiation was mainly obtained within the micromolar range of concentrations (Figure 7).

Discussion

In this study, we provide evidence that 5-HT potentiates the electrically evoked excitatory neuromuscular transmission to isolated detrusor strips from the guinea-pig, in agreement with previous studies on the mouse (Holt *et al.*, 1986) and human bladder (Corsi *et al.*, 1991; Tonini *et al.*, 1994). Since the potentiating effect of 5-HT was insensitive to hexamethonium, this would exclude the interference of 5-HT with excitatory (nicotinic) transmission at ganglionic level.

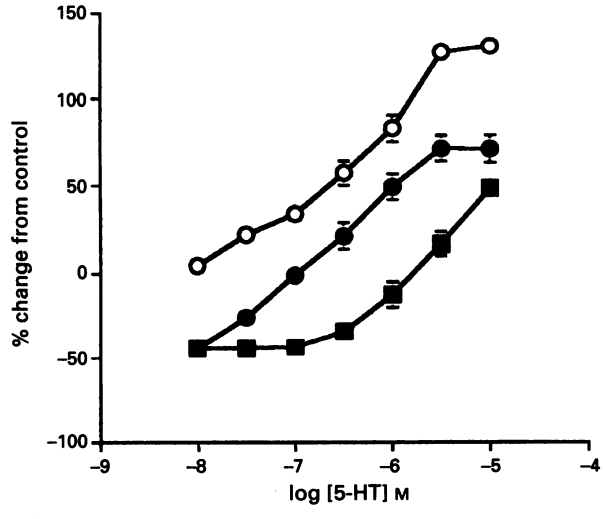

Figure 7 Concentration-response curve for 5-HT in enhancing the height of stimulated detrusor strip contractions under control conditions (○) or in the presence of either 1 μM hyoscine (●) or 300 μM suramin (■). Each antagonist reduced by approximately 50% the height of contractions. Compared to the potentiation produced by 5-HT in suramin pretreated tissues, in the presence of hyoscine 5-HT produced significantly ($P < 0.05$) higher potentiations in the range 30 nM – 3 μM. Values are expressed as means ± s.e.mean, $n = 4$.

As in other isolated smooth muscle tissues of this species, including small intestinal (Buchheit *et al.*, 1985; Eglen *et al.*, 1990; Butler *et al.*, 1990) and tracheal preparations (Lucchelli *et al.*, 1994), concentration-response curves to 5-HT were biphasic in nature, suggesting the participation of more than one class of receptors in its excitatory effect. Based on our findings, both 5-HT_{2A} and 5-HT_4 receptors seem to mediate the high potency phase of the 5-HT curve, while the low potency phase is almost entirely mediated by the 5-HT_3 receptors.

The latter evidence is consistent with the observation that this phase, together with the 5-HT responses obtained after pharmacological isolation of 5-HT_3 receptors with methiothepin and GR 125487, was antagonized by the selective 5-HT_3 receptor antagonist, granisetron (pK_B values: 7.3 and 7.1, respectively). The participation of neural 5-HT_3 receptors in the excitatory effect of 5-HT was further strengthened by the observation that the selective 5-HT_3 receptor agonist, 2-methyl-5-HT enhanced the electrically stimulated neuromuscular transmission in a concentration-dependent manner. This agonist, which displayed a monophasic curve, was less potent than 5-HT, but had comparable intrinsic activity (Figure 1). Even after concomitant blockade of 5-HT_{2A} and 5-HT_4 receptors with ketanserin and GR 125487 (Figure 5), the maximum response to 5-HT was not significantly different from that induced by 2-methyl-5-HT, because of the marked variability in the responses to the highest concentrations of the latter agonist. The nature of such variability is not clear, although one could hypothesize that high 2-methyl-5-HT concentrations may induce partial 5-HT_3 receptor desensitization in a proportion of tissues (i.e., those showing hyporesponsiveness), a phenomenon not observed, however, with 5-HT. The responses to 2-methyl-5-HT were competitively antagonized by granisetron with an estimated pA_2 value of 7.3. Based on these results, the affinity estimate for granisetron in the urinary bladder is somewhat lower than that obtained in other guinea-pig peripheral tissues (e.g. 7.8 – 8.1 in the intestine) (Eglen *et al.*, 1990; Butler *et al.*, 1990). Since this may reflect the existence of 5-HT_3 receptor intertissue variants in the same species (Wong *et al.*, 1992; but see Kilpatrick & Tyers, 1992), further studies with the use of other selective 5-HT_3 receptor agonists and antagonists are required to clarify this issue.

The presence of 5-HT_3 receptors has also been demonstrated in the unstimulated bladder of other animal species, including the cat (Saxena *et al.*, 1985) and the rabbit (Chen, 1990), where they cause indirect contractile responses which are due to neurally released ACh and ATP. With regard to the high potency phase of the 5-HT curve, it was insensitive to granisetron, but inhibited by both ketanserin (or methiothepin) and GR 125487, and virtually abolished by the concomitant administration of these antagonists. This suggests that the potentiation of the neuromuscular transmission caused by sub-micromolar concentrations of 5-HT is mediated by the 5-HT_{2A} receptor subtypes (antagonized by ketanserin) and by the 5-HT_4 receptors (antagonized by GR 125487). In this respect, the slight excitatory effect caused by DOI (a 5-HT_2 receptor agonist) and 5-MeOT (a 5-HT_4 receptor agonist) can be taken as indirect evidence for the presence of both types of receptors, although a quantitative receptor characterization was not undertaken, due to the small magnitude of agonist responses. Furthermore, the inhibition caused by methiothepin, a 5-HT_1/5-HT_2 receptor antagonist which has an affinity value (pK_D) for mammalian brain 5-HT_{2A} sites (8.8) comparable to that of ketanserin (8.9) (Hoyer, 1989), may involve 5-HT_{2A} receptor blockade. This assumption is based on the following observations: (*i*) equimolar concentrations of ketanserin and methiothepin produced equivalent inhibitory effects, and (*ii*) once ketanserin had partially inhibited the potentiation caused by sub-micromolar concentrations of 5-HT, the addition of methiothepin at a concentration sufficiently high (0.3 μM) to block the 5-HT_1 receptors (Hoyer,

1989), failed to reduce further the response to 5-HT. The latter evidence makes unlikely any contribution of the 5-HT_1 receptors to the potentiating effect of 5-HT. In peripheral tissues, the 5-HT_{2A} receptors are usually regarded as post-junctional sites (see Introduction, and Kaumann, 1989). However, these receptors have been recently found to participate in the neurogenic (cholinergic) component of the response to 5-HT in the guinea-pig isolated trachea (Lucchelli *et al.*, 1994), and to enhance the electrically induced cholinergic transmission in the isolated circular muscle of dog colon (Malysz *et al.*, 1993), indicating an additional location on intrinsic excitatory motor neurones.

As far as 5-HT_4 receptors are concerned, these receptors are present in the bladder of guinea-pig (present study), monkey (Waikar *et al.*, 1994) and man (Tonini *et al.*, 1994). However, while in guinea-pigs and man 5-HT_4 receptors are located prejunctionally, where they cause excitatory transmitter release, in monkeys these receptors are located post-junctionally, where they cause a direct muscular relaxation.

In a separate set of experiments we investigated whether, and through which receptors, 5-HT potentiated each of the main excitatory components (cholinergic and purinergic) of detrusor neuromuscular transmission. These components were isolated pharmacologically by pretreating detrusor strips with either the P_2-purinoceptor antagonist suramin (Hoyle *et al.*, 1990; Bailey & Hourani, 1994) or hyoscine, respectively. In agreement with previous findings (Moss & Burnstock, 1985), each component accounted for approximately 45% of total transmission evoked by 10 Hz stimulation. Based on our results, purinergic transmission was mostly sensitive to sub-micromolar concentrations of 5-HT, while cholinergic transmission was preferably enhanced by micromolar concentrations. It follows that potentiation of purinergic transmission is largely (though not exclusively) mediated by 5-HT_{2A} and 5-HT_4 receptors, while 5-HT_3 receptors are mainly involved in enhancing cholinergic transmission.

From literature data it is not clear whether ACh and ATP are stored in separate nerves supplying the bladder (Burnstock *et al.*, 1978; Westfall *et al.*, 1983) or co-stored in and co-released from the same class of neurones (MacKenzie *et al.*, 1982; Theobald, 1982; Brading & Mostwin, 1989). Our study cannot answer this question. However, it is interesting to point out that 5-HT may differentially affect ACh or ATP release from detrusor strips by activating at least three separate excitatory receptors. These receptors are probably located at different levels, including the neural cell body (5-HT_3 receptors) and the axonal processes (5-HT_{2A} and 5-HT_4 receptors) of final excitatory motor neurones, as observed in other peripheral excitable tissues (Kilpatrick *et al.*, 1990; Tonini *et al.*, 1991; Ford & Clarke, 1993; Lucchelli *et al.*, 1994).

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Cytokine-mediated inflammatory hyperalgesia limited by interleukin-10

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1 The effect of interleukin-10 (IL-10) upon the hyperalgesic activities in rats of bradykinin, tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), prostaglandin E_2 (PGE_2) and carrageenin were investigated in a model of mechanical hyperalgesia.

2 Hyperalgesic responses to bradykinin (1 μg) were inhibited in a dose-dependent manner by prior treatment with IL-10 (1–100 ng).

3 Hyperalgesic responses to TNFα (2.5 pg), IL-1β (0.5 pg) and IL-6 (1.0 ng) but not to IL-8 (0.1 ng) and PGE_2 (50 ng and 100 ng) were inhibited by prior treatment with IL-10 (10 ng).

4 Hyperalgesic responses to carrageenin (100 μg) were inhibited by IL-10 (10 ng) when this cytokine was injected before but not after the carrageenin.

5 A monoclonal antibody to mouse IL-10 potentiated the hyperalgesic responses to carrageenin (10 μg) and TNFα (0.025 pg) but not that to IL-8 (0.01 ng).

6 In *in vitro* experiments in human peripheral blood mononuclear cells (MNCs), IL-10 (0.25–4.0 ng ml^{-1}) inhibited in a dose-dependent manner PGE_2 production by MNCs stimulated with IL-1β (1–64 ng ml^{-1}) or endotoxin (lipopolysaccharide, LPS, 1 iu = 143 pg ml^{-1}) but evoked only small increases in IL-1ra production.

7 These data suggest that IL-10 limits the inflammatory hyperalgesia evoked by carrageenin and bradykinin by two mechanisms: inhibition of cytokine production and inhibition of IL-1β evoked PGE_2 production. Our data suggest that the latter effect is not mediated via IL-10 induced IL-1ra and may result from suppression by IL-10 of prostaglandin H synthase-2 (COX-2).

Keywords: Inflammatory hyperalgesia; tumor necrosis factor α; bradykinin; interleukin-1; interleukin-6; interleukin-8; interleukin-10; interleukin-1 receptor antagonist; ELISA; prostaglandin H synthase-2; cyclo-oxygenase-2

Introduction

In a rat paw pressure test, carrageenin-evoked hyperalgesia has been shown to result from the combined effect of the release of cyclo-oxygenase products and sympathomimetic amines (Nakamura & Ferreira, 1987). In this model, carrageenin caused hyperalgesia by releasing bradykinin, which initiated a cascade of cytokine release (Ferreira *et al.*, 1993). The proposed sequence of events was that bradykinin stimulated the release of tumour necrosis factor α (TNFα), which: (i) induced interleukin-1β (IL-1β) and interleukin-6 (IL-6), which stimulated the production of cyclo-oxygenase products and (ii) induced interleukin-8 (IL-8) which stimulated production of sympathomimetics (Cunha *et al.*, 1992; Ferreira *et al.*, 1993).

In recent years a number of cytokines have been described which inhibit the production of TNFα, IL-1β, IL-6 and IL-8 (cytokines that are generally regarded as pro-inflammatory). One such 'antagonist cytokine' is IL-10. IL-10, which is produced by murine Th2 lymphocytes and monocytes, inhibits production of cytokines by murine Th1 lymphocytes (Fiorentino *et al.*, 1989) and is believed to play a role in inhibiting delayed type hypersensitivity responses (Zlotnik & Moore, 1991; Howard & O'Garra, 1992) and to suppress macrophage functions such as class II expression (De Waal Malefyt *et al.*, 1991a), adhesion (Fiorentino *et al.*, 1991) and the synthesis of cytokines including IL-1, IL-6, IL-8, G-CSF, GM-CSF and TNFα (De Waal Malefyt *et al.*, 1991b; Fiorentino *et al.*, 1991; Bogdan *et al.*, 1991; Oswald *et al.*, 1992).

Given the capacity of IL-10 to inhibit the production of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL-8, we have investigated the possibility that IL-10 limits inflammatory hyperalgesia by diminishing the cytokine-mediated hyperalgesic responses to carrageenin and bradykinin.

Methods

Nociceptive test

A constant pressure of 20 mmHg was applied to the hind paws of rats and discontinued when they presented a typical freezing reaction (reaction time). This reaction was characterized by a reduction of escape movements: animals usually made several attempts to escape from the position imposed by the experimental situation. These were followed by alterations in respiratory frequency with the onset of a typical shivering reaction. The intensity of hyperalgesia was quantified as the variation in reaction time (Δ reaction time) obtained by subtracting values measured 3 h after administration of hyperalgesic substances from (control) reaction times measured before injection at zero time (Ferreira *et al.*, 1978).

Experimental protocol

In vivo *measurements* Hyperalgesia was measured 3 h after injection of bradykinin (1–100 μg), TNFα (0.025 and 2.5 pg), IL-1β (0.5 pg), IL-6 (1.0 ng), IL-8 (0.01 or 0.1 ng), PGE_2 (50 and 100 ng) and carrageenin (10 and 100 μg), each injected in

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100 μl, into the hind paws (intraplantar, i.pl.) of rats. IL-10 (1–100 ng in 100 μl, i.pl.) or saline control (100 μl) was injected into the (same) hind paws, 30 min before or 2 h after the hyperalgesic substances. A monoclonal antibody to mouse IL-10 (150 μg in 50 μl, i.pl.) or a control antibody (150 μg in 50 μl, i.pl.) was injected into the (same) hind paws, 30 min before hyperalgesic substances.

In vitro *assays* PGE_2 and IL-1 receptor antagonist (IL-1ra) in media conditioned by human peripheral blood mononuclear cells (MNCs, 5×10^6 cells ml^{-1}) incubated for 16 h with IL-1β, IL-10 and endotoxin (lipopolysaccharide, LPS) were quantified by enzyme immunoassay (Amersham plc, Amersham, UK) and ELISA, respectively. The cells were isolated from the buffy coats of heparinised human blood by density gradient centrifugation (Poole *et al.*, 1989). The protocol for ELISA of IL-1ra was based upon a protocol for ELISA of IL-6 described previously (Taktak *et al.*, 1991). Briefly, microtiter plates (Immunoplate Maxisorp, NUNC) were coated with immunoaffinity purified sheep anti-IL-1ra polyclonal antibodies (100 μl of a 2 μg ml^{-1} solution in PBS buffer) at 4°C for 16–24 h. After washing with assay buffer (0.01 M phosphate, 0.05 M NaCl, 0.1% Tween 20, pH 7.2), 100 μl of 1% ovalbumin (Sigma, Poole, Dorset) in PBS coating buffer were added to each well except reagent blank wells and the wells covered and incubated at 37°C for 1 h. After washing with assay buffer, 100 μl of standard (recombinant IL-1ra, NIBSC preparation 92/644, 7.8–10,000 pg ml^{-1}) or sample were added to each well and incubated at room temperature for 4 h. After washing with assay buffer, 100 μl/well of biotinylated immunoaffinity purified sheep anti-IL-1ra polyclonal antibodies (diluted 1/1000) were added to each well and the wells covered and incubated at room temperature for 1 h. After washing with assay buffer, colour was developed using avidin-peroxidase (Dako Ltd, High Wycombe, UK) and 1, 2-phenylenediamine dihydrochloride (orthophenylene diamine, OPD, Sigma, Poole, Dorset). The enzyme reaction was stopped by adding 150 μl 1 M H_2SO_4 to each well and absorbance at 490 nm was measured.

Results are presented as means with s.e.mean of groups of 5 animals for the *in vivo* measurements and of triplicate wells (using pooled cells from two donors) for the *in vitro* assays. Three independent *in vitro* assays were performed, each using blood from different donors of the same blood group. Differences between responses were evaluated by ANOVA, followed by Bonferroni *t* test. Formal statistical tests are not reported for differences where means differed by more than three times the larger s.e.mean.

Materials

Drugs Recombinant human IL-1β, IL-6, IL-8 (72 amino acids), IL-10, TNFα, IL-1ra and *E. coli* 0113:H10:K- endotoxin (lipopolysaccharide, LPS) were NIBSC preparations coded 86/680, 88/514, 89/520, 92/516, 87/650, 92/644 and 84/650. The specific activities of these materials are IL-1β: 100,000 international units (iu/1 μg/ampoule, IL-6: 100,000 iu/1 μg/ampoule, IL-8: 1,000 units/1 μg/ampoule, IL-10: 5,000 units/1 μg/ampoule, TNFα: 40,000 iu/1 μg/ampoule, IL-1ra: 10 milli-units/10 μg/ampoule, LPS: 14,000 iu/2 μg/ampoule. PGE_2 was a gift from the Upjohn Co (U.S.A.). Carrageenin was a gift from the FMC Corporation (Philadelphia, U.S.A.). Bradykinin was purchased from Sigma (St. Louis, U.S.A.). The monoclonal IgM antibody to mouse IL-10, SXC-1, was provided by Professor F. Liew (University of Glasgow, UK). The control monoclonal antibody was a purified unrelated IgM raised against ovalbumin in our laboratory.

Animals Male Wistar rats, 130–180 g, housed in temperature controlled-rooms (22–25°C) with water and food *ad libitum* until use.

Results

Inhibition by IL-10 of the hyperalgesic responses to bradykinin TNFα, IL-1β and IL-6

Injection of bradykinin (1 μg, i.pl.) into one hindpaw of rats evoked hyperalgesia, measured 3 h after injection (Figure 1a). IL-10 (1–100 ng/paw), injected 30 min before bradykinin (1 μg/paw), dose-dependently inhibited the hyperalgesic response measured 3 h after injection of bradykinin (Figure 1a). Although no effect was seen with IL-10 at 1 ng/paw, IL-10 at

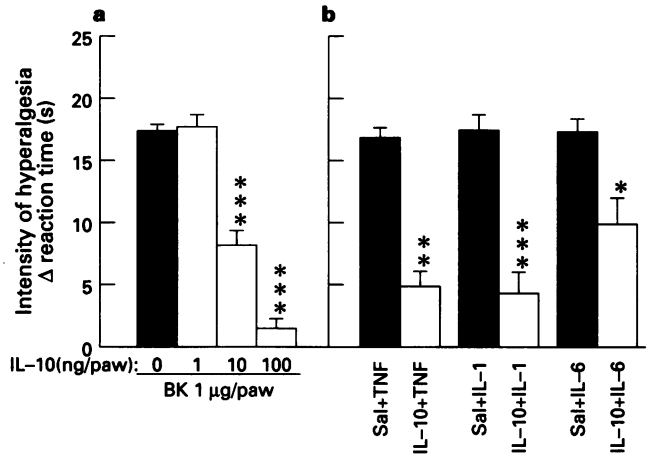

Figure 1 Inhibition by interleukin-10 (IL-10) of the hyperalgesic responses to bradykinin, tumour necrosis factor α (TNFα), IL-1β and IL-6. Responses were measured 3 h after injection (in 100 μl, i.pl.) of bradykinin, TNFα, IL-1β and IL-6. IL-10 (1–100 ng in 100 μl, i.pl., open columns) or saline (Sal, 100 μl, i.pl., solid columns) was given 30 min before hyperalgesic substances. Panel (a) shows the intensity of hyperalgesia in injected paws in response to bradykinin (1.0 μg, solid column) and its inhibition by IL-10 (1–100 ng in 100 μl, i.pl., open columns). Panel (b) shows the effect of saline (Sal) and IL-10 (10 ng in 100 μl, i.pl.) on responses to TNFα (TNF, 2.5 μg), IL-1β (IL-1, 0.5 pg) and IL-6 (1.0 ng). Mean ± s.e.mean in groups of 5 rats are shown; $^*P<0.05$; $^{**}P<0.01$, $^{***}P<0.001$.

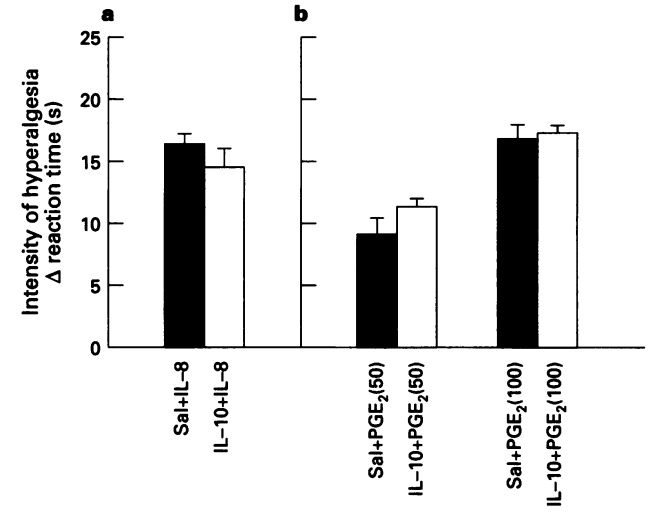

Figure 2 Effect of interleukin-10 (IL-10) on the hyperalgesic responses to IL-8 and prostaglandin E_2 (PGE_2). Responses were measured 3 h after injection (in 100 μl, i.pl.) of IL-8 and PGE_2. IL-10 (10 ng in 100 μl, i.pl., open columns) or saline (100 μl, i.pl., solid columns) was given 30 min before hyperalgesic substances. Panel (a) shows the effect of saline (Sal) and IL-10 (10 ng in 100 μl, i.pl.) on the response to IL-8 (0.1 ng). Panel (b) shows the effect of saline (Sal) and IL-10 (10 ng in 100 μl, i.pl.) on the response to PGE_2 (50 and 100 ng). Mean ± s.e.mean in groups of 5 rats are shown.

10 and 100 ng/paw inhibited responses to bradykinin (1 μg/paw) by 53±7% ($P<0.001$) and 92±4% ($P<0.001$), respectively. IL-10, 1–100 ng/paw, injected 30 min before saline had little effect upon Δ reaction times, which were 2.7±0.7, 0.3±0.7 and 0.8±1.0 s for 1, 10 and 100 ng/paw IL-10. IL-10 (10 ng/paw), injected 30 min before the cytokine, also inhibited hyperalgesic responses to TNFα (2.5 pg/paw, −71±8%, $P<0.01$), IL-1β (0.5 pg/paw, −75±10%, $P<0.001$) and IL-6 (1.0 ng/paw, −43±12%, $P<0.05$, Figure 1b). The Δ reaction time for IL-10, 10 ng/paw, injected 30 min before saline was 1.1±1.6 s.

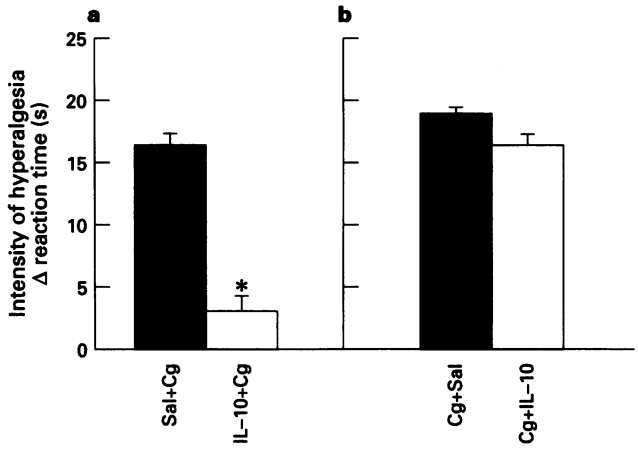

Figure 3 Inhibition by interleukin-10 (IL-10) of the hyperalgesic response to carrageenin. Responses were measured 3 h after injection (in 100 μl, i.pl.) of carrageenin. IL-10 (10 ng in 100 μl, i.pl., open columns) or saline (100 μl, i.pl., solid columns) was given 30 min before or 2 h after carrageenin. Panel (a) shows the effects of saline (Sal) and IL-10 (10 ng in 100 μl, i.pl.) given 30 min before carrageenin on the response to carrageenin (Cg, 100 μg). Panel (b) shows the effects of saline (Sal) and IL-10 (10 ng in 100 μl, i.pl.) given 2 h after carrageenin on the response to carrageenin (Cg, 100 μg). The Δ reaction times for IL-10, 10 ng/paw, injected 30 min before and 2 h after saline were 1.1±1.6 s and 1.0±3.3 s, respectively, data not shown. Mean±s.e.mean in group of 5 rats are shown; *$P<0.001$.

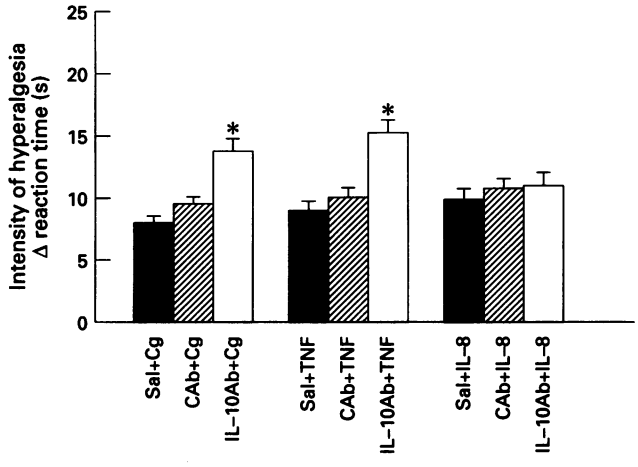

Figure 4 Effect of a monoclonal antibody to interleukin-10 (IL-10) on the hyperalgesic responses to carrageenin, tumour necrosis factor α (TNFα) and IL-8. Responses were measured 3 h after injection (in 100 μl, i.pl.) of hyperalgesic substances. The monoclonal antibody to IL-10 (IL-10Ab, 150 μg in 50 μl, i.pl., open columns) or a control antibody (CAb, 150 μg in 50 μl, i.pl., hatched columns) or saline (50 μl, i.pl., solid columns) was given 30 min before carrageenin (10 μg), TNFα (0.025 pg) and IL-8 (0.1 ng). The Δ reaction times for control antibody and monoclonal antibody to IL-10 were 2.6±0.9 s and 1.7±1.6 s, respectively, data not shown. Mean±s.e.mean in groups of 5 rats are shown; *$P<0.05$.

Failure of IL-10 to inhibit the hyperalgesic responses to IL-8 and PGE_2

IL-10 (10 ng/paw), injected 30 min before the hyperalgesic stimulus, failed to inhibit responses measured 3 h after injection of IL-8 (0.1 ng/paw, Figure 2a) and PGE_2 (50 and 100 ng/paw, Figure 2b).

Inhibition by IL-10 of the hyperalgesic response to carrageenin

Injection of carrageenin into one hindpaw of rats (100 μg/paw) evoked hyperalgesia, measured 3 h after injection (Figure 3a). IL-10 (10 ng/paw) injected 30 min before carrageenin inhibited carrageenin-evoked hyperalgesia by 81±8% ($P<0.001$) whereas IL-10 (10 ng/paw) injected 2 h after the carrageenin had no significant effect upon the response (Figure 3b). The Δ reaction times for IL-10, 10 ng/paw, injected 30 min before and 2 h after saline were 1.1±1.6 s and 1.0±3.3 s, respectively.

Potentiation by a monoclonal antibody to IL-10 of the hyperalgesic response to carrageenin and TNFα but not IL-8

A monoclonal antibody to IL-10 (150 μg/paw) but not a control antibody (150 μg/paw), injected 30 min before the

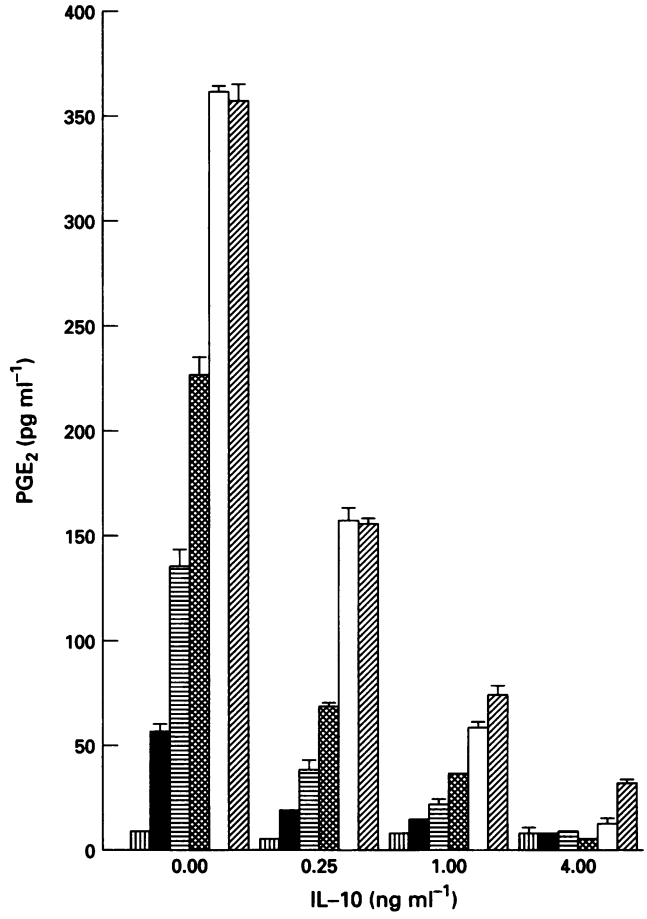

Figure 5 Inhibition by interleukin-10 (IL-10) of IL-1β-evoked and lipopolysaccharide (LPS)-evoked immunoreactive prostaglandin E_2 (PGE_2) production by human peripheral blood mononuclear cells (MNCs, 5×10^6 cells ml^{-1}) incubated for 16 h with IL-10 (0.25–4.0 ng ml^{-1}), IL-1β (1–64 ng ml^{-1}) and LPS (1 iu ml^{-1}). IL-10 (vertically hatched columns), IL-10+IL-1β (1 ng mg^{-1}, solid columns), IL-10+IL-1β (4 ng ml^{-1}, horizontally hatched columns), IL-10+IL-1β (16 ng ml^{-1}, cross hatched columns), IL-10+IL-1β (64 ng ml^{-1}, open columns), and IL-10+LPS (1 iu ml^{-1}, diagonally hatched columns). S.e.mean of triplicate wells using pooled cells from two donors are shown. (Three independent assays were performed.)

stimulus, potentiated hyperalgesic responses measured 3 h after injection of carrageenin (10 μg/paw, +59±23%, $P<0.05$) and TNFα (0.025 pg/paw, +69±11%, $P<0.05$) but not responses measured 3 h after IL-8 (0.1 ng/paw, +11±12%, $P<0.05$, Figure 4). The Δ reaction times for control antibody and monoclonal antibody to IL-10 were 2.6±0.9 s and 1.7±1.6 s, respectively.

Inhibition by IL-10 of IL-1β-evoked PGE_2 production by human peripheral blood mononuclear cells (MNCs)

IL-1β (1–64 ng ml^{-1}) evoked dose-dependent increases in the production of immunoreactive PGE_2 by MNCs; LPS (1 iu = 143 pg ml^{-1}) evoked an increase in the production of immunoreactive PGE_2 similar to that seen with IL-1β (64 ng ml^{-1}, Figure 5). IL-10 (0.25–4.0 ng ml^{-1}) inhibited in a dose-dependent manner responses to IL-1β and LPS, with the highest dose of IL-10 (4.0 ng ml^{-1}) abolishing responses to IL-1β and reducing the response to LPS by over 90% (Figure 5).

Induction of IL-1ra by IL-10, IL-1β and LPS in MNCs

IL-10 (0.25–4.0 ng ml^{-1}) evoked small (dose-dependent) increases in the production of immunoreactive IL-1ra by MNCs (Figure 6). IL-1β (1–64 ng ml^{-1}) and LPS (1 iu ml^{-1}) evoked larger increases in secretion of immunoreactive IL-1ra by MNCs; the effects of IL-10 and IL-1β and of IL-10 and LPS were not additive (Figure 6).

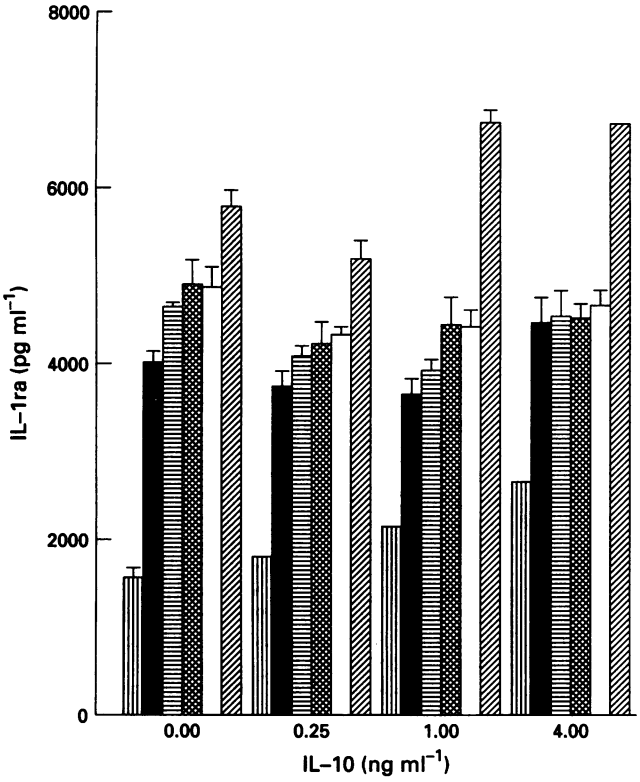

Figure 6 Induction of immunoreactive interleukin-1 receptor antagonist (IL-1ra) by IL-10, IL-1β and lipopolysaccharide (LPS) in MNCs. Immunoreactive IL-1ra production by human peripheral blood mononuclear cells (MNCs, 5×10^6 cells ml^{-1}) incubated for 16 h with IL-10 (0.25–4.0 ng ml^{-1}), IL-1β (1–64 ng ml^{-1}) and LPS (1 iu ml^{-1}). IL-10 (vertically hatched columns), IL-10+IL-1β (1 ng ml^{-1}, solid columns), IL-10+IL-1β (4 ng ml^{-1}, horizontally hatched columns), IL-10+IL-1β (16 ng ml^{-1}, cross hatched columns), IL-10+IL-1β (64 ng ml^{-1}, open columns), and IL-10+LPS (1 iu ml^{-1}, diagonally hatched columns). Vertical bars are s.e.mean of triplicate wells using pooled cells from two donors are shown. (Three independent assays were performed.)

Discussion

IL-10 inhibits the production of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL-8 and upregulates the expression of the IL-1 receptor antagonist, IL-1ra, (Howard & O'Garra, 1992, Jenkins *et al.*, 1994). Consequently IL-10 has therapeutic potential in acute and chronic inflammatory diseases. To investigate the possibility that IL-10 could inhibit inflammatory hyperalgesia, bradykinin was chosen as the first inflammatory mediator against which to test IL-10 since bradykinin initiates the release of a cascade of pro-inflammatory cytokines following administration of carrageenin in a model of inflammatory hyperalgesia (Ferreira *et al.*, 1993).

The capacity of a single injection of IL-10 to abolish hyperalgesia evoked by a potent pro-inflammatory stimulus such as carrageenin confirms the anti-inflammatory activity reported for IL-10 in other systems (Fiorentino *et al.*, 1989, 1991; Bogdan *et al.*, 1991; Zlotnik & Moore, 1991; De Waal Malefyt *et al.*, 1991a,b; Howard & O'Garra, 1992; Oswald *et al.*, 1992; Jenkins *et al.*, 1994). That IL-10 inhibited hyperalgesic responses to TNFα and IL-6 but not to IL-8 and PGE_2 was expected since the hyperalgesic activities of TNFα and IL-6 depend upon induction of cytokines (IL-1β and IL-8, and IL-1β, respectively). In contrast, IL-8 induces hyperalgesic sympathomimetic amines (Cunha *et al.*, 1991) and PGE_2 sensitizes nociceptors (Ferreira, 1972), actions which are not sensitive to inhibition by IL-10. Consequently, the data obtained are consistent with the cascade of mediators proposed for this model, in which the sequence of events appears to be as follows. Carrageenin stimulates release of bradykinin, which stimulates the release of TNFα. This (i) induces IL-6 and IL-1β, which stimulate the production of cyclo-oxygenase products and (ii) induces IL-8 which stimulates local production of sympathetic amines (Cunha *et al.*, 1991, 1992; Ferreira *et al.*, 1993).

The finding that IL-10 inhibited hyperalgesic responses to carrageenin when the IL-10 was injected before but not after the carrageenin are consistent with the reported inhibitory action of IL-10 on the production of TNFα, IL-1β, IL-6 and IL-8 (de Waal Malefyt *et al.*, 1991b; Fiorentino *et al.*, 1991; Bogdan *et al.*, 1991; Oswald *et al.*, 1992), the pro-inflammatory cytokines that transduce the hyperalgesic activity of carrageenin (Ferreira, *et al.*, 1988; Cunha *et al.*, 1991; 1992). That endogenous IL-10 has a significant role in limiting cytokine-mediated inflammatory hyperalgesia is indicated by the finding that, in rats, a monoclonal antibody to mouse IL-10 potentiated hyperalgesic responses to carrageenin and TNFα. The lack of effect of the antibody neutralizing endogenous IL-10 on the response to IL-8 was expected since, as stated above, IL-8 releases hyperalgesic sympathomimetics (Cunha *et al.*, 1991) and therefore does not rely upon IL-10-sensitive cytokine production to evoke hyperalgesia.

A finding that required further study was the capacity of IL-10 to inhibit IL-1β-evoked hyperalgesia. IL-1β stimulates the production of cyclo-oxygenase products, such as PGE_2 (Seibert *et al.*, 1990) which sensitize nociceptors (Ferreira, 1972), i.e., IL-1β evokes hyperalgesia by a mechanism that does not require IL-10-sensitive production of pro-inflammatory cytokines. Therefore IL-10 must have inhibited IL-1β-evoked hyperalgesia by other means. Since IL-10 can induce the IL-1 receptor antagonist, IL-1ra (Howard *et al*, 1992; Jenkins *et al.*, 1994), experiments were performed to investigate the possibility that induction of IL-1ra by IL-10 contributed to the inhibition by IL-10 of IL-1β evoked hyperalgesia. These experiments were performed *in vitro* using human peripheral blood mononuclear cells (MNCs), cells that are a major source of IL-10 (Howard & O'Garra, 1992) and which are sensitive to suppression by IL-10 of inflammatory cytokine production (Ralph *et al.*, 1992). IL-10 abolished IL-1β-induced PGE_2 production by MNCs while having little effect upon IL-1β-induced production of IL-1ra, suggesting that IL-10 induction of IL-1ra contributed little to the inhibition by IL-10 of IL-1β-induced PGE_2 production.

In addition to its potent inhibitory effect on IL-1β-induced PGE_2 production, IL-10 inhibited (by more than 90%) LPS-evoked PGE_2 production. Similarly, IL-10 inhibited, by 80–95%, concanavalin A (ConA)-induced monocyte prostaglandin H synthase-2 (PGHS-2/COX-2) and completely inhibited detectable PGHS-2 protein (Mertz *et al.*, 1994). Taken together, these data support the notion that suppression by IL-10 of PGHS-2, rather than some activity of IL-10 against IL-1β, accounted for the inhibition by IL-10 of PGE_2 production evoked by IL-1β and LPS, although it should be noted that in the *in vitro* studies of Mertz *et al.* (1994) optimal expression of COX-2 mRNA and protein occurred at 4 h and 18 h, respectively after stimulation with ConA, whereas the *in vivo* hyperalgesic response to IL-1β was maximal at 1 h (Ferreira *et al.*, 1988). A proportion of this difference could be accounted for by the time required for ConA to induce endogenous mediators such as IL-1β. It is conceivable that inhibition by IL-10 of an effect of IL-1β on phospholipase A_2 (PLA_2) may have contributed to the diminished PGE_2 production since IL-1 upregulates cytosolic (>>secretory) PLA_2 gene expression in human synovial cells (Angel *et al.*, 1994).

In summary, the data reported above suggest that IL-10 limits the inflammatory hyperalgesia evoked by carrageenin and bradykinin by two mechanisms: inhibition of cytokine production and inhibition of IL-1β evoked PGE_2 production. The latter effect does not appear to be mediated via IL-10-induced IL-1ra and may result from suppression by IL-10 of prostaglandin H synthase-2 (COX-2). The capacity of IL-10 to inhibit concomitantly both IL-1β production and IL-1β-stimulated PGE_2 production helps to ensure that the cascade of mediators that cause inflammatory hyperalgesia is closely regulated.

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Comparison of the interaction of agmatine and crude methanolic extracts of bovine lung and brain with α_2-adrenoceptor binding sites

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1 In the present study we have evaluated whether α_2-adrenoceptor binding sites on bovine cerebral cortex membranes labelled by [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 can distinguish between known agonists and antagonists. This model has then been used to compare the binding profiles of the putative non-catecholamine, clonidine-displacing substance (CDS), agmatine and crude methanolic extracts of bovine lung and brain.

2 Saturation studies carried out in the presence and absence of noradrenaline, 10 μmol l^{-1}, revealed that the maximum number of binding sites on bovine cerebral cortex membranes for [^{3}H]-idazoxan and [^{3}H]-RX-821002 were approximately 60-80% greater than those for [^{3}H]-clonidine (62.6 fmol mg^{-1} protein). Rauwolscine, the selective α_2-adrenoceptor antagonist, was approximately 100 fold more potent against each of the ligands than the selective α_1-adrenoceptor diastereoisomer, corynanthine. Also, the pK_i value for the selective α_1-adrenoceptor prazosin against each ligand was less than 6.

3 Adrenaline, UK-14034, rauwolscine, corynanthine, RX-811059 and prazosin produced concentration-dependent inhibition of binding of all three ^{3}H-ligands. The agonists, adrenaline and UK-14304, were approximately 5 and 10 fold less potent against [^{3}H]-idazoxan and [^{3}H]-RX-821002, respectively, than against [^{3}H]-clonidine. In marked contrast, the antagonists, rauwolscine, corynanthine, RX-811059 and prazosin exhibited a different profile, being approximately 2-3 fold more potent against sites labelled by [^{3}H]-RX-821002 and [^{3}H]-idazoxan compared to sites labelled by [^{3}H]-clonidine.

4 Agmatine and histamine produced a concentration-dependent displacement of [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes. The pK_i values for agmatine and histamine were independent of the ^{3}H-ligand employed, approximately 4.8 and 4.5, respectively.

5 Crude methanolic extracts of bovine brain and lung produced a concentration-dependent inhibition of [^{3}H]-clonidine binding to bovine cerebral cortex membranes (>90%). Based on the volume of the extract that caused 50% inhibition of [^{3}H]-clonidine binding, bovine lung contains 3 fold more CDS than bovine brain. Both extracts were at least 5 fold more potent against α_2-adrenoceptor sites labelled by [^{3}H]-clonidine than those labelled by [^{3}H]-idazoxan and [^{3}H]-RX-821002.

6 All three ^{3}H-ligands label the same population of α_2-adrenoceptor binding sites on bovine cerebral cortex membranes, but [^{3}H]-clonidine appears to label selectively the 'agonist' state of the sites: for which known agonists, adrenaline and UK-14304, exhibit a higher affinity. Our results indicate that neither agmatine nor histamine can account for the CDS activity present in crude extracts of bovine brain and lung. Moreover, these extracts appear to possess a binding profile similar to that of adrenaline and UK-14304, suggesting that they may possess agonist activity.

Keywords: α_2-Adrenoceptors; agmatine; clonidine-displacing substances; [^{3}H]-clonidine; [^{3}H]-idazoxan

Introduction

Atlas & Burstein (1984a,b) coined the term 'clonidine-displacing substance' (CDS) to describe the ability of a non-catecholamine substance(s), contained in a partially purified extract of rat and bovine brain, to displace [^{3}H]-clonidine from α_2-adrenoceptor binding sites on bovine cerebral cortex membranes. In subsequent experiment these workers reported that the extract was able to recognize non-adrenoceptor, imidazoline binding sites labelled by [^{3}H]-idazoxan, and activate α_2-adrenoceptors in human platelets and the rat vas deferens to produce a pro-aggregatory response and to inhibit neurogenic contractions, respectively (Diamant & Atlas, 1986; Diamant *et al.*, 1987; Parini *et al.*, 1989). The ability of a similarly prepared, crude brain extract to recognize α_2-adrenoceptor and non-adrenoceptor, imidazoline binding sites labelled by [^{3}H]-*para*-aminoclonidine has also been described by Ernsberger *et al.* (1988), although no data were presented to show that this extract could activate α_2-adrenoceptors. We have recently described the presence of a non-catecholamine activity in crude extracts of bovine brain and lung, capable of selective interaction with both α_2-adrenoceptor and non-adrenoceptor, imidazoline binding sites (Singh *et al.*, 1995). However, it was not possible to examine whether these extracts were able to activate α_2-adrenoceptors because of the presence of many impurities (eg., catecholamines, histamine and monovalent cations).

Li *et al.* (1994) recently proposed that agmatine, which could be extracted from bovine brain and which is able to displace [^{3}H]-clonidine from α_2-adrenoceptor binding sites, may represent a CDS. We have confirmed that agmatine can recognize α_2-adrenoceptor binding sites, but in functional studies it failed to activate or inhibit prejunctional α_2-adrenoceptors in the rat vas deferens (Pinthong *et al.*, 1995). Thus, agmatine cannot account for the CDS activity originally described by Atlas & Burnstein (1984a). Furthermore, since agmatine also failed to exhibit biological activity, even in preparations in which it recognized the associated binding site (in the rat cerebral cortex), this raises serious questions about the appropriateness of radioligand binding assay as the principal detection system for 'CDS'.

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Whilst the ability of an agent to activate a receptor cannot be gleaned from radioligand binding studies involving a single radioligand, the potency of agonists and antagonists at α_2-adrenoceptor binding sites are known to be differentially affected by the affinity state of the site. For example, α_2-adrenoceptor agonists possess a higher affinity for $\alpha_{2A/D}$-sites labelled by ^{3}H-agonists (e.g., [^{3}H]-adrenaline or [^{3}H]-UK-14304) compared to the same sites labelled by ^{3}H-antagonists (e.g., [^{3}H]-rauwolscine). Conversely, antagonists appear to possess a higher affinity for α_2-adrenoceptors labelled by ^{3}H-antagonists compared to the corresponding site labelled by an ^{3}H-agonist (Gleason & Hieble, 1991; MacKinnon *et al.*, 1993; see also Wilson *et al.*, 1991). Examples of preparations possessing α_2-adrenoceptors that exhibit this type of behaviour include human platelets (Garcia-Seville & Fuster, 1986; MacKinnon *et al.*, 1993). HT-29 cells (Turner *et al.*, 1985; Bylund *et al.*, 1988; Gleason & Hieble, 1991) and rat cerebral cortex membranes (Wallace *et al.*, 1994): preparations that belong to the $\alpha_{2A/D}$ subtypes.

The aims of the present study were two fold. First, to assess whether α_2-adrenoceptor binding sites on the bovine cerebral cortex membranes, when labelled by [^{3}H]-clonidine (a selective agonist U'Prichard & Synder, 1980), [^{3}H]-RX-821002 and [^{3}H]-idazoxan (selective antagonists; Brown *et al.*, 1990; Mallard *et al.*, 1992), can distinguish between known agonists and antagonists. Secondly, to determine whether the putative, non-catecholamine endogenous ligands for α_2-adrenoceptors, agmatine (Li *et al.*, 1994) and bovine brain and lung 'CDS' (Singh *et al.*, 1995), exhibit 'agonist-like' or 'antagonist-like' characteristics in this system.

Methods

Preparation of bovine brain and lung methanolic CDS

Bovine brain and lung were obtained from a local abattoir immediately after slaughter of the animal; 100 g wet weight of brain (minus cerebellum) and 80–100 g wet weight of lung were finely chopped and placed in 10 volumes (w/v) of boiling distilled water. Both tissues were homogenized in an OMNI-GEN sealed homogenizer (Setting 5 for 3 × 3 min) and the resulting homogenized material centrifuged at 65,000 *g* for 30 min at 4°C (MSE Superspeed 65). The supernatant was then removed, boiled for approximately 15 min to precipitate soluble protein and then allowed to cool to room temperature. The resulting solution was centrifuged at 65,000 *g* for 30 min at 4°C and the supernatant removed, frozen at −20°C and then freeze-dried. The lyophysylate was then extracted by sonication (5 min) with 2 × 20 volumes (w/v) of Analar grade methanol at room temperature. The methanolic extracts were combined and centrifuged at 4,000 r.p.m. for 5 min (MSE Mistral 3000) to remove any particulate matter and then evaporated to dryness at low pressure. The residual material was dissolved in 10 volumes (w/v) of twice-distilled water and stored at −20°C until required for use.

Membrane preparation

Bovine brains were obtained from the local abattoir immediately after the slaughter of the animal and the cerebral cortices homogenized in 20 volumes of ice cold Tris buffer (50 mmol l^{-1} Tris HCl; pH 7.7 at 25°C) with an OMNI-GEN sealed macro-homogenizer (setting 5; 120 s), to minimize potential health risks associated with aerosol formation. The homogenate was then centrifuged at 20,000 r.p.m. for 10 min at 4°C (MSE Europa 24M). The pellet was resuspended in 20 volumes (w/v) of Tris buffer and re-centrifuged. The final pellet was resuspended in 4.9 volumes (w/v) of 50 mmol l^{-1} Tris buffer for direct use in the binding assay or stored at −20°C. Resuspension of the centrifuged pellet was achieved with an Ultra-turrax homogenizer sited in a laminar airflow hood.

Binding assays

Bovine cerebral cortex membranes (200–300 μg protein) were incubated with increasing concentrations of the three radioligands in the presence and absence of noradrenaline (10 μmol l^{-1}). In another series of experiments the cortical membranes were incubated with either 0.5 nmol l^{-1} [^{3}H]-clonidine, 1 nmol l^{-1} [^{3}H]-idazoxan or 0.2 nmol l^{-1} [^{3}H]-RX-821002

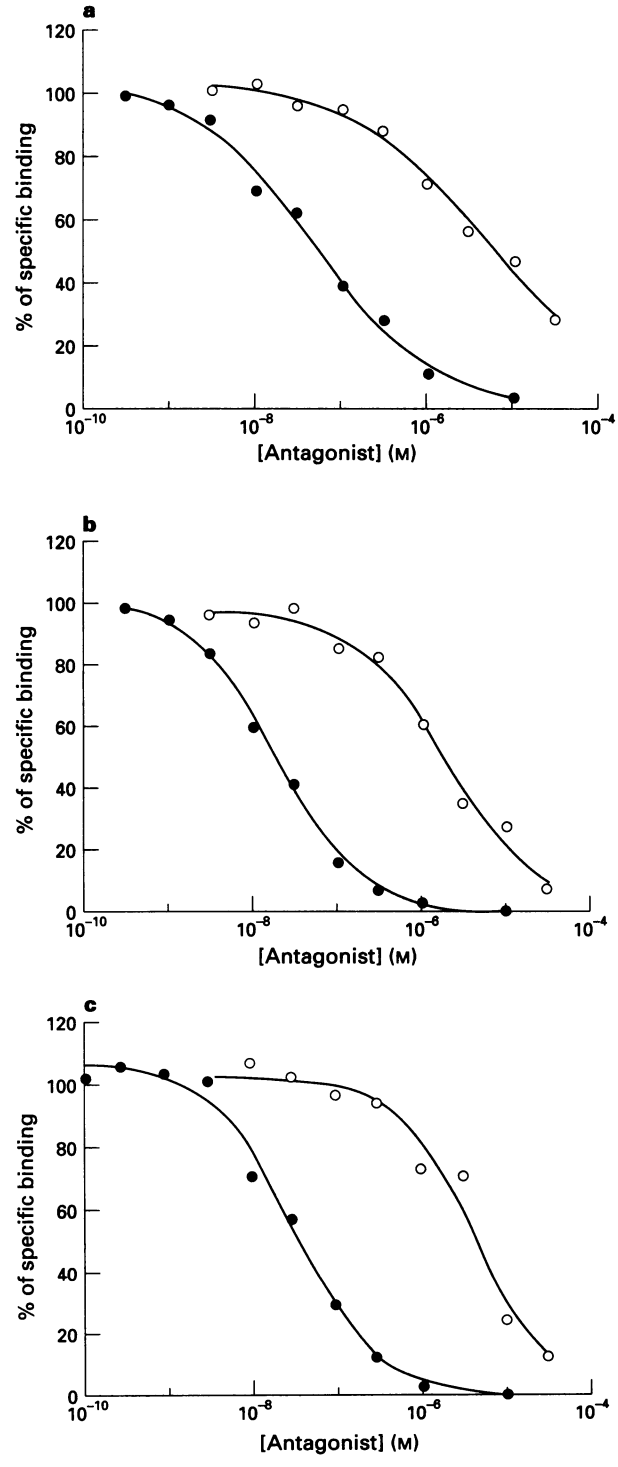

Figure 1 The effect of rauwolscine (●) and corynanthine (○) against the specific binding of (a) 0.5 nmol l^{-1} [^{3}H]-clonidine, (b) 1 nmol l^{-1} [^{3}H]-idazoxan and (c) 0.2 nmol l^{-1} [^{3}H]-RX-821002 to bovine cerebral cortex membranes. Non-specific binding was determined by effect of 10 μmol l^{-1} noradrenaline. The results shown are from duplicate determinations in a single experiment which was repeated on 2 further occasions with the same results.

in the presence and absence of various concentrations of known displacing agents in a final volume of 0.5 ml assay buffer (50 mM Tris HCl; pH 7.4 at 25°C). In addition, the effects of the crude methanolic extracts of bovine brain and bovine lung were also examined against the three selective α_2-adrenoceptor ligands. Non-specific binding was determined in the presence of noradrenaline (10 μmol l^{-1}) and ranged between 5% ([^{3}H]-RX821002) to 15% ([^{3}H]-clonidine) of the total binding. After an incubation period of 60 min at 25°C, bound radioactivity was separated from free by vacuum filtration over Whatman GF/B glass fibre filters using a Brandel cell harvester followed by 2 x 3 ml washes with ice-cold assay buffer. In all experiments the filters were suspended in 4 ml of scintillation cocktail and bound ligand determined by scintillation spectrometry.

Data analysis

Saturation data were analysed with the computer programme InPlot (Graphpad, California, U.S.A.) using a non-linear equation for a rectangular hyperbolic curve. The logarithm of the concentration of either the brain extract, the lung extracts, or the displacing agents, producing 50% inhibition of the radioligand binding (pIC_{50}) was determined by the non-linear least squares method described by DeLean *et al.* (1978) using Kaleidagraph (Synergy Software) on a MacIntosh computer. One unit of CDS is the volume of extract that produces 50% inhibition of 0.5 nmol l^{-1} [^{3}H]-clonidine binding to bovine cerebral cortex membranes in a 1 ml assay (see Atlas & Burstein, 1984a, b), and this was used to calculate the units per wet weight of tissue for each organ. With the exception of the extracts, apparent inhibition constants (K_i) were calculated from the radioligand binding data using the Cheng-Prusoff transformation (Cheng & Prusoff, 1973) and the mean Hill slope (n_H) of the displacement of either [^{3}H]-idazoxan or [^{3}H]-RX-821002 compared with that observed against [^{3}H]-clonidine using Student's unpaired t test. The difference was considered statistically significant if $P<0.05$. All values are shown as the mean ± s.e.mean of n observations.

Drugs

The following drugs and radioligands were used: [^{3}H]-clonidine (2.5 TBq mmol^{-1}, Amersham, UK); [^{3}H] - RX - 821002 (2-(2-methoxy - 1,4 - benzodioxan - 2 - yl) - 2 - imidazoline) 2.11 TBq mmol^{-1}, Amersham, UK); [^{3}H]-idazoxan (1.59 TBq mmol^{-1}, Amersham, UK); (-)-adrenaline bitartrate (Sigma), (-)-noradrenaline bitartrate (Sigma); histamine (Sigma); prazosin hydrochloride (Pfizer), rauwolscine HCl (Roth); UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer), RX-811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline, Reckitt and Coleman), agmatine sulphate (Sigma).

Results

From saturation studies carried out in the presence and absence of noradrenaline (10 μmol l^{-1}) the maximum number of binding sites on bovine cerebral cortex membranes for [^{3}H]-idazoxan (112.3 ± 3.9 fmol mg^{-1} protein, $n=3$) and [^{3}H]-RX-821002 (103.6 ± 9.2 fmol mg^{-1} protein, $n=3$) were significantly greater than that for [^{3}H]-clonidine (62.6 ± 10.7 fmol mg^{-1} protein, $n=3$). Based on the dissociation constant, the rank order of affinity (K_d) for the ligands was [^{3}H]-RX-821002 (0.28 ± 0.07 nM, $n=3$) > [^{3}H]-idazoxan (0.88 ± 0.14 nM, $n=3$) > [^{3}H]-clonidine (1.32 ± 0.49 nM, $n=3$). At these concentrations the non-specific binding varied from 5% ([^{3}H]-RX-821002) to 15% ([^{3}H-clonidine]). As shown in Figure 1 and Table 1 the selective α_2-adrenoceptor antagonist rauwolscine was approximately 100 fold more potent than the selective α_1-adrenoceptor diastereoisomer, corynanthine, against each of the ligands. Also, the pK_i values for the selective α_1-adrenoceptor antagonist, prazosin, against [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002, were less than 6. Taken together, these observations suggest that these [^{3}H]-ligands label prazosin-insensitive, α_2-adrenoceptor binding sites on bovine cerebral cortex membranes.

Table 1 shows the interaction between known agonists

Table 1 pK_i and Hill Slope (n_H) values for various agents against [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes

	[^{3}H]-Clonidine		*[^{3}H]-Idazoxan*		*[^{3}H]-RX-821002*	
	pK_i RP	n_H	pK_i RP	n_H	pK_i RP	n_H
Adrenaline	8.30 ± 0.13 1	-0.69 ± 0.06	7.53 ± 0.17 0.17	-0.47 ± 0.04*	7.16 ± 0.18 0.07	-0.49 ± 0.02
UK-14304	8.56 ± 0.04 1	-0.89 ± 0.05	7.93 ± 0.08 0.23	-0.63 ± 0.02*	7.51 ± 0.01 0.09	-0.55 ± 0.03**
Rauwolscine	7.37 ± 0.05 1	-0.70 ± 0.03	7.81 ± 0.01 2.75	-0.95 ± 0.04**	7.57 ± 0.04 1.58	-0.94 ± 0.07*
Corynanthine	5.16 ± 0.07 1	-0.52 ± 0.03	5.74 ± 0.03 3.8	-0.81 ± 0.03**	5.32 ± 0.06 1.44	-0.80 ± 0.05*
RX-811059	8.73 ± 0.11 1	-0.80 ± 0.06	9.26 ± 0.03 3.36	-1.02 ± 0.07	9.04 ± 0.02 2.04	-1.00 ± 0.09
Prazosin ($n=2$)	5.25 1	-0.95	5.81 3.6	-0.95	5.62 2.30	-1.09
Agmatine	4.77 ± 0.38 1	-0.82 ± 0.05	4.95 ± 0.17 1.51	-0.84 ± 0.06	4.70 ± 0.19 0.85	-0.90 ± 0.05
Histamine	4.61 ± 0.19 1	-0.84 ± 0.09	4.64 ± 0.09 1.07	-0.88 ± 0.07	4.41 ± 0.10 0.63	-0.75 ± 0.08

Except where indicated the values shown represent the mean ± s.e. mean of 3-5 seperate experiments carried out in duplicate. RP is the potency of the displacing agent against each ligand relative to that observed against [^{3}H]-clonidine. *$P<0.05$ and **$P<0.01$ denote that the Hill slope (n_H) for the displacement of either [^{3}H]-idazoxan or [^{3}H]-RX-821002 from bovine cerebral cortex membranes is significantly different (unpaired Student's t test) from that observed for the ligand against [^{3}H]-clonidine.

and antagonists against [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes. Adrenaline, UK-14304, rauwolscine, corynanthine, RX-811059 and prazosin produced concentration-dependent inhibition of binding of all three ^{3}H-ligands. Adrenaline and UK-14304 were approximately 5 and 10 fold less potent against [^{3}H]-idazoxan and [^{3}H]-RX-821002, respectively, than against [^{3}H]-clonidine. Also, the Hill slopes of the interaction were significantly lower against [^{3}H]-idazoxan and [^{3}H]-RX-821002 compared to that observed against [^{3}H]-clonidine (Table 1). In contrast, rauwolscine, corynanthine, RX-811059 and prazosin were approximately 2–3 fold more potent against [^{3}H]-RX-821002 and [^{3}H]-idazoxan binding compared to [^{3}H]-clonidine binding. In addition, the Hill slope was steeper against [^{3}H]-RX-821002 and [^{3}H]-idazoxan compared to that observed against [^{3}H]-clonidine; a difference which was statistically significant for both rauwolscine and corynanthine (Table 1). The qualitative difference between the interaction of an agonist (adrenaline) and an antagonist (RX-811059) with the three ^{3}H-ligands is highlighted in Figure 2.

Agmatine and histamine produced a concentration-dependent displacement of [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes. (Figure 3). As shown in Table 1, the pK_i values, and also the Hill Slope, for agmatine and histamine were independent of the ^{3}H-ligand employed.

The crude methanolic extract from 100 g wt of bovine brain and lung was reconstituted in 10 vol of distilled water. As shown in Figure 4, both extracts produced a concentration-dependent inhibition of [^{3}H]-clonidine binding to bovine cerebral cortex membranes (>90%), with a Hill slope close to unity (see Table 2). Based upon the volume of the extract that produced 50% inhibition of [^{3}H]-clonidine binding (1 unit of CDS, see Table 2), bovine lung contains 3 fold more CDS (17.4 ± 1.7 units g^{-1} wet wt, $n=4$ batches of the extracts) than bovine brain (5.4 ± 0.6 units g^{-1} wet wt, $n=4$ batches of the extract). In contrast to the effect observed against [^{3}H]-clonidine binding, 100 μl ml^{-1} of the lung extract produced only $71.3 \pm 3.0\%$ ($n=4$) and $38.7 \pm 5.2\%$ ($n=4$) inhibition of [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding, respectively. Similarly, the bovine brain extract was not effective against [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding, the inhibition produced by 100 μl ml^{-1} of the extract was $26.5 \pm 4.6\%$ ($n=4$) and $12.3 \pm 1.3\%$ ($n=4$), respectively (Figure 4). As shown in Table 2, both extracts were at least 5 fold more potent against sites labelled by [^{3}H]-clonidine compared to those labelled by [^{3}H]-idazoxan and [^{3}H]-RX-821002.

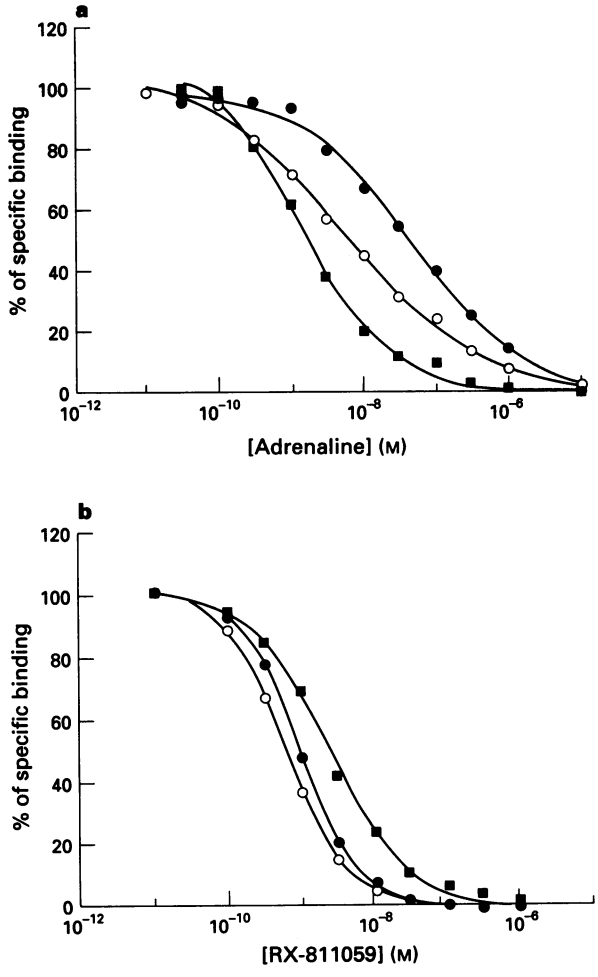

Figure 2 The effect of (a) adrenaline and (b) RX-810059 against the specific binding of 0.5 nmol l^{-1} [^{3}H]-clonidine (■), 1 nmol l^{-1} [^{3}H]-idazoxan (○) and 0.2 nmol l^{-1} [^{3}H]-RX-821002 (●) to bovine cerebral cortex membranes. Non-specific binding was determined by the effect of 10 μmol l^{-1} noradrenaline. The results shown are from duplicate determinations in a single experiment which was repeated on 2 (RX-810059) to 4 (adrenaline) further occasions with similar results.

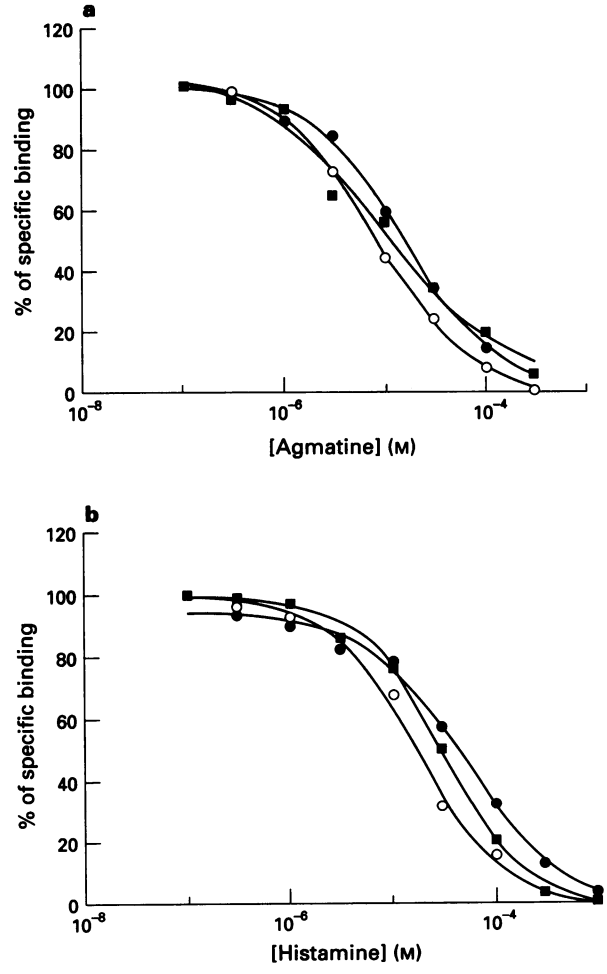

Figure 3 The effect of (a) agmatine and (b) histamine against the specific binding of 0.5 nmol l^{-1} [^{3}H]-clonidine (■), 1 nmol l^{-1} [^{3}H]-idazoxan (○) and 0.2 nmol l^{-1} [^{3}H]-RX-821002 (●) to bovine cerebral cortex membranes. Non-specific binding was determined by the effect of 10 μmol l^{-1} noradrenaline. The results shown are from duplicate determinations in a single experiment which was repeated on 2 (histamine) to 4 (agmatine) further occasions with similar results.

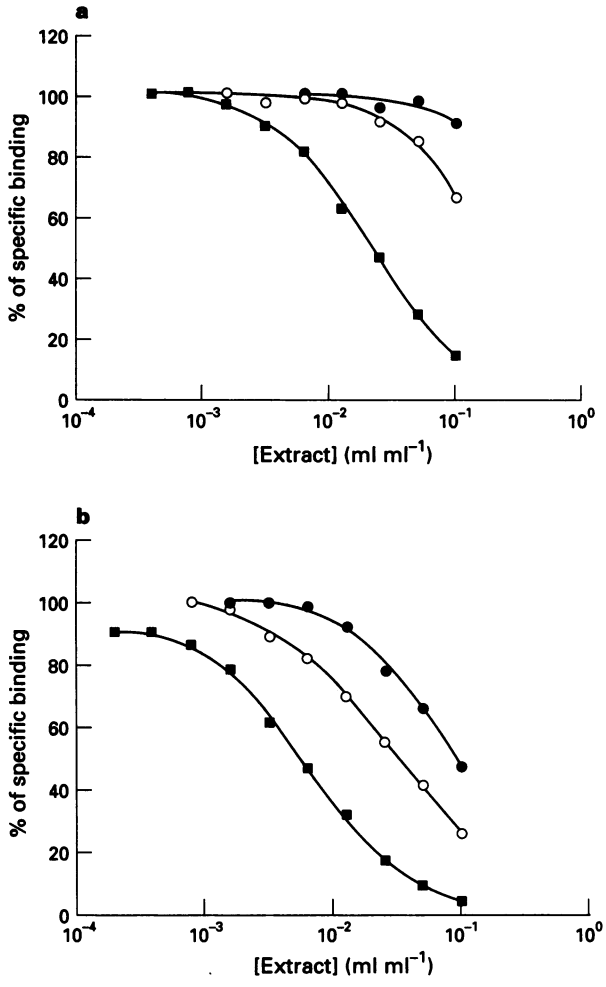

Figure 4 The effect of (a) crude methanolic extracts of bovine brain and (b) crude methanolic extracts of bovine lung against the specific binding of 0.5 nmol l^{-1} [^{3}H]-clonidine (■), 1 nmol l^{-1} [^{3}H]-idazoxan (○) and 0.2 nmol l^{-1} [^{3}H]-RX-821002 (●) to bovine cerebral cortex membranes. Non-specific binding was determined by the effect of 10 μmol l^{-1} noradrenaline. The results shown are from duplicate determinations in a single experiment with the one batch of the lung and brain extract. This was repeated on 3 further occasions, with 3 different batches of the extracts, with similar results. Assays on each batch of the extract were performed in duplicate on three separate occasions. The ordinate scale shows the logarithm of the concentration of the extract (ml) in a 1 ml assay volume.

Discussion

The pharmacological identity of the sites labelled by [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002

The working hypothesis underpinning the present study is that radioligand binding assays, involving the use of a ^{3}H-agonist and a ^{3}H-antagonist, can predict the pharmacological properties of recognized agonists and antagonists (Wilson *et al.*, 1991; MacKinnon *et al.*, 1993). For this to be possible, it is necessary to establish that the radioligands employed identify a pharmacologically homogeneous population of sites. Three observations suggest that [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 label α_2-adrenoceptor binding sites on bovine cerebral cortex membranes that belong to the $\alpha_{2A/D}$ subtype. First, 10 μM noradrenaline displaced 80% to 95% of the total binding of the three ligands, indicating that even for [^{3}H]-idazoxan, non-adrenoceptor, imidazoline binding sites make a minor contribution in this preparation. This contrasts with the rat cerebral cortex, for example, where [^{3}H]-idazoxan, also labels a sizable population of the non-adrenoceptor, imidazoline binding sites (Brown *et al.*, 1990; Hussain *et al.*, 1993; Wallace *et al.*, 1994). Secondly, the selective α_2-adrenoceptor, rauwolscine (Weitzell *et al.*, 1979) caused 100% displacement of the specific binding, and was 100 fold more potent than its diastereoisomer corynanthine, at the sites labelled by each ligand (Starke, 1981). Thirdly, the selective α_1-adrenoceptor antagonist, prazosin exhibited low potency ($pK_i < 6.3$) against each ligand; this makes it unlikely that the sites belong to the B/C (prazosin-sensitive) subgroups of α_2-adrenoceptors (Bylund *et al.*, 1994). Too few agents have been employed in the present study to distinguish conclusively between the A and D subtypes but, as suggested by MacKinnon *et al.* (1994) and Bylund *et al.* (1994), these may simply represent species homologues of the same subtype. If the latter view is correct, then two additional observations lend support to the presence of the D subtype on bovine cerebral cortex membranes. First, the pK_i for rauwolscine (7.57) against [^{3}H]-RX-821002 is consistent with the D subtype (Bylund *et al.*, 1994). Secondly, the α_{2D}-subtype and not the α_{2A}, has been reported on membranes of bovine pineal gland (Simmoneaux *et al.*, 1991).

In an earlier study we reported that the B_{max} for [^{3}H]-clonidine on bovine cerebral cortex membranes was significantly less than that for [^{3}H]-idazoxan, and that the former was more affected by the presence of guanine triphosphate (GTP) (Hussain *et al.*,1993). This, we argued, was consistent with the possibility that [^{3}H]-clonidine preferentially labelled the 'high' affinity state of the α_2-adrenoceptor binding site, while [^{3}H]-idazoxan, an apparently less efficacious partial agonist, was less able to distinguish between the 'low' and 'high' affinity states. Qualitatively similar observations have been made in

Table 2 The effect of crude methanolic extracts of bovine brain and bovine lung against [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes

	[^{3}H]-clonidine (µl of extract-50% inhibition) RP	n_H	*[^{3}H]-idazoxan* (µl of extract-50% inhibition) RP	*[^{3}H]-RX-821002* (µl of extract-50% inhibition) RP
Brain extract	19.4 ± 2.3 1	-1.01 ± 0.11	NP <0.2	NP <0.2
Lung extract	6.2 ± 0.9 1	-1.08 ± 0.1	44.2 ± 7.6 <0.14	NP <0.06.

NP - not possible to determine a value because 100 µl ml^{-1} of the extract failed to cause more than 50% inhibition. RP - relative potency of the extract against each ligand compared to that observed against [^{3}H]-clonidine. Where the maximum concentration (100 µl ml^{-1}) of extract failed to produce 50% inhibition this value has been estimated. The values shown are the mean ± s.e. mean of a single experiment (performed in duplicate) with 4 different batches of the lung and brain extract.

the present study, where the B_{max} values for both [^{3}H]-idazoxan and [^{3}H]-RX-821002 were significantly greater than that obtained for [^{3}H]-clonidine. However, the B_{max} value for [^{3}H]-RX-82100 was not significantly different from that for [^{3}H]-idazoxan but, in a separate study, was unaffected by the addition of 300 μM GTP (unpublished observation). At present we are unable to reconcile, on the one hand, the ability of the GTP to reduce selectively [^{3}H]-idazoxan binding to bovine cerebral cortex membranes (Hussain *et al.*, 1993), with the similar B_{max} values for [^{3}H]-idazoxan and [^{3}H]-RX-821002 in this preparation (present study). However, for the purposes of the interpretation of the results in this study, we suggest that [^{3}H]-clonidine selectively labels the high affinity state of the population of $\alpha_{2(D)}$-adrenoceptors, while [^{3}H]-idazoxan and [^{3}H]-RX-821002, as antagonists, do not discriminate between the high and low affinity states of the binding site. It is noteworthy that qualitatively similar results with [^{125}I]-*p*-aminoclonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 have been reported on the rat cerebral cortex membranes (Wallace *et al.*, 1994).

The value of the radioligand binding assays in predicting agonist or antagonist activity

The three radioligands used to label $\alpha_{2(D)}$-adrenoceptors on bovine cerebral cortex membranes were able to discriminate successfully between known agonists, and antagonists. Adrenaline and UK-14304, exhibited a 5–10 fold greater affinity for the sites labelled by [^{3}H]-clonidine than for those labelled by [^{3}H]-idazoxan and [^{3}H]-RX-821002. On the other hand, the selective α_2-adrenoceptor antagonists rauwolscine and RX-810059 (Mallard *et al.*, 1992) were 2–3 fold more potent against [^{3}H]-idazoxan and [^{3}H]-RX-821002 than against [^{3}H]-clonidine. The predictive value of this model is further underlined by the finding that a similar profile was observed even for antagonists with selectivity for α_1-adrenoceptors (prazosin and corynanthine). These observations are consistent with several earlier findings with platelets (MacKinnon *et al.*, 1993), HT-29 cells (Turner *et al.*, 1985; Bylund *et al.*, 1988; Gleason & Hieble, 1991) and rat cerebral cortex membranes (Wallace *et al.*, 1994), membranes that possess $\alpha_{2A/D}$-adrenoceptor binding sites (see also Wilson *et al.*, 1991). This suggests that the known preference of agonists for the high affinity state of the binding sites can be exploited to identify potential agonists.

The stimulus for the present investigations was our finding that agmatine, a putative clonidine-displacing substance (Li *et al.*, 1994), failed to display detectable biological activity at peripheral and central α_2-adrenoceptors, even though it was able to displace [^{3}H]-clonidine from α_2-adrenoceptor binding sites on both rat and bovine cerebral cortex membranes (Pinthong *et al.*, 1995). Taken together, these observations question the value of employing [^{3}H]-clonidine binding to α_2-adrenoceptors alone as a means of detecting a biologically-active non-catecholamine CDS; as demonstrated by Atlas and coworkers this should be complemented by functional studies to show that this substance has the predicted activity (Diamant & Atlas, 1986; Diamant *et al.*, 1987). Data from this study adds further weight to this view, since agmatine failed to discriminate between [^{3}H]-clonidine and [^{3}H]-RX-821002 binding (Table 1) and, therefore, exhibited a profile unlike that produced by the α_2-adrenoceptor agonists, adrenaline and UK-14304. Furthermore, relative to the affinity exhibited at the sites labelled by [^{3}H]-clonidine, agmatine was less potent against [^{3}H]-idazoxan and [^{3}H]-RX-821002 than might have been expected from the findings with the known antagonists (see Tables 1 and 2). At present we have no satisfactory explanation for the detectable affinity of agmatine for α_2-adrenoceptor binding sites labelled by a ^{3}H-agonist and ^{3}H-antagonists failing to translate into either agonist or antagonist activity in biologically-intact systems. One strategy for examining this paradox may be to examine events intimately linked to receptor activation, e.g. GTPγS binding to membranes (Ito *et al.*, 1994). It is equally possible, however, that agmatine may interact with an allosteric site associated with α_2-adrenoceptors which, under conditions yet to be identified, is of biological significance.

The crude methanolic extracts of bovine lung and brain, previously shown to possess CDS activity (Singh *et al.*, 1995), were significantly more potent against α_2-adrenoceptors labelled by [^{3}H]-clonidine, than against those labelled by [^{3}H]-idazoxan and [^{3}H]-RX-821002. It should be noted, however, that since the IC_{50} values have not been corrected to account for the radioligand concentration, the possibility exists that the estimate of the relative potency (5–15 fold greater affinity for the [^{3}H]-clonidine sites) may have been overestimated. Nonetheless, these observations underline the similar nature of the CDS activity in these extracts, and supports the view that they contain a substance which cannot be accounted for by either agmatine or histamine (Singh *et al.*, 1995), the latter a known contaminant of the crude methanolic extract of bovine lung. Also, the profile exhibited by the crude extracts was remarkably similar to that for the known agonists adrenaline and UK-14304, which raises the possibility that the CDS-activity may possess agonist activity at α_2-adrenoceptors. On a cautionary note, the Hill slope for the extracts against [^{3}H]-clonidine binding to α_2-adrenoceptors was closer to unity than that observed for either of the two known agonists, and this may indicate that the unidentified substance interacts with α_2-adrenoceptors in a novel fashion.

In conclusion, we have demonstrated that α_2-adrenoceptor binding sites on bovine cerebral cortex membranes, when labelled with a ^{3}H-agonist and ^{3}H-antagonists, can successfully discriminate between known agonists and antagonists. Using this model, the putative CDS, agmatine (Li *et al.*, 1994) exhibited a profile qualitatively dissimilar to that observed for either adrenaline or UK-14304, which supports functional studies indicating that agmatine is not an agonist at α_2-adrenoceptors (Pinthong *et al.*, 1995). On the other hand, CDS activity in crude methanolic extracts of bovine brain and lung exhibited a profile consistent with the possibility that it is an agonist at α_2-adrenoceptors and, therefore, not attributable to agmatine. Further purification of the extract, to permit examination of the effect of CDS on functional α_2-adrenoceptors, appears to be warranted.

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Effects of mastoparan upon the late stages of the ACTH secretory pathway of AtT-20 cells

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1 The mouse AtT-20/D16-16 anterior pituitary tumour cell line was used as a model system for the study of the effects of mastoparan upon the late stages of the adrenocorticotrophin (ACTH) secretory pathway.

2 Mastoparan ($10^{-8}-10^{-5}$ M), an activator of heterotrimeric guanosine 5'-triphosphate binding proteins (G-proteins), stimulated ACTH secretion from electrically-permeabilized AtT-20 cells in a concentration-dependent manner in the effective absence of calcium ions with a threshold of 10^{-6} M. Guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) ($10^{-8}-10^{-4}$ M) also stimulated ACTH secretion from electrically-permeabilized AtT-20 cells in a concentration-dependent manner in the effective absence of calcium ions with a threshold of 10^{-6} M. This GTP-γ-S-evoked secretion is consistent with previous studies which demonstrated that a G-protein, termed G_E, mediates calcium evoked ACTH secretion from AtT-20 cells. GTP-γ-S-evoked secretion however was not as great as that obtained in response to mastoparan.

3 Both mastoparan (10^{-5} M) and GTP-γ-S (10^{-4}M) stimulated ACTH secretion from electrically-permeabilized AtT20 cells in a time-dependent manner. A time of 30 min was adopted as the standard incubation period for the study of both mastoparan and GTP-γ-S-stimulated ACTH secretion from permeabilized AtT-20 cells.

4 Mastoparan ($10^{-8}-10^{-5}$ M) stimulated ACTH secretion from permeabilized AtT-20 cells to the same extent in the presence and absence of the protein kinase C (PKC) inhibitor, chelerythrine chloride (10^{-5} M).

5 Mastoparan ($10^{-8}-10^{-5}$ M)-stimulated ACTH secretion from permeabilized AtT-20 cells was significantly reduced in the presence of guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S, 10^{-4} M).

6 The mastoparan analogue, Mas-7 ($10^{-8}-10^{-5}$ M) stimulated ACTH secretion from permeabilized AtT-20 cells to a greater extent than mastoparan ($10^{-8}-10^{-5}$ M) however, the mastoparan analogue Mas-17 ($10^{-8}-10^{-5}$ M) had no effect upon ACTH secretion from permeabilized AtT-20 cells.

7 Mastoparan ($10^{-8}-10^{-5}$ M) stimulated ACTH secretion from permeabilized AtT-20 cells in the presence and absence of ATP, normally present in the standard permeabilization medium at a concentration of 5 mM. Mastoparan ($10^{-8}-10^{-5}$ M)-stimulated ACTH secretion as well as control secretion was reduced when ATP was omitted.

8 The results of the present study demonstrate that mastoparan stimulated ACTH secretion from permeabilized AtT-20 cells and displayed characteristics consistent with calcium ion- and GTP-γ-S-stimulated ACTH secretion from permeabilized AtT-20 cells. This suggests that in permeabilized AtT-20 cells, mastoparan directly activates G_E and that this G-protein may be a heterotrimeric G-protein. This study also suggests mastoparan may be a useful alternative to GTP-γ-S as a means of directly activating G_E.

Keywords: Heterotrimeric G-protein; mastoparan; anterior pituitary cell line; ACTH

Introduction

Increasing the concentration of free calcium ions in the cytosol of secretory cells has long been established as a trigger to exocytosis (Douglas, 1968) with much supporting evidence emerging from the use of a variety of permeabilized cell types in which the cytosolic free calcium ion concentration can be controlled (for review see Knight & Scrutton, 1986). One such cell type used in permeabilization studies is the adrenocorticotrophin (ACTH)-secreting mouse AtT-20/D16-16 anterior pituitary tumour cell line, a model system for the study of the normal corticotroph (Luini & DeMatteis, 1988; 1990; Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994). Permeabilized AtT-20 cells release ACTH in response to increasing cytosolic free calcium ion levels (Luini & DeMatteis, 1988; 1990; Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994); however, the mechanism by which calcium ions stimulate secretion from AtT-20 cells is not fully understood. Calcium ions have been shown to mediate their effects upon hormone secretion via stimulation of a guanosine 5'-triphosphate binding protein (G-protein) in a variety of permeabilized cell systems (for review see Gomperts, 1990). This has been demonstrated in permeabilized AtT-20 cells by the ability of the non-hydrolysable GTP analogue guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) to stimulate ACTH secretion in the effective absence of calcium ions (Luini & DeMatteis, 1988; 1990; Guild, 1991; McFerran & Guild, 1994). This G-protein mediating calcium ion-stimulated hormone secretion has been termed G_E by Gomperts (1990).

Two families of G-protein have been proposed as candidates for G_E. These are heterotrimeric G-proteins composed of three distinct subunits termed α, β and γ (for reviews see Gilman, 1987; Taylor, 1990) and small molecular weight monomeric Ras-like G-proteins (for review see Hall, 1990). Both heterotrimeric G-proteins (Toutant *et al.*, 1987) and monomeric G-proteins (Burgoyne & Morgan, 1989; Darchen *et al.*, 1990; Fischer von Mollard, 1991) have been located on intracellular membranes and implicated in vesicular traffic. In AtT-20 cells monomeric Ras-like proteins, in particular rab 3, have been shown to play a role in localization, sequestration

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and storage of secretory vesicles (Ngsee *et al.*, 1993). In addition an inhibitory form of G_E (G_{Ei}), which mediates somatostatin inhibition of ACTH secretion, has been identified in AtT-20 cells and is thought to be heterotrimeric (Luini & DeMatteis, 1988; 1990). The object of this study therefore was to characterize further the stimulatory form of G_E (G_{Es}) which mediates calcium ion-stimulated ACTH secretion from AtT-20 cells.

The G-protein which mediates calcium ion-stimulated ACTH secretion from AtT-20 cells was further investigated by use of mastoparan and related peptides. Mastoparan is an amphiphilic tetradecapeptide originally isolated from wasp venom with a wide variety of actions some of which have been attributed to the activation of heterotrimeric G-proteins by a mechanism similar to that of agonist-bound receptors (Higashijima *et al.*, 1988; 1990; Weingarten *et al.*, 1990). This study demonstrates that mastoparan is able to stimulate secretion from permeabilized AtT-20 cells independently of changes in the cytosolic calcium ion level and protein kinase C (PKC) and that this action is mediated, at least partly, by a G-protein. Mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells displayed characteristics consistent with previous studies investigating calcium ion- and GTP-γ-S-stimulated ACTH secretion from permeabilized AtT-20 cells which originally established the existence of G_E in AtT-20 cells (Guild, 1991; McFerran & Guild, 1994). The results of this study suggest that, in AtT-20 cells, mastopaparan is acting by a direct action upon G_E and are consistent with G_E present in this cell line being a heterotrimeric G-protein. This study also suggests that mastoparan may be a useful alternative to GTP-γ-S as a means of directly activating G_E.

Methods

Culture of AtT-20 cells

Cells of the mouse AtT-20/D16-16 anterior pituitary tumour cell line were grown and subcultured in Dulbecco's modified Eagle's medium (DMEM) (4500 mg glucose l^{-1}) supplemented with 10% (v/v) foetal calf serum as previously described (Reisine, 1984). Cells were plated in 75 cm^2 flasks (Nunc, Gibco, U.K.) at an initial density of 2×10^6 cells/flask and were used upon reaching 80–90% confluency.

Preparation of AtT-20 cells

The culture medium was removed, cells adhering to the substrate were liberated with trypsin (0.05% w/v)/EDTA (1 mM). The cells were washed twice by centrifugation (200 *g*, 5 min)/resuspension in a balanced salt solution of the following composition (mM): NaCl 145, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 0.5, glucose 5.6, HEPES 5, sodium ascorbate 0.5, bovine serum albumin (BSA) 0.1% (w/v), pH 7.4. After washing, the cells were suspended at a density of 10^6 cells ml^{-1} in this buffer and incubated for a further 30 min at 37°C. The cell suspension was then centrifuged (200 *g*, 5 min) and the cell pellet washed twice by resuspension/centrifugation (200 *g*, 5 min) in the standard permeabilization buffer of the following composition (mM): potassium glutamate 129, PIPES (potassium salt) 20, glucose 5, ATP 5, EGTA 5, $MgCl_2$ 1, BSA 0.1% (w/v), pH 6.6. The cells were finally resuspended in this buffer at a density of 4×10^7 cells ml^{-1} and electrically permeabilized by subjection to intense electric fields of brief duration (Knight & Baker, 1982). Optimum permeabilization parameters were determined as previously described (Guild, 1991) and were found to be 10 discharges each of 3000 V cm^{-1}. These parameters were adopted in this study.

Measurement of stimulated ACTH secretion from permeabilized AtT-20 cells

Permeabilized cells were suspended at a density of 10^5 cells ml^{-1} in calcium/EGTA buffers designed to establish and maintain the desired cytosolic free calcium concentration as previously described (Guild, 1991). The standard permeabilization medium was designed to maintain a cytosolic free calcium level of 10^{-9} M (effectively zero calcium). All experiments involving permeabilized cells were carried out in this standard permeabilization medium. These incubations were supplemented, as indicated in the figure legends, with the G-protein activators guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S), mastoparan and mastoparan analogues. The stable guanosine 5'-diphosphate (GDP) analogue guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S), used as a means of inhibiting G-proteins by its ability to compete with GTP, was also investigated. At this point zero time incubations were centrifuged (200 *g*, 5 min) and samples of the supernatant stored for subsequent measurement of ACTH content. The cell suspensions were incubated at 37°C for 30 min (with the exception of time course experiments) at which point incubations were terminated by centrifugation (200 *g*, 5 min, 4°C) and samples of the supernatant stored for subsequent measurement of ACTH content. In each experiment six samples were run for each condition and the ACTH content measured by radioimmunoassay.

The effect of protein kinase C inhibition upon mastoparan-evoked ACTH secretion from permeabilized AtT-20 cells

GTP-γ-S is able to stimulate ACTH secretion from permeabilized AtT-20 cells independently of protein kinase C (PKC). The PKC inhibitor, chelerythrine chloride (Herbert *et al.*, 1990), was used to investigate whether mastoparan-evoked ACTH secretion is similarly independent of PKC. Mastoparan-evoked ACTH secretion was measured (as described above) in the presence and absence of chelerythrine chloride (10^{-5} M). At this concentration, chelerythrine chloride selectively inhibits PKC (Herbert *et al.*, 1990) and has been shown to be effective in inhibiting PKC-stimulated ACTH secretion from permeabilized AtT-20 cells (McFerran & Guild, 1994). In addition, the effect of omitting ATP from the permeabilization media upon mastoparan-stimulated ACTH secretion was also investigated.

Radioimmunoassay

ACTH secretion was measured by radioimmunoassay (RIA) based upon the previously described method of Antoni *et al.* (1983). Dilutions of sample and antiserum were made in RIA buffer of the following composition: sodium phosphate 0.05M, polyethylene glycol 8000 6% (w/v), BSA, 0.1% (w/v), Triton-X 100 0.1% (v/v), EDTA 2.5 mM, pH 7.4. The incubation mixture contained a total volume of 300 μl RIA buffer, consisting of 100 μl of human ACTH 1-39 standard or unknown sample, 100 μl antiserum (rabbit antihuman-ACTH) at a final dilution of 1:32000 in RIA buffer, and approximately 10000 c.p.m. [^{125}I]-ACTH in 100 μl RIA buffer. [^{125}I]-ACTH was produced using the iodogen reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) which was first described as a reagent for iodination by Fraker & Speck (1978). Samples were then incubated for 16–24 h at 4°C. Donkey anti-rabbit IgG (100 μl), at a dilution of 1:10 in RIA buffer containing 0.5% normal rabbit serum, was added to each tube and samples were further incubated at room temperature for 3 h; 1 ml 3% (w/v) polyethylene glycol was added to each tube and samples were centrifuged at 4°C, 3200 *g* for 30 min. The supernatants were then decanted and the precipitates counted by means of a gamma counter. The amount of ACTH released during experimental procedures was expressed as the amount present at the end of the specified incubation period less the amount present at zero time (with the exception of time course experiments).

Statistics

In each experiment six determinations for each experimental condition were made and each experiment was repeated three

times on different days. ACTH secretion is expressed as the mean ± s.e. mean from these three experiments. Statistical significance was determined by use of ANOVA tests with Scheffe's *F*-test *post hoc* analysis. The statistical significance of a particular treatment was determined by a two-way ANOVA test. In both cases a *P* value less than or equal to 0.05 was considered significant.

Materials

The following substances (with their sources) were used: ATP, bovine serum albumin (fraction V), mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2) from Sigma, U.K.; guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) and guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) from Boehringer Mannheim, U.K.; Mas-7 and Mas-17 from Peninsula Laboratories; chelerythrine chloride from Calbiochem-Novabiochem, U.K.; DMEM, foetal calf serum and trypsin EDTA from GIBCO, UK; human ACTH antiserum and human ACTH standards were a gift of the National Hormone and Pituitary programme, Baltimore, MD, U.S.A.; anti-rabbit IgG was a gift of the Scottish antibody production unit, Carluke, Lanarkshire, U.K.; iodogen iodination reagent from Pierce & Warriner. All other chemicals were of Analar grade and readily commercially available.

Results

The effects of mastoparan and GTP-γ-S upon ACTH secretion from permeabilized AtT-20 cells

GTP-γ-S (10^{-6}–10^{-4} M) significantly stimulated ACTH secretion from electrically-permeabilized AtT-20 cells, in the effective absence of calcium (free calcium concentration of 10^{-9} M), in a concentration-dependent manner with a threshold of 10^{-6}M (Figure 1) but lower concentrations (10^{-8}–10^{-7} M) were ineffective in this regard as previously reported (Guild, 1991; McFerran & Guild, 1994). Mastoparan (10^{-6}–10^{-5} M) also stimulated ACTH secretion, in the effective absence of calcium (free calcium concentration of 10^{-9} M), in a concentration-dependent manner with a threshold of 10^{-6} M but lower concentrations (10^{-8}–10^{-7} M) were ineffective in this regard (Figure 1). ACTH secretion evoked by mastoparan was significantly ($P<0.001$) greater than that evoked by GTP-γ-S.

Mastoparan (10^{-5} M) (Figure 2a) significantly ($P<0.001$) stimulated ACTH secretion from permeabilized AtT-20 cells in

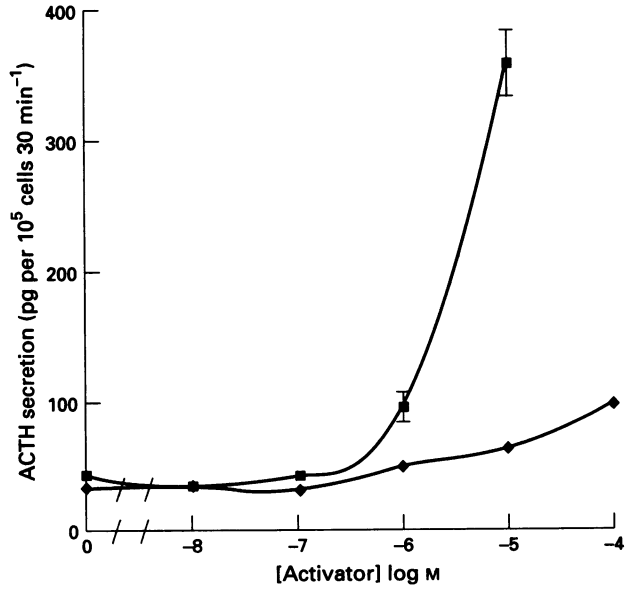

Figure 1 Effect of mastoparan (■) and GTP-γ-S (◆) on adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of mastoparan and GTP-γ-S and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

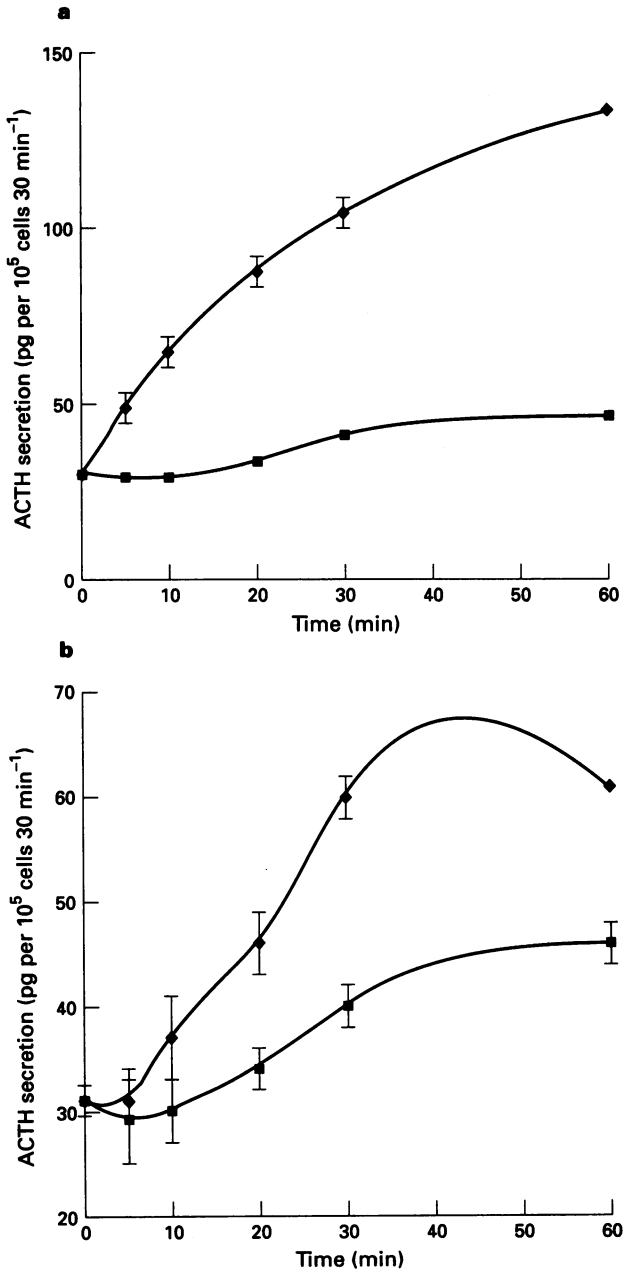

Figure 2 Time course of adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. (a) Permeabilized cells were incubated for the indicated time periods in standard permeabilization medium in the presence (◆) or absence (■) of 10^{-5} M mastoparan and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicate that they lie within the symbol. (b) Permeabilized cells were incubated in standard permeabilization medium for the indicated time periods in the presence (◆) or absence (■) of 10^{-4}M GTP-γ-S and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

the effective absence of calcium in a time-dependent manner. Secretion in response to mastoparan (10^{-5} M) after 30 min was 104 ± 6 pg per 10^5cells compared to a control secretion of 40 ± 3 pg per 10^5 cells. GTP-γ-S (10^{-4} M) (Figure 2b) also significantly ($P<0.01$) stimulated ACTH secretion from permeabilized AtT-20 cells in the effective absence of calcium in a time-dependant manner. Secretion to GTP-γ-S (10^{-4} M) after 30 min was 60 ± 2 pg per 10^5 cells compared to a control secretion of 40 ± 2 pg per 10^5 cells. In both cases 30 min was chosen as the standard incubation period for subsequent experiments.

Characterization of mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells

Mastoparan (10^{-5} M) significantly stimulated ACTH secretion from permeabilized AtT-20 cells in both the presence and absence of the stable GDP analogue GDP-β-S (10^{-4} M). However, GDP-β-S significantly ($P>0.001$) reduced mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells. Secretion evoked by 10^{-5} M mastoparan was reduced from 340 ± 9 pg per 10^5 cells to 211 ± 23 pg per 10^5 cells (Figure 3). GDP-β-S at a higher concentration of 10^{-3} M did not reduce ACTH secretion evoked by mastoparan 10^{-5} M to a greater extent than 10^{-4} M GDP-β-S (data not shown) indicating that this was the maximal inhibition obtained by the use of this guanine nucleotide analogue.

Chelerythrine chloride (10^{-5} M), a potent protein kinase C inhibitor, had no significant effect upon control secretion or mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells (data not shown). ACTH secretion in response to 10^{-5} M mastoparan was 321 ± 11 pg per 10^5 cells in the absence of chelerythrine chloride and 307 ± 22 pg per 10^5 cells in the presence of chelerythrine chloride.

Mastoparan significantly stimulated ACTH secretion from permeabilized AtT-20 cells in both the presence and absence of ATP normally present in the standard permeabilization medium at a concentration of 5 mM (Figure 4). Omitting ATP from the standard permeabilization medium however significantly ($P<0.01$) reduced secretion in response to mastoparan. Secretion obtained in the absence of mastoparan was reduced from 31 ± 3 pg per 10^5 cells to 13 pg per 10^5 cells. ACTH secretion in response to 10^{-5} M mastoparan was reduced from 274 ± 13 pg per 10^5 cells in the presence of ATP to 128 ± 11 pg per 10^5 cells in the absence of ATP (Figure 4).

The effects of mastoparan analogues, Mas-7 and Mas-17, upon ACTH secretion from permeabilized AtT-20 cells

The effects of two analogues of mastoparan upon ACTH secretion from permeabilized AtT-20 cells were investigated. The mastoparan analogue Mas-7 has been reported to be a highly active G-protein activator whereas the mastoparan analogue Mas-17 is unable to activate G-proteins (Higashijima *et al.*, 1990). Mas-7 (10^{-7} M – 10^{-5} M) stimulated ACTH secretion, in the effective absence of calcium (free calcium concentration of 10^{-9} M), in a concentration-dependent manner with a threshold of 10^{-7} M (Figure 5) but lower concentrations (10^{-8} M) were ineffective in this regard. ACTH secretion in response to Mas-7 (10^{-5} M) was 430 ± 13 pg per 10^5 cells compared to 291 ± 11 pg per 10^5 cells obtained in response to mastoparan (10^{-5} M). In contrast Mas-17 (10^{-8} M – 10^{-5} M) was unable to stimulate ACTH secretion from permeabilized AtT-20 cells (Figure 5). No significant difference was observed between control secretion and secretion in response to any concentration of Mas-17.

Discussion

The aim of this study was to determine whether mastoparan is able to evoke ACTH secretion from AtT-20 cells by an action at a late stage in the secretory pathway distal to changes in cytosolic calcium ion levels. Electrical permeabilization provided a means of directly manipulating the intracellular environment and studying the effects of mastoparan at this late stage in the secretory pathway. Electrical permeabilization has

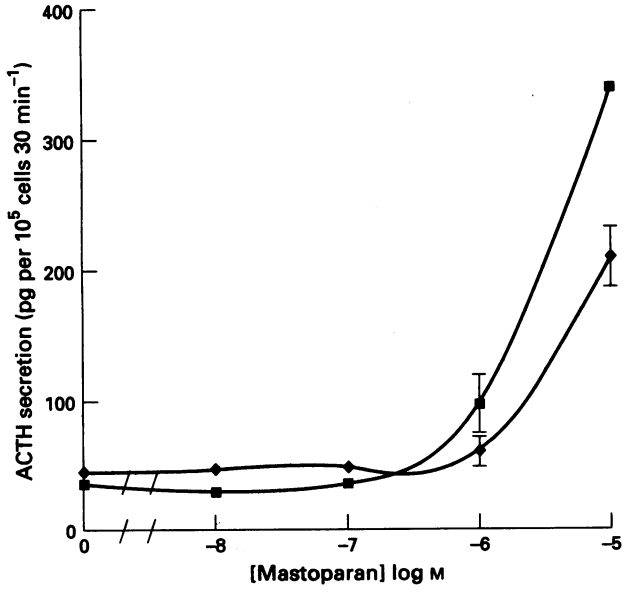

Figure 3 Effect of GDP-β-S upon mastoparan-evoked adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of mastoparan in the absence (■) or presence (◆) of 10^{-4}M GDP-β-S and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

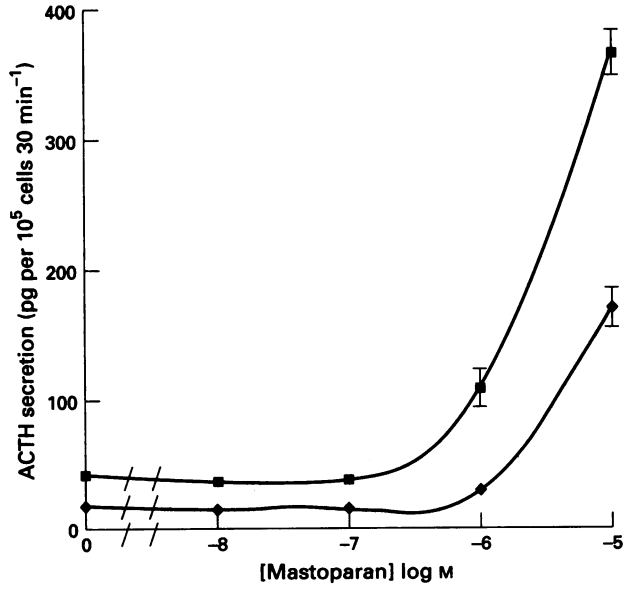

Figure 4 Mastoparan-evoked adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells in the presence and absence of ATP. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of mastoparan in the absence (◆) or presence (■) of 5 mM ATP and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

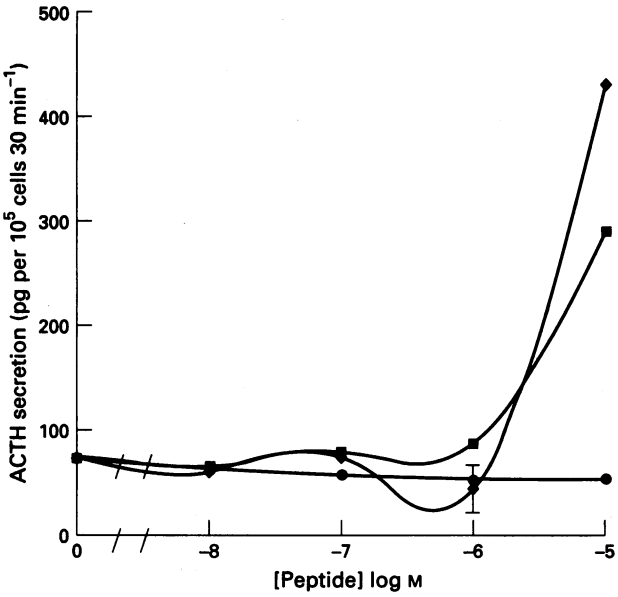

Figure 5 Effect of mastoparan (■), Mas-7 (◆) and Mas-17 (●) on adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of peptide and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

previously been used to gain access to the cytosol of AtT-20 cells without impairing the ability of these cells to undergo exocytosis (Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994). Mastoparan stimulated ACTH secretion from permeabilized AtT-20 cells in a concentration-dependent manner in the effective absence of free calcium ions which are themselves able to stimulate ACTH secretion from electrically-permeabilized AtT-20 cells (Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994). Any changes in free calcium ion concentrations induced by mastoparan would have been buffered by the calcium/EGTA buffers designed to establish and maintain the required cytosolic free calcium ion concentration which in the case of this study was 10^{-9} M (effectively zero); therefore mastoparan evoked ACTH secretion from permeabilized AtT-20 cells is calcium-independent.

Adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA) is also known to stimulate ACTH secretion from permeabilized AtT-20 cells; however, this evoked secretion does not occur in the absence of either calcium ions or guanine nucleotides (Guild, 1991). Since mastoparan stimulated secretion in the absence of guanine nucleotides and the effective absence of calcium ions it is probable that activation of PKA by means of cyclic AMP generation does not contribute to these actions of mastoparan.

In contrast to cyclic AMP, phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, has been shown to stimulate ACTH secretion from permeabilized AtT-20 cells independently of calcium ions and guanine nucleotides (McFerran & Guild, 1994). The possibility that mastoparan was acting via PKC activation was therefore investigated. Mastoparan was able to stimulate ACTH secretion from permeabilized AtT-20 cells to the same extent in the presence or absence of chelerythrine chloride, a potent inhibitor of PKC (Herbert *et al.*, 1990). Chelerythrine chloride, at the concentration used in this study, has been shown to be an effective PKC inhibitor in AtT-20 cells by completely inhibiting PMA-evoked ACTH secretion from permeabilized AtT-20 cells (McFerran & Guild, 1994). Mastoparan is therefore able to stimulate ACTH secretion from AtT-20 cells independently of PKC.

Mastoparan stimulates secretion from a number of cell types including histamine from mast cells (Argiolas & Pisano, 1984; Mousli *et al.*, 1989; Bueb *et al.*, 1990) (after which the peptide is named), catecholamines from chromaffin cells (Kuroda *et al.*, 1980), insulin from pancreatic islets (Komatsu *et al.*, 1992; Yokokawa *et al.*, 1989) and the RINm5F β-cell line (Komatsu *et al.*, 1993), prolactin from anterior pituitary lactotrophs (Kurihara *et al.*, 1986), 5-hydroxytryptamine from platelets (Ozaki *et al.*, 1990) and surfactant from pulmonary alveolar cells (Joyce-Brady *et al.*, 1991). Some of these actions of mastoparan have recently been attributed to the activation of heterotrimeric G-proteins by the ability of this protein to form a highly structured α-helix in the phospholipid membrane which resembles the intracellular loops of G-protein-coupled receptors and as a result is able to activate heterotrimeric G-proteins in a similar fashion to that of agonist-bound receptors (Higashijima *et al.*, 1988; 1990; Weingarten *et al.*, 1990).

However, mastoparan has been reported to have a number of actions which may not be mediated by G-proteins. These actions include; non-specific cell lysis in chromaffin cells (Wilson, 1989); binding to calmodulin (Malenick & Anderson, 1983); direct activation of phospholipases A_2 (Argiolas & Pisano, 1983); and C (Wallace & Carter, 1989); stimulation of nucleoside diphosphate kinase (Kikkawa *et al.*, 1992); inhibition of PKC, Ca/CaM kinase II, Na-K ATPase and the Na pump (Raynor *et al.*, 1991). Some of these actions will not be relevant in the permeabilized cell system used here where changes in the cytosolic free calcium concentration, calmodulin activity, membrane-bound ion channels and pumps are circumvented. It is apparent from this diversity of action that great caution must be exerted when interpreting the sites and mechanism of action of mastoparan in any secretory system.

It was important, therefore, to establish the degree to which the effects of mastoparan in AtT-20 cells were due to G-protein activation as opposed to a non G-protein-mediated event. The stable GDP analogue GDP-β-S, which inhibits GTP activation of G-proteins by means of competitive antagonism, partially inhibited (50%) mastoparan-evoked ACTH secretion from permeabilized AtT-20 cells. Mastoparan stimulated ACTH secretion from AtT-20 cells is therefore mediated, at least partly, by a G-protein. GDP-β-S was used to assess G-protein contribution to the actions of mastoparan in pancreatic β-cells where the results (showing a similar degree of attenuation of mastoparan's actions by GDP-β-S to those seen here) indicate that mastoparan stimulates insulin secretion by a mechanism that is independent of changes in cytosolic calcium ions or PKC activation and is dependent, at least partly, upon activation of a G-protein at a late stage in the secretory pathway (Jones *et al.*, 1993). The results of the present study are consistent with previous studies showing that calcium ion-evoked ACTH secretion from permeabilized AtT-20 cells is also inhibited by GDP-β-S (Guild, 1991). In addition Mas-17, an analogue of mastoparan unable to activate G-proteins (Higashijima *et al.*, 1990), was unable to stimulate ACTH secretion from permeabilized AtT-20 cells. This is in contrast to the mastoparan analogue Mas-7, a highly active G-proteins activator (Higashijima *et al.*, 1990), which stimulated ACTH secretion to an even greater extent than mastoparan. This again suggests that the ability of mastoparan to stimulate ACTH secretion from permeabilized AtT-20 cells independently of changes in calcium and PKC is, at least partly, due to a direct activation of a G-protein. It is not possible at this stage to identify the mechanism(s) underlying the apparent G-protein-independent actions of mastoparan in these cells.

This study is consistent with previous studies using the same system in which guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) (a non hydrolysable GTP analogue) similarly stimulated ACTH secretion independently of calcium and PKC by activation of G_E (Luini & DeMatteis, 1988; 1990; Guild, 1991; McFerran & Guild, 1994). It can therefore be concluded that mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells is also partly mediated by G_E. Mastoparan has been postulated to activate G_E directly in a number of other

secretory cells including pancreatic β-cells (Jones *et al.*, 1993), mast cells (Aridor *et al.*, 1990) and platelets (Wheeler-Jones *et al.*, 1992). In addition the ability of mastoparan to stimulate ACTH secretion from permeabilized AtT-20 cells may suggest that this G-protein may belong to the heterotrimeric family of G-proteins.

Both small molecular G-proteins and heterotrimeric G-proteins are present on intracellular organelles and are thought to play an important role in intracellular membrane trafficking (for review see Pfeffer, 1992). Small molecular weight G-proteins participating in membrane traffic are postulated to cycle between a GTP and a GDP bound state (for review see Pfeffer, 1992) therefore the non-hydrolysable GTP analogue, GTP-γ-S, would arrest this cycle in the GTP bound conformation and result in an inhibition of this process. In contrast heterotrimeric G-proteins upon binding GTP-γ-S become persistently activated (for reviews see Gilman, 1987; Taylor, 1990). This and other reports from this laboratory (Guild, 1991; McFerran & Guild, 1994) demonstrate that GTP-γ-S is able to stimulate ACTH secretion from AtT-20 cells and is thought to do so by a direct action upon G_E. This evidence is therefore consistent with G_E belonging to the heterotrimeric and not the small molecular weight family of G-proteins.

Mastoparan-evoked ACTH secretion from permeabilized AtT-20 cells was significantly, but not completely, reduced when ATP was omitted from the permeabilization medium. This is again consistent with calcium ion-evoked ACTH secretion from permeabilized AtT-20 cells which displayed a similar ATP-dependency (Guild, 1991) suggesting that calcium and mastoparan are acting through similar mechanisms. One possible explanation for this ATP-dependency is that ATP, via conversion by the ubiquitous enzyme nucleoside diphosphate kinase, provides a source of GTP which is otherwise absent from the permeabilization medium (Gomperts, 1990) and as a result mastoparan or calcium can activate G_E. However, it should be noted that there remained a significant mastoparan-stimulated ACTH secretion in the absence of ATP. This may be due to mastoparan-induced activation of nucleoside diphosphate kinase (Kikkawa *et al.*, 1992) and subsequent generation of GTP from residual cellular ATP to permit G_E-evoked ACTH secretion or may be due to an action completely independent of G_E.

Mastoparan evoked ACTH secretion from permeabilized AtT-20 cells with characteristics consistent with previous studies investigating calcium ion- and GTP-γ-S-evoked ACTH secretion from the same system (Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994). The results of this study therefore confirm G_E is present in AtT-20 cells and are also consistent with G_E belonging to the heterotrimeric family of G-proteins. Therefore mastoparan may provide a useful alternative to GTP-γ-S as a means of directly activating G_E, with the advantages that it appears to stimulate secretion to a greater extent and may be more selective for G_E than GTP-γ-S.

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Increase of noradrenaline release in the hypothalamus of freely moving rat by postsynaptic 5-hydroxytryptamine$_{1A}$ receptor activation

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1 5-Hydroxytryptamine (5-HT) plays a role in the regulation of noradrenergic neurones in the brain, but the precise mechanism of regulation of noradrenaline (NA) release by 5-HT$_{1A}$ receptors has not been defined. The present study describes the effect of a highly potent and selective 5-HT$_{1A}$ receptor agonist, 5-{3-[[(2S)-1,4-benzodioxan-2-ylmethyl]amino]propoxy}-1,3-benzodioxole HCl (MKC-242), on NA release in the hypothalamus using microdialysis in the freely moving rat.

2 Subcutaneous injection of MKC-242 (0.5 mg kg^{-1}) increased extracellular levels of NA and its metabolite, 3-methoxy-4-hydroxyphenylglycol, in the hypothalamus and hippocampus.

3 The 5-HT$_{1A}$ receptor agonists, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) (0.2 mg kg^{-1}) and buspirone (3 mg kg^{-1}) mimicked the effect of MKC-242 in increasing NA release in the hypothalamus.

4 The effects of MKC-242 and 8-OH-DPAT in the hypothalamus were antagonized by pretreatment with WAY100135 (10 mg kg^{-1}), a silent 5-HT$_{1A}$ receptor antagonist.

5 Local administration of 8-OH-DPAT (10-100 μM), citalopram (1 μM), a 5-HT reuptake inhibitor, and MDL72222 (10 μM), a 5-HT$_3$ receptor antagonist, into the hypothalamus, had no effect on NA release.

6 Intracerebroventricular injection with 5,7-dihydroxytryptamine caused a marked reduction in brain 5-HT content, but the treatment affected neither basal NA levels nor the MKC-242-induced increase in NA release.

7 The effect of MKC-242 in increasing NA release was not attenuated by repeated treatment with the drug (0.5 mg kg^{-1}, once a day for 2 weeks).

8 The present results suggest that activation of postsynaptic 5-HT$_{1A}$ receptors increases NA release in the hypothalamus.

Keywords: 5-Hydroxytryptamine (5-HT); 5-HT$_{1A}$ receptors; postsynaptic; MKC-242; noradrenaline (NA) release; microdialysis

Introduction

Neuroanatomical (Pickel *et al.*, 1977; Steinbusch, 1981) and biochemical (Crespi *et al.*, 1980; McRae-Degueurce *et al.*, 1985; Reader *et al.*, 1986; Tian *et al.*, 1993) studies indicate that noradrenergic neurones are functionally connected with 5-hydroxytryptaminergic neurones in the brain. The regulation of noradrenergic neurones by 5-hydroxytryptamine (5-HT) may be mediated by an activation of specific 5-HT receptor subtypes such as 5-HT$_{1A}$ (Fuller & Perry, 1989; Done & Sharp, 1994), 5-HT$_{1B}$ (Clement *et al.*, 1992), 5-HT$_{1C}$ (Blandina *et al.*, 1991), 5-HT$_2$ (Rasmussen & Aghajanian, 1986; Gorea & Ardrien, 1988; Done & Sharp, 1992) and 5-HT$_3$ (Blandina *et al.*, 1991). The involvement of 5-HT$_{1A}$ receptors in this regulation is considered to be responsible for an antidepressant-like effect of 5-HT$_{1A}$ receptor agonists, in view of the previous attempts to explain the effects of antidepressant treatments in terms of changes in function of brain NA. However, the possibility remains that 5-HT$_{1A}$ agonists as indicated in previous studies (Fuller & Perry, 1989; Clement *et al.*, 1992; Tian *et al.*, 1993; Done & Sharp, 1994) activate noradrenergic neurones by directly blocking α_2-adrenoceptors, since 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (Crist & Superenant, 1987) and the metabolite of azapirone, 1-(2-pyrimidinyl)-piperazine, (Bianchi *et al.*, 1988; Gobbi *et al.*, 1990) have α_2-adrenoceptor antagonist properties. Furthermore, it is not known whether the regulation of noradrenergic neurones is mediated by presynaptic or postsynaptic 5-HT$_{1A}$ receptors. In this paper, we examined the effect of 5-{3-[[(2S)-1,4-benzodioxan-2-ylmethyl]amino]propoxy}-1,3-benzodioxole HCl (MKC-242), a novel 5-HT$_{1A}$ receptor agonist with anticonflict and antidepressant-like effects (Egawa *et al.*, 1993), on NA release in the hypothalamus of the freely moving rat. A preliminary account of these findings was presented to the Japanese Pharmacological Society (Suzuki *et al.*, 1994).

Methods

Animals and drugs

Male Wistar rats were maintained under controlled environmental conditions (22±1°C; 12-12 h light-dark cycle, lights on at 08 h 00 min food and water *ad libitum*) for at least 1 week before being used for the experiments during which time they were accustomed to being handled.

MKC-242, citalopram HBr and WAY100135 (N-tert-butyl-3-(4-(2-methoxyphenyl)piperazin-1-yl)-2-phenyl-propariamide) were gifts from Mitsubishi Chemical Co. (Japan), H. Lundbeck A/S (Denmark) and Wyeth Research (U.K.) Ltd., respectively. All other chemicals used were of the highest commercially available purity. All drugs were freshly prepared. MKC-242 and WAY100135 for s.c. injection (1 ml kg^{-1}) were suspended in 0.5% carboxymethylcellulose (CMC). 5,7-dihydroxytryptamine (5,7-DHT) was dissolved in 0.9% NaCl containing 0.2% ascorbic acid. Other drugs for peripheral (1 ml kg^{-1}) and the locus coeruleus (LC) injections were dissolved in 0.9% NaCl. For direct administration into the hy-

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pothalamus via dialysis probe, drugs were dissolved in artificial cerebrospinal fluid (aCSF) (composition, mM: NaCl 140, KCl 3.35, $MgCl_2$ 1.15, $CaCl_2$ 1.26, Na_2HPO_4 1.20 and NaH_2PO_4 0.30, pH 7.4).

Microdialysis procedure

Rats (250–350 g) were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and stereotaxically implanted with guide cannulae for the dialysis probes (Eicom, Japan) at the hypothalamus (A −1.8 and L 0.7, with the tip of the probe 10.5 mm deep from the top of the skull) or the hippocampus (A −5.3 and L 4.4, with the tip of the probe 6.5 mm deep from the top of the skull) (Paxinos & Watson, 1986). The active probe membranes were 2 mm (hypothalamus) and 3 mm (hippocampus) in length. The day after surgery the probes were perfused at a constant flow rate of 2 μl min^{-1} with aCSF containing 1 μM desipramine (for NA and its metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), assays) or 1 μM citalopram (for 5-HT assay). A 4 h stabilization period was

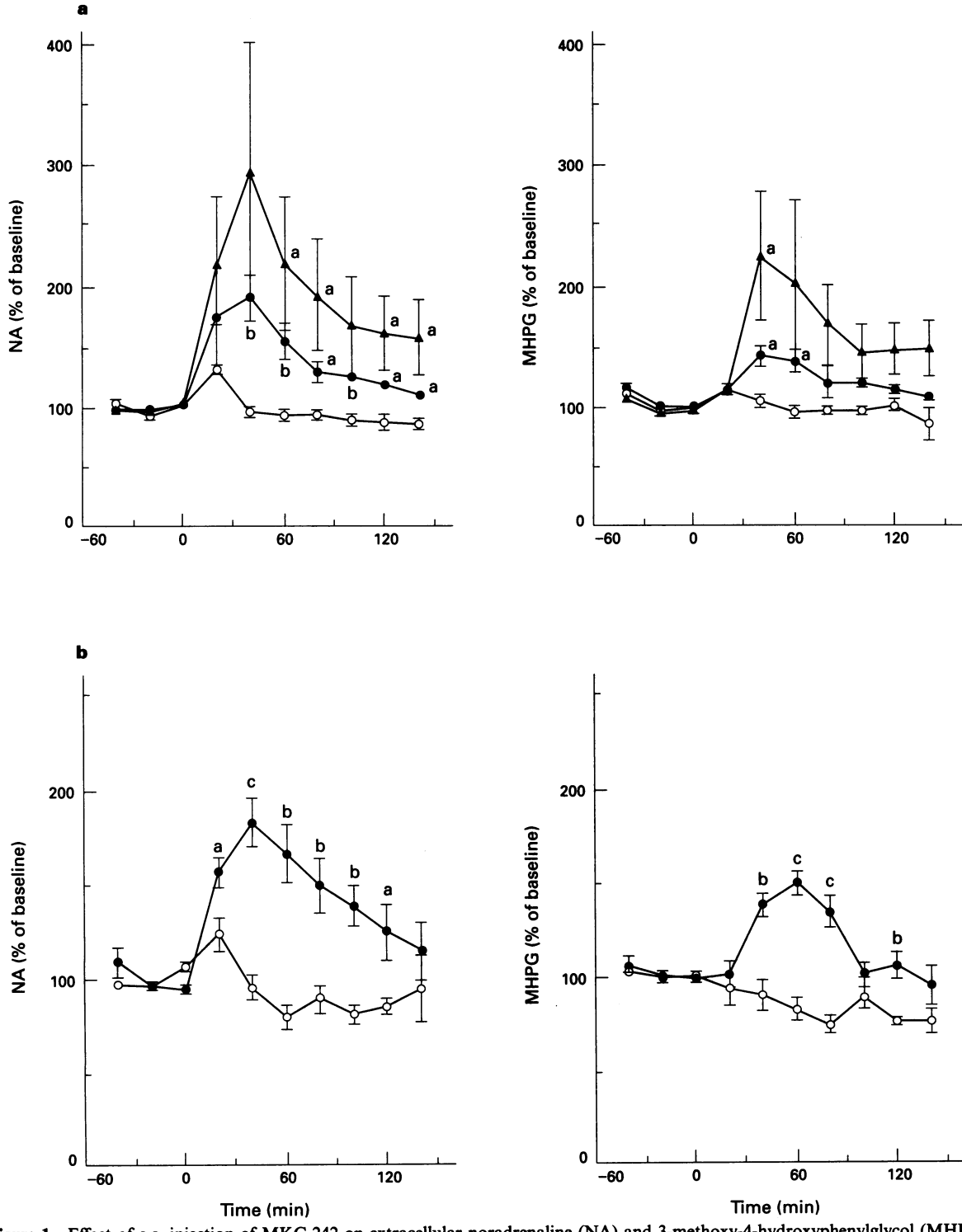

Figure 1 Effect of s.c. injection of MKC-242 on extracellular noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in the hypothalamus (a) and hippocampus (b) of freely moving rats. Rats were treated with carboxymethylcellulose (CMC, ○) and MKC-242 at 0.5 (●) and 2.0 (▲) mg kg^{-1} at zero time. Points are means ± s.e.mean of four to six determinations. $^aP<0.05$, $^bP<0.01$, $^cP<0.001$, compared with the corresponding value of CMC-treatment (two-way ANOVA followed by Student's t test).

allowed before 20 min microdialysis samples (40 μl) were taken and immediately injected onto an h.p.l.c. column for subsequent assay of amines. The probe recoveries (means ± s.e.) for NA and MHPG were 19.0 ± 0.9% and 7.7 ± 0.3%, respectively. For experiments of microinjection, guide cannulae were implanted in the locus coeruleus (A −9.8, L 1.2, and V 5.9 from the top of the skull) under pentobarbitone anaesthesia (40 mg kg^{-1}, i.p.) the day before the experiment.

Drugs were administered at 0 time. The LC injection (0.5 μl per rat) and perfusion with drugs was carried out for a period of 2 min and 1 h (three fractions), respectively.

Analysis of dialysates

Dialysates were assayed by h.p.l.c. with electrochemical detection: Eicompak CA-ODS column (4.6 mm i.d. × 150 mm:

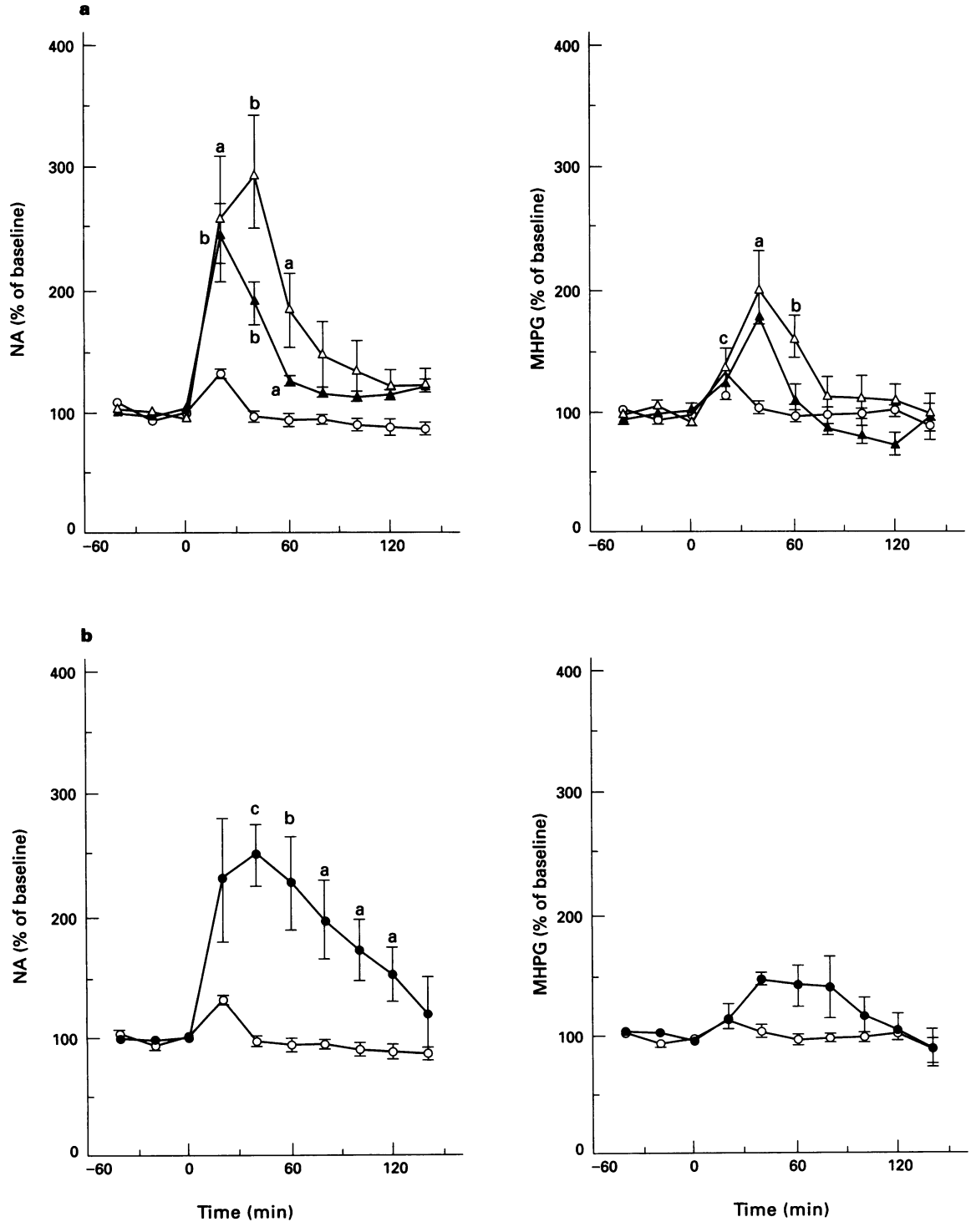

Figure 2 Effect of s.c. injection of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (a) and buspirone (b) on extracellular noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in the hypothalamus of freely moving rats. Rats were treated with saline (○), 8-OH-DPAT at 0.2 (▲) and 1.0 (△) mg kg^{-1}, and buspirone at 3.0 mg kg^{-1} (●) at zero time. Points are means ± s.e. of four to five rats. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, compared with the corresponding value of saline treatment (two-way ANOVA followed by Student's *t* test).

Eicom, Japan) and graphite electrode (Eicom) set at +550 mV (for NA and MHPG) or +450 mV (for 5-HT) against an Ag/AgCl reference electrode were used as previously described (Matsuda *et al.*, 1989). The mobile phase for NA and MHPG assays contained 95 mM sodium phosphate buffer (pH 6.0), 1.85 mM sodium octanesulphonic acid, 134 μM EDTA and 5% (v/v) methanol, and that for 5-HT assay contained 80 mM sodium phosphate buffer (pH 6.0), 1.39 mM sodium octanesulphonic acid, 134 μM EDTA and 20% v/v methanol.

Lesions of 5-HT neurones with 5,7-DHT

Rats (220–250 g) were anaesthetized with pentobarbitone (40 mg kg^{-1}, i.p.). Lesions of 5-HT neurones were carried out by injection of 5,7-DHT (150 μg as free base, 20 μl per rat, over a period of 2 min) into the lateral ventricles (A −0.8, L −1.5 and V 4.5 from skull surface). Desipramine at 25 mg kg^{-1}, i.p. was injected 30 min before 5,7-DHT to protect noradrenergic neurones. The animals were used 2 weeks after the i.c.v. injection.

Data analysis

The average of three fractions before drug administration was defined as 100% (control), and the subsequent perfusate levels were expressed as a percentage of the control. Statistical analyses were conducted by two-way ANOVA followed by Student's *t* test. *P* values of 5% or less were considered statistically significant.

Results

Basal levels of NA and MHPG, corrected with probe recoveries, in the rat hypothalamus, were 200 ± 12 and 287 ± 13 pg per fraction, respectively (means ± s.e., $n = 56$). The

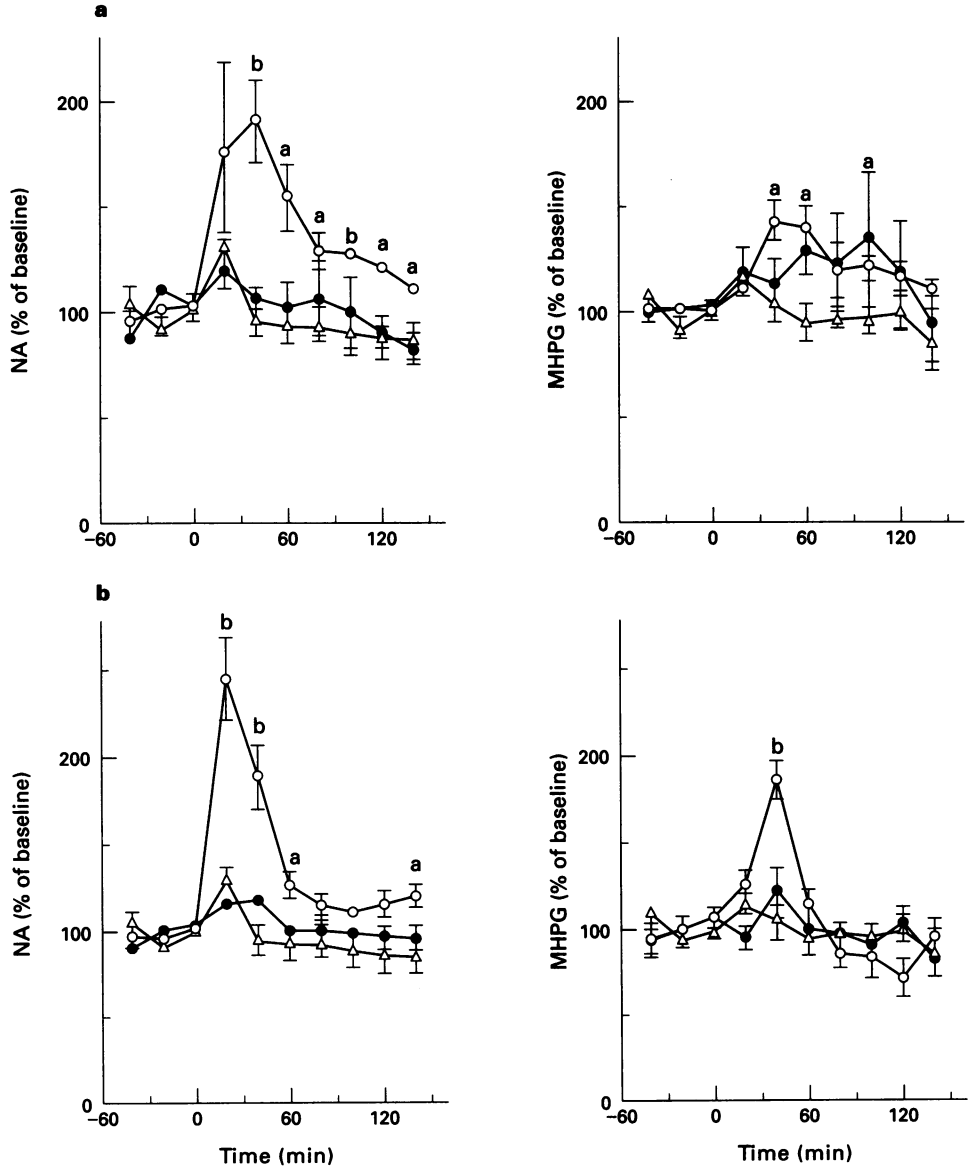

Figure 3 Effects of WAY100135 on MKC-242- and 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT)-induced increases in extracellular noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in the hypothalamus. MKC-242 at 0.5 mg kg^{-1} (a) and 8-OH-DPAT at 0.2 mg kg^{-1} (b) were injected s.c. at zero time. WAY100135 (10 mg kg^{-1}) was administered s.c. 30 min before the agonists: (○) the agonists; (●) WAY100135/the agonists; (△) vehicle control. Points are means ± s.e. of four to six rats. $^{a}P < 0.05$, $^{b}P < 0.01$, compared with the corresponding value of CMC treatment (two-way ANOVA followed by Student's *t* test). Two-way ANOVA analysis showed that the effects of WAY100135 on MKC-242- and 8-OH-DPAT-induced NA release were significant (MKC-242 vs. WAY100135/MKC-242, $P = 0.042$; 8-OH-DPAT vs. WAY100135/8-OH-DPAT, $P = 0.001$).

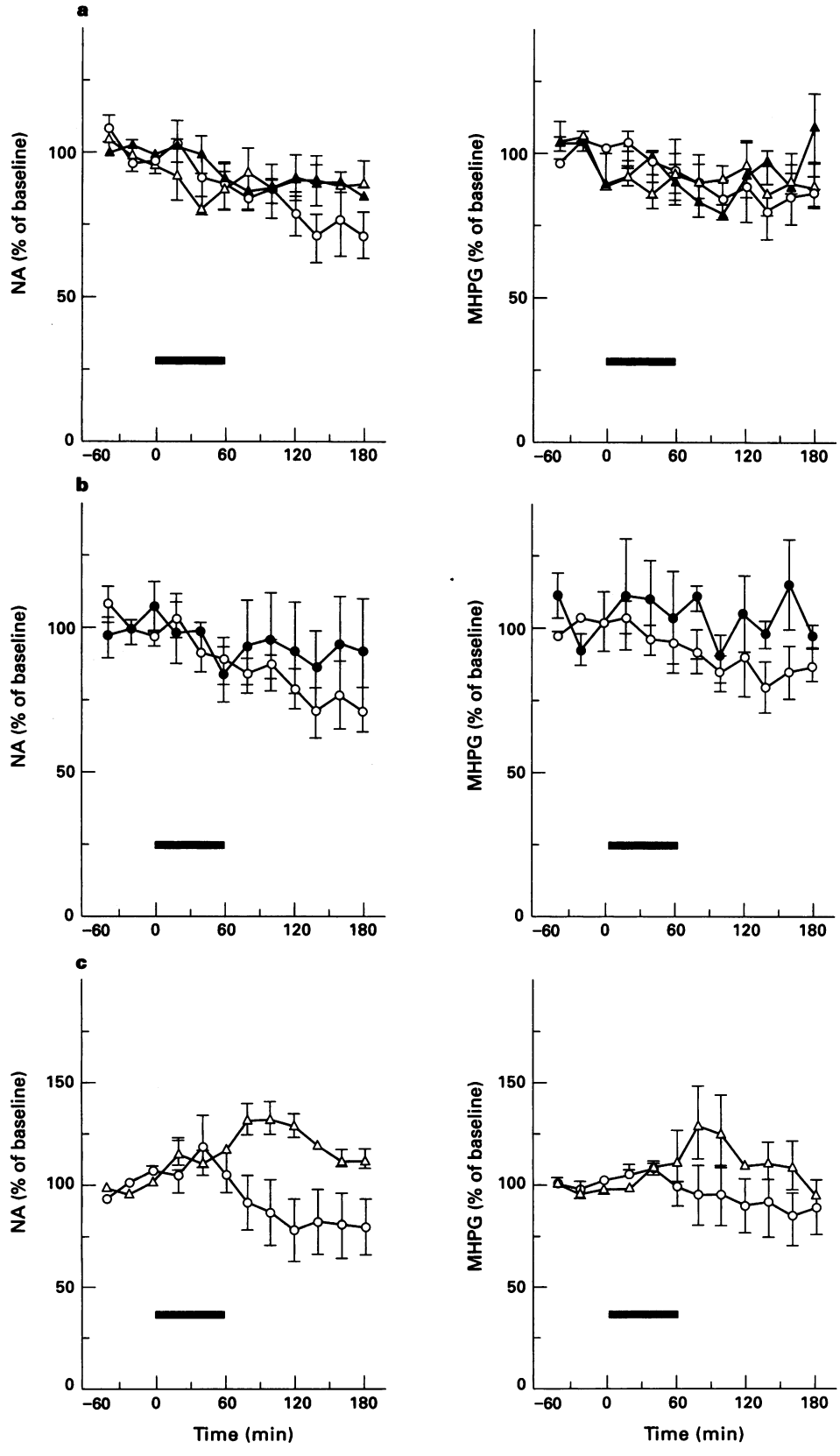

Figure 4 Effect of perfusion with 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), citalopram or MDL7222 on extracellular noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in the hypothalamus. 8-OH-DPAT (a), citalopram (b) and MDL72222 (c) were infused into the hypothalamus via the dialysis probe for 1 h period indicated by the horizontal bar. The concentrations of drugs were: 0 (○), 1 (●), 10 (△) and 100 (▲) μM. Points are means ± s.e. of three to five. Two-way ANOVA analysis revealed no significant effect of either 8-OH-DPAT, citalopram or MDL72222 vs aCSF controls.

Table 1 Effect of 5-hydroxytryptamine (5-HT) neuronal lesions with 5,7-dihydroxytrypamine (5,7-DHT) on noradrenaline (NA), dopamine (DA), 5-HT and their metabolite levels in rat whole brain (except for cerebellum)

	NA	*MHPG*	*DA*	*DOPAC*	*HVA*	*5-HT*	*5-HIAA*
Vehicle	474±14	141±2	1505±47	145±6	189±17	748±28	492±48
5,7-DHT	484±22	130±2	1237±54*	138±7	165±5	48±5***	28±3***

Rats were injected i.c.v. with 20 μl of 5,7-DHT creatinine sulphate (314.8 μg) and vehicle 14 days before the experiment. Results (ng g^{-1} tissue) are mean±s.e.mean from three to five rats. *$P<0.05$, ***$P<0.01$, compared with vehicle (Student's t test).

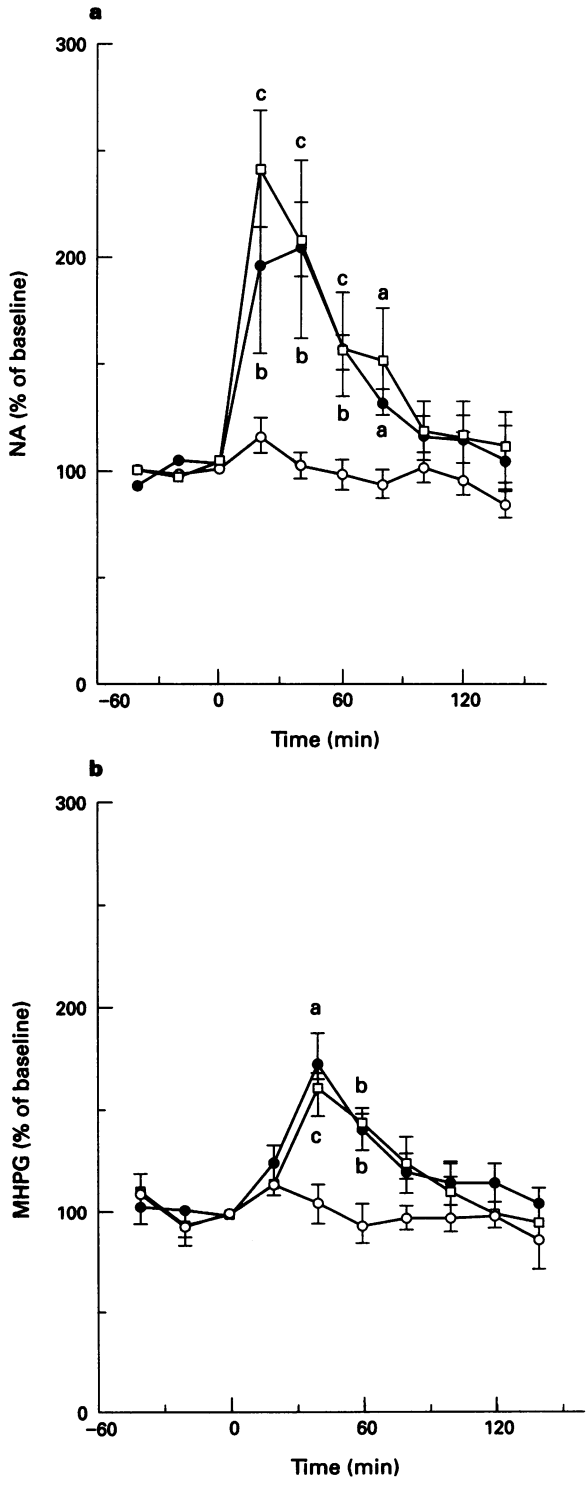

Figure 5 Effect of pretreatment with 5,7-dihydroxytryptamine (5,7-DHT) on MKC-242-induced increase in extracellular noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in the hypothalamus. MKC-242 at 0.5 mg kg^{-1} was injected s.c. 14 days after rats were injected i.c.v. with saline (●) and 5,7-DHT at 150 μg (□). Only saline/CMC controls (○) are shown, since there was no difference in the effect of CMC between saline- and 5,7-DHT-treated rats. Points are means±s.e. of three to five rats. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$, compared with the corresponding value of CMC treatment (two-way ANOVA followed by Student's t test). Two-way ANOVA analysis revealed no significant difference in the effects of MKC-242 on NA and MHPG release between 5,7-DHT- and saline-treated groups.

basal level of NA in the present study was similar to that reported previously in the hypothalamus (Qadri *et al.*, 1991).

Subcutaneous injection of MKC-242 caused a significant dose-dependent increase in extracellular NA level in the hypothalamus of the freely moving rat (Figure 1a). The extracellular level of MHPG, a metabolite of NA, was also increased by MKC-242, but the increase in MHPG concentration was smaller than that of NA. At a dose of 0.1 mg kg^{-1}, MKC-242 was ineffective (data not shown). A similar effect of MKC-242 on NA release was also observed in the hippocampus (Figure 1b). The effect of MKC-242 was mimicked by the 5-HT$_{1A}$ receptor agonists, 8-OH-DPAT and buspirone: 8-OH-DPAT at 0.2 and 1.0 mg kg^{-1} and buspirone at 3.0 mg kg^{-1} significantly increased extracellular levels of NA and MHPG in the hypothalamus (Figure 2). 8-OH-DPAT-induced increase in NA release in the hypothalamus was also observed even when the perfusion was carried out with aCSF lacking desipramine (data not shown). In rats pretreated with WAY100135 at 10 mg kg^{-1}, MKC-242 and 8-OH-DPAT did not increase extracellular levels of NA and MHPG (Figure 3). WAY100135 alone did not affect the basal release of NA in the hypothalamus (data not shown), in agreement with previous data (Routledge *et al.*, 1993). When 8-OH-DPAT (10, 100 μM), citalopram (1 μM) and MDL 72222 (10 μM) were administered locally via the dialysis probe, they had no effect on hypothalamic NA release (Figure 4).

Lesions of 5-HT neurones with 5,7-DHT caused a marked reduction in 5-HT (by 94%) and 5-hydroxyindoleacetic acid (5-HIAA) (by 95%) levels and a slight reduction in the dopamine level (by 18%), while it did not affect NA, MHPG, HVA and DOPAC levels in the brain (Table 1). In 5,7-DHT-treated rats, MKC-242 (0.5 mg kg^{-1}) significantly increased extracellular NA and MHPG levels (Figure 5). There was no difference in basal NA release between control and 5,7-DHT-treated groups: the NA levels (means±s.e., $n=3-5$) in the hypothalamus of vehicle- and 5,7-DHT-treated rats were 165±31 and 168±26 pg per fraction, respectively. The LC injection of 8-OH-DPAT at 2 μg did not cause a significant increase in NA release in the hypothalamus, although it showed a tendency to increase (Figure 6).

Repeated treatment with MKC-242 (once a day for 2 weeks at 0.5 mg kg^{-1}) did not affect the increase in NA release in the hypothalamus caused by MKC-242. While the chronic treatment showed a tendency to attenuate the effect of MKC-242 on 5-HT release, the decrease in 5-HT release was not significant in rats chronically treated with the agonist (Figure 7).

Discussion

We have recently developed a novel compound, MKC-242, which is a potent and selective 5-HT$_{1A}$ receptor agonist with a K_i value in the subnanomolar range in [^{3}H]-8-OH-DPAT binding and with a low or negligible affinity for 5-HT transporter and other neurotransmitter receptors (Yoshikawa *et al.*, 1994). In the present study, we examined, by *in vivo* micro-

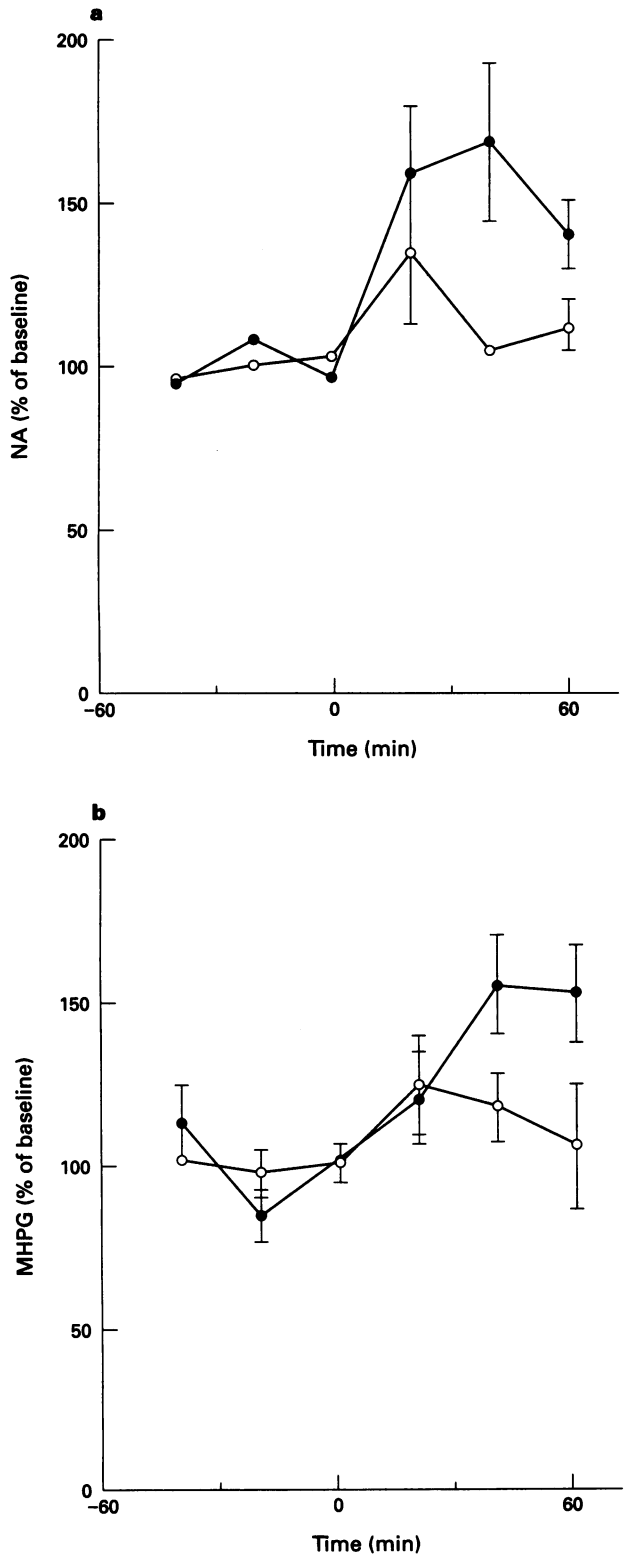

Figure 6 Effect of microinjection of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) into the LC on noradrenaline (NA) release in the hypothalamus. Saline (○) and 8-OH-DPAT at 2 μg (●) were injected into the LC, and the perfusion was carried out in the hypothalamus. Points are means ± s.e. of three rats. Two-way ANOVA analysis revealed no significant differences in the effects of MKC-242 on NA and 3-methoxy-4-hydroxyphenylglycol (MHPG) release between saline and 8-OH-DPAT.

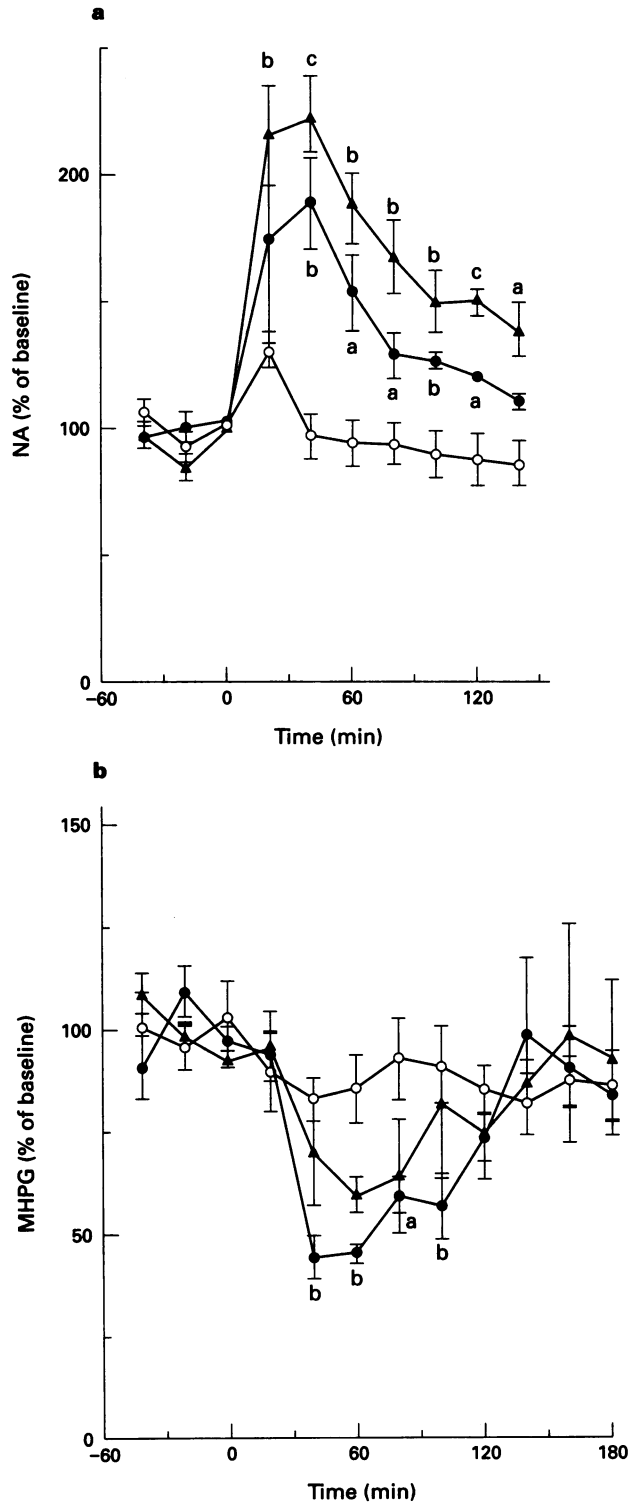

Figure 7 Effects of chronic treatment with MKC-242 on noradrenaline (NA) release (a) and 5-hydroxytryptamine (5-HT) release (b) in the hypothalamus. MKC-242 at 0.5 mg kg^{-1} was injected s.c. in rats treated once a day for 14 days with carboxymethyl cellulose (CMC) (●) and MKC-242 at 0.5 mg kg^{-1} (▲). Only acute CMC controls (○) are shown, since the effect of chronic CMC was similar to that of acute CMC. Points are means ± s.e. of three to five rats. $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$, compared with the corresponding value of CMC control (two-way ANOVA followed by Student's *t* test). Two-way ANOVA analysis revealed no significant differences in the effects of MKC-242 on NA and 5-HT release between acute and chronic groups.

dialysis, the effect of MKC-242 on rat hypothalamic NA release, in order to clarify the 5-HT$_{1A}$ receptor-mediated regulation of noradrenergic neurones. Systemic administration of MKC-242, as well as other 5-HT$_{1A}$ receptor agonists such as 8-OH-DPAT and buspirone, increased extracellular NA and its metabolite, MHPG, in the hypothalamus and hippocampus. It is unlikely that the increase in NA release by the drugs is due to an inhibition of the reuptake, since desipramine, an inhibitor of NA reuptake, was included in the perfusion fluid in this study. We also observed that the effects of MKC-242 and 8-OH-DPAT on the extracellular NA level were almost completely blocked by pretreatment with WAY100135, a selective antagonist at presynaptic and postsynaptic 5-HT$_{1A}$ receptors (Fletcher *et al.*, 1993). These results suggest that activation of 5-HT$_{1A}$ receptors increases NA release. This was also supported by the previous observation that systemic administration of 8-OH-DPAT and buspirone increased NA turnover in the hypothalamus (Fuller & Perry, 1989). In contrast, Broderick & Piercey (1991) reported that buspirone and ipsapirone depressed NA release in the hippocampus, although they found that the 5-HT$_{1A}$ receptor agonists excited NA neuronal cell firing rates in the LC. The discrepancy might be due to a difference in the method used to detect NA release: Broderick & Piercey (1991) used *in vivo* voltammetric recording of monoamine release.

The results of the present study are consistent with the findings of Done & Sharp (1994) who have shown that 8-OH-DPAT increased the release of NA in the hippocampus as measured by microdialysis. They further suggested that the effect of 8-OH-DPAT might be due to activation of presynaptic 5-HT$_{1A}$ autoreceptors, since the effect was mimicked by NAN-190 and MDL 73005EF, 5-HT$_{1A}$ receptor antagonists with agonist properties at presynaptic 5-HT$_{1A}$ receptors (Gartside *et al.*, 1990; Hjorth & Sharp, 1990; Sprouse, 1991; Greuel & Glaser, 1992). In contrast with this proposal, the present study demonstrates that treatment with 5,7-DHT which destroys presynaptic 5-hydroxytryptaminergic nerve fibres does not alter the effect of MKC-242 in increasing NA release. This finding suggests that NA release is modulated in part by postsynaptic 5-HT$_{1A}$ receptors, although it does not rule out the possibility that the presynaptic 5-HT$_{1A}$ receptors play a role in NA release as previously suggested by Done & Sharp (1994). In view of the earlier finding that NA release is under inhibitory control by 5-HT (Done & Sharp, 1992), it may be considered that 5,7-DHT lesions eliminate the inhibitory tone on noradrenergic neurones, and increase NA release. However, the present study showed that there was no difference in basal NA release between vehicle- and 5,7-DHT-treated rats. This may be explained by 5,7-DHT-induced supersensitivity of 5-HT$_2$ receptors as previously reported (Butler *et al.*, 1990; Heal *et al.*, 1990), since 5-HT$_2$ receptors can exert an inhibitory influence on NA release (Done & Sharp, 1992).

Feuerstein & Hertting (1986) showed that the 5-HT receptor agonists, 5-HT, 2-methyl-5-HT and 5-carboxamidotryptamine, increased [^{3}H]-NA release in hippocampal slices. Furthermore, Blandina *et al.* (1991) reported that 5-HT$_3$ receptors mediated an inhibition of NA release from hypothalamic slices in the presence of ritanserin, a 5-HT$_2$/5-HT$_{1C}$ receptor antagonist. However the present *in vivo* study showed that local administration of 8-OH-DPAT, citalopram and MDL72222 did not affect NA release in the hypothalamus. The lack of effect of 8-OH-DPAT suggests that the 5-HT$_{1A}$ receptors controlling NA release are not present in noradrenergic nerve terminals in the hypothalamus. Earlier studies (Renaud *et al.*, 1975; Crespi *et al.*, 1980; Reader *et al.*, 1986) suggest that the LC which receives a rich 5-HT innervation (Pickel *et al.*, 1977; Steinbusch, 1981) may be a possible site for the action of the 5-HT$_{1A}$ agonist. In this connection, Broderick & Piercey (1991) found that azapirones increased NA neuronal firing rate in the LC. In contrast, Gorea *et al.* (1991) reported that microiontophoretic application of 8-OH-DPAT did not modify the spontaneous firing rate of neurones in the LC. The present study showed that microinjection of 8-OH-DPAT into the LC did not increase NA release in the hypothalamus. It remains to be determined where the postsynaptic 5-HT$_{1A}$ receptors controlling NA release are localized in the brain.

It is known that the effects of 5-HT$_{1A}$ agonists on body temperature and corticosterone secretion are attenuated by repeated treatment with the agonists (Nash *et al.*, 1989; Larsson *et al.*, 1990; Nakano *et al.*, 1992). In contrast to these postsynaptic 5-HT$_{1A}$ receptor-mediated responses, the desensitization of 5-HT$_{1A}$ autoreceptors is not confirmed. Electrophysiological studies (Blier & Montigny, 1987; Godbout *et al.*, 1991) suggest that 5-HT$_{1A}$ autoreceptors are desensitized by chronic 5-HT$_{1A}$ receptor agonists, while neurochemical observations using *in vivo* microdialysis (Beer *et al.*, 1990; Kreiss & Lucki, 1992; Sharp *et al.*, 1993; Soderpalm *et al.*, 1993; Hjorth & Auerback, 1994) are controversial. The present study suggests that the postsynaptic 5-HT$_{1A}$ receptors controlling NA release are not desensitized following the repeated treatment. As regards the desensitization of 5-HT$_{1A}$ autoreceptors, we could not reach any conclusion: the significant effect of MKC-242 in decreasing 5-HT release was not observed in rats chronically treated with the drug, while there was no difference in the effect of MKC-242 on 5-HT release between the acute and chronic treatment.

In conclusion, the present study indicates that activation of postsynaptic 5-HT$_{1A}$ receptors causes NA release. The 5-HT$_{1A}$ receptors controlling NA release are possibly localized on the NA cell body rather than the nerve terminals, and the regulation may be involved in the pharmacology of 5-HT$_{1A}$ receptor agonists.

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