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SPECIAL REPORT

# Receptors for endothelin-1 in asthmatic human peripheral lung

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[$^{125}$I]-endothelin-1 ([$^{125}$I]-ET-1) binding was assessed by autoradiography in peripheral airway smooth muscle and alveolar wall tissue in human non-asthmatic and asthmatic peripheral lung. Levels of specific binding to these structures were similar in both non-asthmatic and asthmatic lung. The use of the receptor subtype-selective ligands, BQ-123 ($ET_A$) and sarafotoxin S6c ($ET_B$), demonstrated the existence of both $ET_A$ and $ET_B$ sites in airway smooth muscle and in alveoli. In airway smooth muscle from both sources, the great majority of sites were of the $ET_B$ subtype. Quantitative analyses of asthmatic and non-asthmatic alveolar wall tissue demonstrated that 29–32% of specific [$^{125}$I]-ET-1 binding was to $ET_A$ sites and 68–71% was to $ET_B$ sites. Thus, asthma was not associated with any significant alteration in the densities of $ET_A$ and $ET_B$ receptors in peripheral human lung.

**Keywords:** Endothelin receptor subtypes; BQ-123; sarafotoxin S6c; human peripheral lung; asthma

**Introduction** Endothelin-1 (ET-1) is a potent spasmogen in human airway smooth muscle *in vitro* (Henry *et al.*, 1990b), accelerates the growth of airway smooth muscle cells in culture (Noveral *et al.*, 1992) and stimulates mucous gland secretion (Mullol *et al.*, 1993). Importantly, human asthma, which characteristically involves subepithelial oedema, airway smooth muscle hyperplasia and elevated tone and mucous gland hypersecretion, is also associated with increased levels of immunoreactive ET-1 in both bronchial epithelial cells (Springall *et al.*, 1991) and bronchial lavage fluid (Sofia *et al.*, 1993). Taken together, these findings suggest a link between the pathogenesis of asthma and endogenous ET-1 levels in the airways (Hay *et al.*, 1993).

Since little is known concerning ET receptors in asthmatic lung, we have compared the densities, distributions and proportions of $ET_A$ and $ET_B$ receptor subtypes in human non-asthmatic and asthmatic peripheral lung using qualitative and quantitative autoradiography.

**Methods** Lung tissue was obtained from 6 non-asthmatic (3 male, 3 female, aged 27 ± 10 years) and 6 moderate/severe allergic asthmatic (2 male, 4 female, aged 29 ± 6 years) organ donors. Each of the asthmatics had been treated with an inhaled β-adrenoceptor agonist bronchodilator. One of these subjects was also prescribed oral theophylline and prednisone. Dry, thaw-mounted peripheral lung frozen sections (10 μm) (Henry *et al.*, 1990a) were incubated at 22°C in Tris-HCl buffer (170 mM, pH 7.6) containing 0.25% bovine serum albumin, 10 μM phenylmethylsulphonylfluoride, 10 U ml$^{-1}$ catalase and 0.2 nM [$^{125}$I]-ET-1 in the absence or presence of the $ET_A$ receptor-selective ligand, BQ-123 (1 μM) or the $ET_B$ receptor-selective ligand, sarafotoxin S6c (Stx S6c; 100 nM) for 180 min. Non-specific binding was assessed in the combined presence of BQ-123 and Stx S6c. [$^{125}$I]-ET-1 binding density in alveolar wall tissue was estimated by computer-assisted image analysis of autoradiographic grains (Henry *et al.*, 1990b) in 6 fields from each of 3 tissue sections from each subject. Results are presented as means or mean ratios ± s.e.mean ($n = 6$). Data were assessed by 2-way ANOVA and a modified $t$ statistic (Wallenstein *et al.*, 1980). $P$ values less than 0.05 were considered statistically significant.

The following drugs were used: [$^{125}$I]-endothelin-1, endothelin-1, sarafotoxin S6c, BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]) (Auspep), phenylmethylsulphonylfluoride (Calbiochem), catalase (Sigma).

**Results** Figure 1 depicts the autoradiographic distribution of binding sites for [$^{125}$I]-ET-1 in non-asthmatic (a–e) and asthmatic (f–j) human peripheral lung. Specific autoradiographic grains were in highest numbers over alveolar wall tissue and airway smooth muscle of small bronchi and bronchioles in both non-asthmatic and asthmatic tissue (Figure 1b,g). Stx S6c markedly reduced the specific grain density in peripheral airway smooth muscle and alveolar septae from both tissue sources (Figure 1d,i), demonstrating that these elements contained large populations of $ET_B$ receptors. BQ-123 (1 μM), caused only a small reduction in specific binding in peripheral airway smooth muscle (Figure 1c,h), indicating the co-existence of a minor but detectable population of $ET_A$ sites. Specific binding to small blood vessels and to neuronal tissue in the periphery of a bronchus was abolished by BQ-123 (Figure 1c), suggesting the predominance of $ET_A$ receptors at these foci.

Quantitation of specific autoradiographic grains in the alveolar wall indicated that the density of specific [$^{125}$I]-ET-1 binding was similar in asthmatic and non-asthmatic lung alveoli ($P > 0.05$) (Figure 2). Furthermore, asthma was not associated with a significant change in ET receptor subtype proportions. On average, the ratio of $ET_A$ to $ET_B$ sites in asthmatic lung was 29:71 (± 4; $n = 6$ subjects) and in non-asthmatic lung was 32:68 (± 4; $n = 6$ subjects) (Figure 2).

**Discussion** In the current study, asthma was not associated with any significant alteration in the densities of $ET_A$ or $ET_B$ binding sites in asthmatic and non-asthmatic human lung alveoli or peripheral airway smooth muscle. The great majority of ET receptors in peripheral airway smooth muscle were of the $ET_B$ subtype, consistent with recent findings in human bronchial smooth muscle (Goldie *et al.*, 1994). $ET_B$ receptors were also the predominant subtype in alveolar wall tissue. Although alveolar capillary endothelial cells in rat lung contained specific ET-1 binding sites (Furuya *et al.*, 1992), identification of the specific cellular locations of human alveolar wall $ET_A$ and $ET_B$ receptors must await further electron microscopic autoradiographic studies. $ET_B$ receptors in human airway smooth muscle are linked to contraction (Goldie *et al.*, 1994) and it is possible that they may mediate the vascular permeability enhancing effects of ET-1 resulting in local oedema and airway thickening. However, little is known at present concerning the functional significance of the binding sites for ET-1 in the alveolar wall of asthmatic and non-asthmatic subjects. ET-1 may be a contributory mediator in asthma (Hay *et al.*, 1993), although any pathophysiological effects cannot be attributed to altered airway receptor density, subtype proportions or distribution in the peripheral lung.

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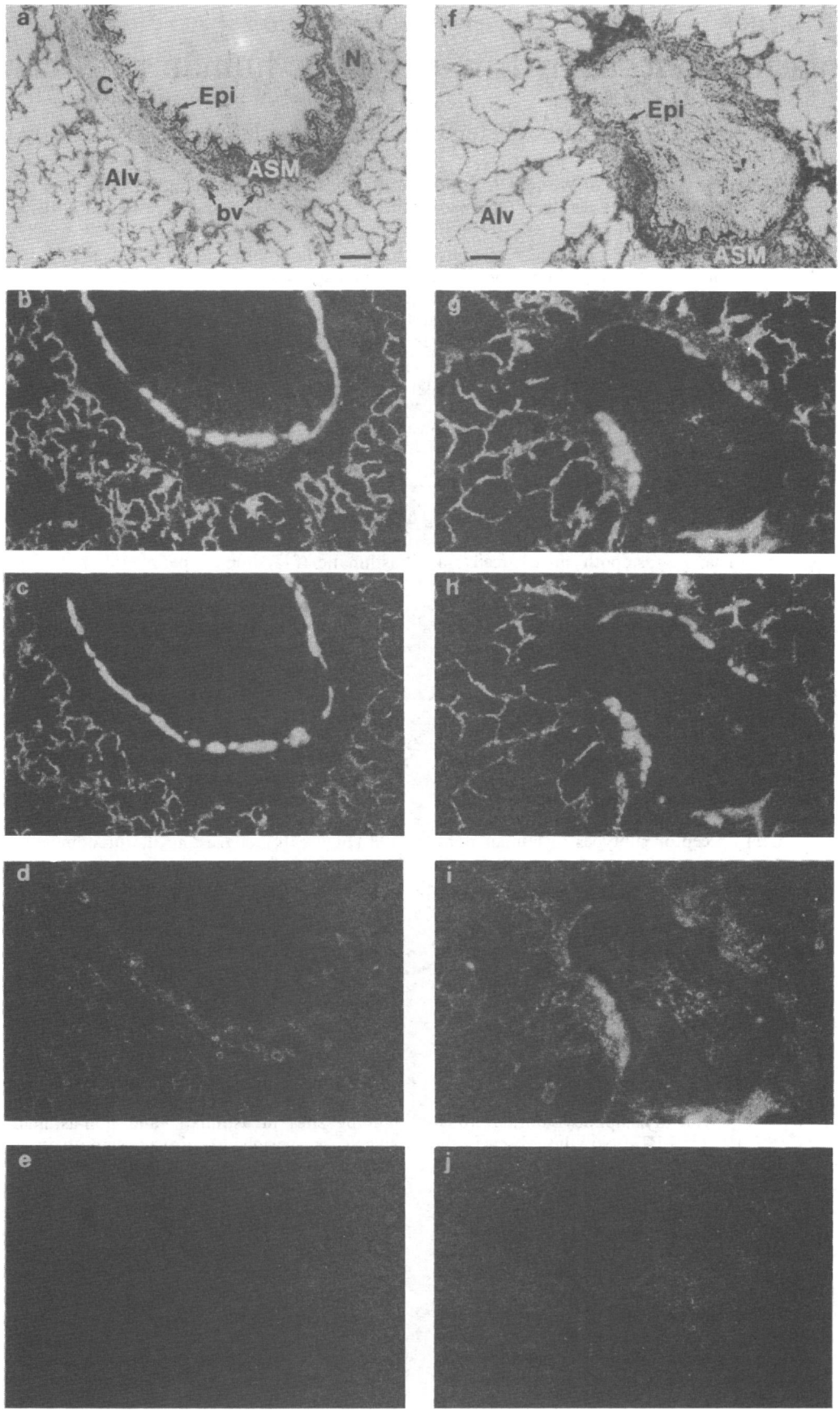


**Figure 1** Distribution of [$^{125}$I]-endothelin-1 ([$^{125}$I]-ET-1) autoradiographic binding sites in non-asthmatic (a–e) and asthmatic peripheral lung (f–j). (a, f) Bright field photomicrographs of 10 μm frozen tissue sections. Alv = alveolar wall, ASM = airway smooth muscle, bv = blood vessel, N = nerve, C = cartilage Epi = epithelium Bar = 200 μm. (b, g) Dark-field photomicrographs of the respective sections (a) and (f) showing the distribution of autoradiographic grains derived from total [$^{125}$I]-ET-1 binding. Dark-field photomicrographs of serial frozen sections showing [$^{125}$I]-ET-1 labelling (c, h) of $ET_B$ receptors (i.e. residual specific binding in the presence of the $ET_A$ receptor-selective antagonist BQ-123, 1 μM) and (d, i) of $ET_A$ receptors (i.e. residual specific binding in the presence of the $ET_B$ receptor-selective agonist sarafotoxin S6c (Stx S6c) 100 nM). (e, j) Non-specific binding in the combined presence of BQ-123, 1 μM and Stx S6c, 100 nM.

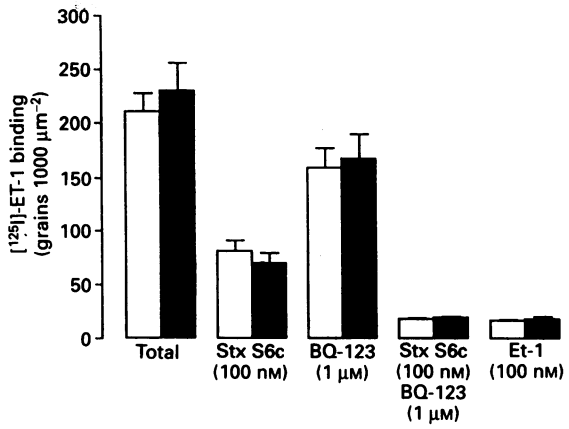


**Figure 2** [$^{125}$I]-endothelin-1 ([$^{125}$I]-ET-1, 0.2 nM) binding at 180 min in the alveolar wall from human non-asthmatic (open columns) and asthmatic (solid columns) peripheral lung in the absence and presence of sarafotoxin S6c (Stx S6c, 100 nM), BQ-123 (1 μM), unlabelled ET-1 (100 nM) or in the combined presence of Stx S6c (100 nM) and BQ-123 (1 μM) (non-specific binding). Each column represents the mean ± s.e.mean of observations from tissue from $n = 6$ subjects.

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SPECIAL REPORT

# Potentiation by tumour necrosis factor-α of calcium signals induced by bradykinin and carbachol in human tracheal smooth muscle cells

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The effect of tumour necrosis factor-α (TNFα) on the increase in cytosolic free calcium ($[Ca^{2+}]_i$) induced by carbachol and bradykinin (BK) was investigated in human tracheal smooth muscle cells in culture (TSMC). BK ($10^{-12}$–$10^{-9}$ M) and carbachol ($10^{-7}$–$10^{-3}$ M) produced a concentration-dependent increase in $[Ca^{2+}]_i$ ($pD_2 = 10.73 \pm 0.05$ and $5.57 \pm 0.03$ respectively). The increase in $[Ca^{2+}]_i$ was significantly enhanced for both agonists in TNFα-treated cells (10 ng ml$^{-1}$ for 24 h). However, $pD_2$ values were not modified ($10.78 \pm 0.03$ and $5.62 \pm 0.04$ for BK and carbachol, respectively) suggesting that no change in the apparent receptor affinity occured. Thus, TNFα induced-alterations in $Ca^{2+}$ homeostasis in human TSMC may be a key mechanism underlying airway hyperreactivity.

**Keywords:** Bradykinin; calcium; human tracheal smooth muscle cells; tumour necrosis factor-α; carbachol

**Introduction** *In vivo*, inhalation of tumour necrosis factor-α (TNFα) caused bronchial hyperresponsiveness in rats (Kips *et al.*, 1992). The precise mechanism underlying this enhancement of airway responsiveness remains to be elucidated, but an *in vitro* study indicates that TNFα can have a direct effect on tracheal smooth muscle (Pennings *et al.*, 1993). Such a mechanism may include alterations in the contractile properties of airway smooth muscle by TNFα and this is the basic hypothesis of the present study.

We have investigated whether TNFα modifies the subsequent response of human tracheal smooth muscle cells (TSMC) to bronchoconstrictors agonists. Since $Ca^{2+}$ is a second messenger common to all airway smooth muscle cell activators, including bradykinin and carbachol (Murray & Kotlikoff, 1991; Marsh & Hill, 1993; Yang *et al.*, 1993), we studied the effect of pretreatment of human TSMC with TNFα on the $[Ca^{2+}]_i$ response to these agonists.

**Methods** Primary cultures of human TSMC were prepared as described previously (Kullman *et al.*, 1993). TSMC were cultured in DMEM/F12 medium supplemented with 10% foetal calf serum, 2 mM glutamine, 1% non-essential amino-acids, 5 μg ml$^{-1}$ insulin, penicillin (100 U ml$^{-1}$) and streptomycin (100 μg ml$^{-1}$). All products were obtained from Gibco BRL (Cergy Pontoise, France).

Human TSMC at confluency were treated for 24 h with 10 ng ml$^{-1}$ TNFα (Genzyme, Dako SA, Trappes) in the above described medium. Cells were washed with HEPES buffer containing (mM): NaCl 137.5, $CaCl_2$ 1.25, $MgCl_2$ 1.25, $NaH_2PO_4$ 0.4, KCl 6, glucose 5.6, HEPES 10, 0.1% BSA (w/v); pH = 7.4 and incubated with 3 μM fura-2/AM in HEPES buffer (Sigma, St Louis, MO, U.S.A.) at 37°C for 45 min. The cells were trypsinized, washed and resuspended at $5 \times 10^5$ cells ml$^{-1}$. $[Ca^{2+}]_i$ was calculated with a Hitachi F2000 spectrofluorometer from the fluorescence intensities measured at 510 nm after excitation at both 340 and 380 nm. Statistical analysis was calculated by Student's *t* test.

**Results** Figure 1 shows the effect of BK on the increase in $[Ca^{2+}]_i$ in human TSMC treated or not with 10 ng ml$^{-1}$ of TNFα. In control cells, $10^{-10}$ M BK induced a typical biphasic increase in $[Ca^{2+}]_i$ consisting of a fast transient phase followed by a sustained phase, i.e. $[Ca^{2+}]_i$ did not return to the basal level (Figure 1a). In TNFα pretreated

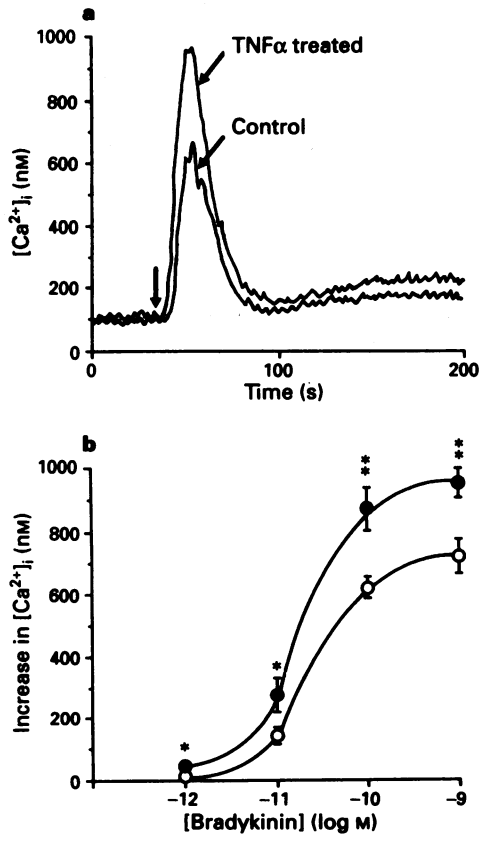


**Figure 1** Effects of tumour necrosis factor-α (TNFα) pretreatment of human TSMC on the increase in $[Ca^{2+}]_i$ induced by BK. (a) Typical traces obtained with $10^{-10}$ M BK in untreated cells (Control) and TNFα-treated cells (10 ng ml$^{-1}$ for 24 h). The arrow indicates the addition of BK (b) concentration-response curves to BK in untreated (○) and in TNFα-treated cells (●). Results are expressed as the net increase in $[Ca^{2+}]_i$ at the peak and values are means ± s.e.mean of four separate experiments. Significantly different from control: $P<0.05$*; $P<0.01$**.

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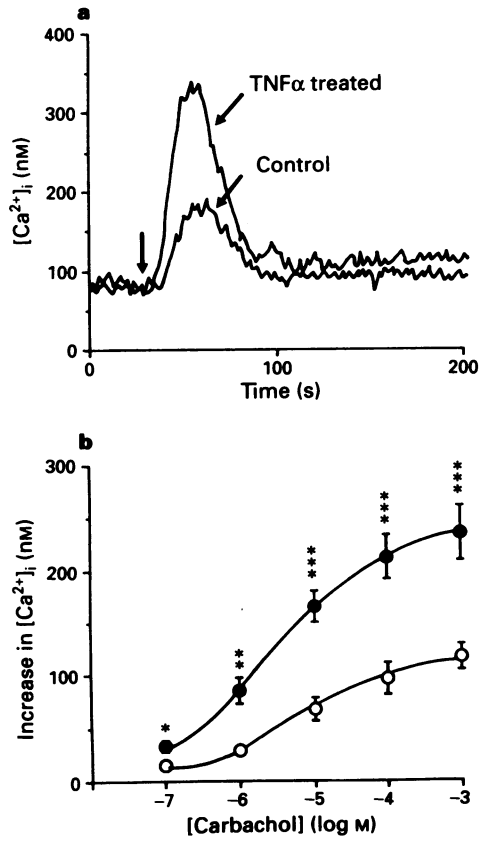


**Figure 2** Effects of tumour necrosis factor-α (TNFα) pretreatment of human TSMC on the increase in $[Ca^{2+}]_i$ induced by carbachol. (a) Typical traces obtained with $10^{-3}$ M carbachol in untreated cells (Control) and TNFα-treated cells (10 ng ml$^{-1}$ for 24 h). The arrow indicates the addition of carbachol. (b) Concentration-response curves to carbachol in untreated (○) and in TNFα-treated cells (●). Results are expressed as the net increase in $[Ca^{2+}]_i$ at the peak and values are means ± s.e.mean of four separate experiments. Significantly different from control: $P<0.05$*; $P<0.01$**; $P<0.001$***.

TSMC, both phases were increased (Figure 1a). BK induced a concentration-dependent increase in $[Ca^{2+}]_i$ between $10^{-12}$ and $10^{-9}$ M in the transient phase (Figure 1b) which was significantly potentiated upon TNFα treatment (Figure 1b). The maximum response in treated and untreated cells was reached at $10^{-9}$ M. $pD_2$ values were 10.73 ± 0.05 in control and 10.78 ± 0.03 in treated cells, showing that TNFα did not modify the sensitivity of the concentration-response curves. In addition, the sustained phase was also potentiated by 81, 60 and 38% at $10^{-11}$, $10^{-10}$ and $10^{-9}$ M BK, respectively (results not illustrated).

Figure 2 shows the effect of carbachol on the increase in $[Ca^{2+}]_i$ in human TSMC treated or not with TNFα. Carbachol at $10^{-3}$ M induced a biphasic increase in $[Ca^{2+}]_i$ which was potentiated (for both phases) in TNFα-treated cells (Figure 2a). As shown in Figure 2b, TNFα significantly potentiated the transient phase for all concentrations of carbachol that were tested. The maximum increase was obtained at $10^{-3}$ M carbachol. $pD_2$ values were 5.57 ± 0.03 in control and 5.62 ± 0.04 in treated cells indicating that the sensitivity of the concentration-response curves are not modified. The sustained phase was also potentiated in TNFα-treated cells by 144, 106 and 86% for $10^{-6}$, $10^{-4}$ and $10^{-3}$ M carbachol, respectively (results not illustrated).

**Discussion** To our knowledge, this study is the first demonstration of a direct effect of TNFα on human TSMC in culture by enhancing the $[Ca^{2+}]_i$ response to BK and carbachol. The $pD_2$ values remained unmodified suggesting that the cell sensitivity to these agonists is not affected by TNFα. Interestingly, a parallel can be drawn with the work of Pennings *et al.* (1993) who showed that pretreatment of guinea-pig trachea with TNFα increases the maximum contractile response to methacholine without changing the $pD_2$ values.

Such a potentiation could involve an over-expression of cell surface receptors, but this would imply an increase in the number of both BK and muscarinic receptors. Surprisingly, we found that a 24 h TNFα pretreatment of human TSMC decreased the specific binding sites number of [$^3$H]-quinuclidinylbenzilate (a muscarinic antagonist) by 37.8 ± 1.3% ($n = 3$) (Amrani, unpublished observations) ruling out the hypothesis of an increase in the number of receptors. Alternatively, TNFα might modify receptor-linked transduction mechanisms, such as G-protein and subsequent effector systems such as phospholipase C, both involved in the increase in $[Ca^{2+}]_i$ in response to carbachol and BK (Pyne & Pyne, 1993; Yang *et al.*, 1993). TNFα might also affect the expression of proteins involved in the regulation of intracellular $Ca^{2+}$ stores and/or $Ca^{2+}$-influx.

In conclusion, our results demonstrate that human airway smooth muscle cells are a new target for TNFα. Alterations in $Ca^{2+}$ handling by TNFα may represent a key mechanism underlying airway hyperreactivity.

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SPECIAL REPORT

# Potentiation by aminopeptidase P of blood pressure response to bradykinin

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We examined whether a specific aminopeptidase P (APP) inhibitor, apstatin, increases vasodepressor responses to bradykinin in anaesthetized rats, and whether it would augment blood pressure responses further after treatment with the angiotensin-converting enzyme inhibitor (ACEi), lisinopril. Apstatin doubled the maximum blood pressure response to bradykinin. The area under the curve (AUC), which incorporates both peak blood pressure changes and duration of response, was doubled in apstatin-treated rats *vs* controls and in the apstatin + lisinopril group *vs* lisinopril alone. These data demonstrate that APP is an important kininase *in vivo*.

**Keywords:** Bradykinin; peptidases; kininases; aminopeptidase P; angiotensin-converting enzyme inhibitors; blood pressure

**Introduction** Bradykinin is believed to play a role in the regulation of blood pressure and renal and cardiac function. Inhibition of bradykinin metabolism by kininases is usually needed to uncover the effects of kinins (Carretero & Scicli 1990), suggesting that responses to endogenous kinins are modulated by peptidases. The only kininase inhibitors known to potentiate responses to exogenous bradykinin significantly are ACEi, and some of the effects of ACEi are mediated by kinins (Carretero & Scicli, 1990; Wiemer *et al.*, 1994). After inhibition of ACE *in vivo* the half life of plasma kinins remains unchanged, suggesting that other peptidases are also important (Ishida *et al.*, 1989).

Aminopeptidase P (EC 3.4.11.9) is known to metabolize bradykinin *in vitro* (Ward *et al.*, 1991; Simmons & Orawski, 1992), but it is not clear whether it is an important kininase *in vivo*. Simmons' group (Prechel *et al.*, 1994) developed an inhibitor of APP called apstatin [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-prolyl-L-prolyl-L-alaninamide]. Apstatin inhibits rat lung membrane-bound APP ($K_i = 2.6\,\mu M$) but fails to inhibit other enzymes known to degrade bradykinin, such as ACE, neutral endopeptidases 24.11 and 24.15, and prolyl endopeptidase. We examined whether blood pressure responses to bradykinin in rats treated with lisinopril are potentiated further by the addition of apstatin.

**Methods** Male Wistar rats (200–250 g, Charles River, Wilmington, MA) were given a standard diet and water *ad libitum* for 2 days before the experiment. After sodium pentobarbitone anaesthesia (50 mg kg$^{-1}$, i.p.), heparinized indwelling catheters (PE10 fused to PE50) were inserted into the abdominal aorta *via* the left femoral artery for direct blood pressure measurements, and into the inferior vena cava *via* the left femoral vein for i.v. injections. All drugs were dissolved in 0.9% NaCl and given as a bolus (100 µl, followed by 100 µl 0.9% NaCl).

After a 30 min stabilization period, blood pressure responses to bradykinin (50, 100, 200 and 400 ng kg$^{-1}$ in random order), angiotensin I (400 ng kg$^{-1}$), and acetylcholine (400 ng kg$^{-1}$) were monitored. Sixty minutes later, rats were divided into four groups as follows: G1: saline; G2: apstatin (80 µg kg$^{-1}$); G3: lisinopril (400 µg kg$^{-1}$); G4: lisinopril + apstatin. After 5 min, blood pressure responses to bradykinin, angiotensin I and acetylcholine were repeated in G1 and G2. G3 and G4 received bradykinin alone. For each dose, the maximum change in mean blood pressure (ΔBP) and the area under the curve (AUC) were determined. The ACEi lisinopril was selected because of reports that nM concentrations failed to inhibit pig APP *in vitro* (Hooper *et al.*, 1992).

Lisinopril was a gift from Merck (Rahway, NJ) and apstatin was provided by Dr R.J. Shebuski of Upjohn (Kalamazoo, MI, U.S.A.).

Univariate repeated-measures ANOVA with the Greenhouse-Geisser sphericity correction was used to evaluate ΔBP responses across both dose and treatment. ANOVA was used to test for changes across the four bradykinin doses within each treatment, and paired *t* tests were used to test for changes across the two treatment categories at each dose.

**Results** BP was not altered by apstatin alone but was reduced from 109 ± 2 to 100 ± 2 mmHg by lisinopril and from 104 ± 2 to 96 ± 3 mmHg by lisinopril plus apstatin ($P < 0.05$).

Apstatin pretreatment roughly doubled responses to bradykinin. Treatment with lisinopril resulted in marked potentiation of the ΔBP response to bradykinin, which was significantly higher than that induced by apstatin ($P < 0.01$). Responses to bradykinin tended to be higher in rats given both lisinopril and apstatin than in those given lisinopril alone, but the differences were not statistically significant (Figure 1). ΔBP responses to angiotensin I and acetylcholine were not affected by apstatin: for angiotensin I, ΔBP was 35.3 ± 3.3 and 38.2 ± 3.4 mmHg ($n = 2$) for controls and apstatin, respectively (NS), while for acetylcholine the values were −26.2 ± 3.2 and −25.2 ± 2.8 ($n = 5$) (NS). The AUC was increased significantly by apstatin: $P < 0.01$ for apstatin *vs* controls and $P < 0.025$ for apstatin + lisinopril *vs* lisinopril alone (Figure 2).

**Discussion** Although plasma and tissue ACE and carboxypeptidase N play an important role in kinin degradation *in vivo*, other peptidases also contribute to kinin metabolism (Ishida *et al.*, 1989). One may be APP, which is widely distributed in the body (Holtzman *et al.*, 1987). Inhibition of APP with apstatin significantly potentiated the vasodepressor effect of bradykinin, heightening both the ΔBP and the duration of the effect. Potentiation of the response to bradykinin was not due to ACE inhibition, since responses to angiotensin I were not affected by apstatin; nor was it due to some nonspecific alteration in response to a vasodepressor,

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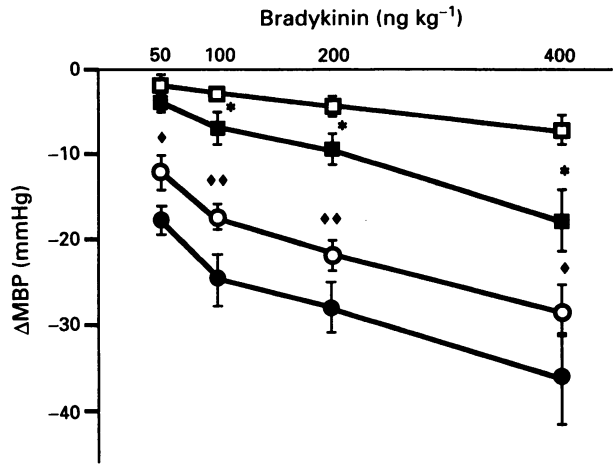


**Figure 1** Changes in the maximum hypotensive response to an i.v. bolus of bradykinin in controls and rats pretreated with either apstatin (80 μg kg$^{-1}$), lisinopril (400 μg ml$^{-1}$) or both: (□) controls ($n = 6$); (■) apstatin ($n = 6$); (○) lisinopril ($n = 6$); (●) apstatin + lisinopril ($n = 8$). Values are mean ± s.e.mean. *$P < 0.025$, apstatin *vs* controls; ◆$P < 0.05$ and ◆◆$P < 0.001$, apstatin *vs* lisinopril.

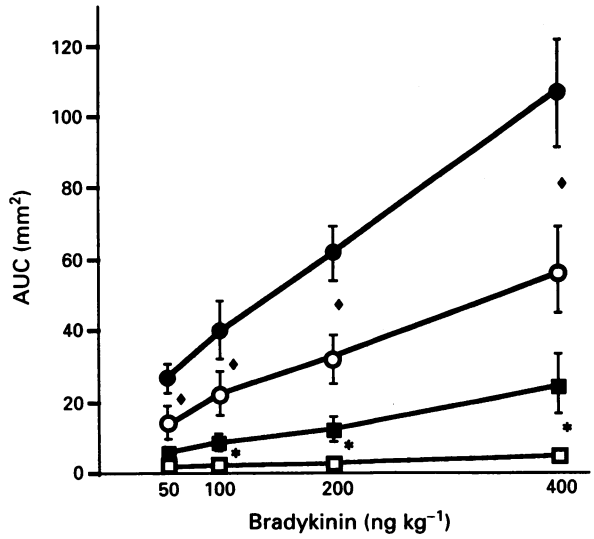


**Figure 2** Effect of treatment with apstatin, lisinopril or both on the area under the curve (AUC) representing the hypotensive response to bradykinin, (AUC includes both maximum response and duration): (□) controls ($n = 6$); (■) apstatin (80 μg kg$^{-1}$; $n = 6$); (○) lisinopril (400 μg kg$^{-1}$; $n = 6$); (●) apstatin + lisinopril ($n = 8$). Values are mean ± s.e.mean. *$P < 0.01$, apstatin *vs* controls; ◆$P < 0.025$, lisinopril + apstatin vs lisinopril alone. AUC was significantly higher after lisinopril than after apstatin at all doses ($P < 0.01$).

since the reaction to acetylcholine was also unaffected. We gave bradykinin intravenously; if the primary effect of apstatin were diminished metabolism by the lung, more kinins should enter the arterial side, resulting in an increased response, which was the case for apstatin alone.

Potentiation of the vasodepressor response to bradykinin by apstatin was markedly less than that induced by lisinopril, indicating that APP plays a lesser role than ACE as a kininase. Vasodepressor responses to bradykinin in rats treated with both lisinopril and apstatin were 5 to 8 mmHg higher than in the group treated with lisinopril alone. The differences were not significant, probably due to the dispersion of the data and/or relatively small sample size. In contrast, the AUC was doubled by adding apstatin to the ACEi.

Thus inhibition of APP added significantly to the reduction in bradykinin metabolism rate. This suggests that ACE is a more important kininase than APP in the lung; however, inhibition of APP in other organs may contribute to the prolonged response. The kidney is one candidate, since it plays an important role in bradykinin degradation (Ishida *et al.*, 1989) and is rich in APP (Holtzman *et al.*, 1987). We conclude that APP contributes to bradykinin degradation *in vivo*, suggesting that combining APP and ACE inhibitors may augment the bradykinin-mediated effects of ACEi.

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# Endotoxin inhibition of distension-stimulated gastric acid secretion in rat: mediation by NO in the central nervous system

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1 The involvement of nitric oxide in the acute inhibitory effects of low doses of endotoxin, following intracerebroventricular (i.c.v.) or intravenous (i.v.) administration, on gastric acid secretion stimulated by distension or i.v. infusion of pentagastrin has been investigated in the continuously perfused stomach of the anaesthetized rat.

2 The i.c.v. administration of *E. coli* endotoxin (800 ng $kg^{-1}$) abolished the acid secretory response induced by gastric distension (20 cm water intragastric pressure) within 30 min of administration.

3 By contrast, submaximal rates of acid secretion induced by i.v. infusion of pentagastrin (8 $\mu g\ kg^{-1}\ h^{-1}$) were not inhibited by i.c.v. administration of endotoxin (800 ng $kg^{-1}$).

4 Prior i.c.v. administration of the NO synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME, 800 $\mu g\ kg^{-1}$) restored the acid secretory responses to distension in rats treated with endotoxin (i.c.v.).

5 Likewise, i.v. administration of endotoxin (5 $\mu g\ kg^{-1}$) abolished the acid secretory response induced by gastric distension within 30 min of administration. Prior i.c.v injection of L-NAME (800 $\mu g\ kg^{-1}$) or its i.v. administration (10 mg $kg^{-1}$) restored acid secretory responses in rats receiving i.v. endotoxin.

6 The reversal by L-NAME (i.v.) of the acid inhibitory effects of endotoxin (i.v.) was prevented by L-arginine (12 mg $kg^{-1}$, i.c.v. or 100 mg $kg^{-1}$, i.v), but not by its enantiomer D-arginine.

7 The present results imply the existence of an acute response to endotoxin involving NO synthesis in the brain. NO may act as a neuromodulator or neurotransmitter in a nervous reflex leading to the inhibition of acid secretion stimulated by gastric distension.

**Keywords:** NO; gastric acid; endotoxin; central nervous system; brain-gut axis; L-NAME

## Introduction

Nitric oxide (NO) is formed when L-arginine by a variety of cell types which include the endothelium, neural tissue or immune cells (Moncada *et al.*, 1991; Moncada & Higgs, 1993). This biosynthetic process is carried out by distinct types of NO synthase, with constitutive $Ca^{2+}$ and calmodulin-dependent enzymes of endothelial and neuronal origin being characterized (Knowles *et al.*, 1989; Bredt *et al.*, 1990; Forstermann *et al.*, 1991), along with a $Ca^{2+}$-independent isoenzyme that can be induced by endotoxin or cytokines (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Busse & Mülsch, 1990; Salter *et al.*, 1991) whose expression is inhibited by glucocorticoids (Radomski *et al.*, 1990; Knowles *et al.*, 1990).

There is now considerable evidence that NO can act as a transmitter in some non-adrenergic non-cholinergic nerves, particularly in the regulation of sphincter and smooth muscle tone in gastrointestinal tissue (Sanders & Ward, 1992; Whittle, 1993). Studies with the NO synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME) have shown that acute inhibition by bolus i.v. administration of endotoxin of gastric acid secretion requires the release or synthesis of NO (Martinez-Cuesta *et al.*, 1992) and the integrity of the peripheral nervous system (Martinez-Cuesta *et al.*, 1994). Such findings raise the possibility that endotoxin could inhibit acid production by triggering a nervous reflex involving NO. The present study analyses the role of NO in the inhibitory action of endotoxin on stimulation of acid production following gastric distension, a stimulus known to be neurally mediated (Esplugues *et al.*, 1990; 1992). In addition, the involvement of NO synthesis in the brain in the acute response to endotoxin, administered either peripherally or centrally, has also been investigated. A preliminary account of this study has been presented to the American Gastroenterological Association (Esplugues *et al.*, 1993b).

## Methods

Wistar rats of either sex (200-250 g) were fasted for 24 h before the experiments but allowed drinking water. Under urethane anaesthesia (1.5 g $kg^{-1}$, i.p.) the trachea was intubated, a jugular vein cannulated and, in some experiments, systemic arterial blood pressure measured from a cannula inserted into a carotid artery. Two soft catheters were inserted into the stomach through incisions made in the cervical oesophagus and the duodenum. When the surgical preparation had been completed, the gastric lumen was flushed with 50-100 ml of saline to remove any solid content. After a 1 h stabilization period, the stomach lumen was perfused continuously (0.9 ml $min^{-1}$) via the oesophageal catheter with saline at room temperature. The gastric effluent was collected every 30 min and the $H^+$ output determined by automatic titration (Radiometer Copenhagen, Denmark) of 8 ml portions of the perfusate to pH 7 with 0.01 M NaOH.

When acid output had remained constant for 60 min it was considered as the basal acid secretion. Thereafter, distension of the stomach was obtained by placing the tip of the antroduodenal cannula 20 cm above the perfused stomach, where it was maintained for the remainder (2 h) of the experimental period. In a further group of experiments acid secretion was stimulated for 2 h by the i.v. infusion (1.25 ml $h^{-1}$) of a submaximal dose of pentagastrin (8 $\mu g\ kg^{-1}\ h^{-1}$). Rats were treated with endotoxin (*E. coli* lipopolysaccharide) either by intracerebroventricular (i.c.v.; 800 ng $kg^{-1}$) or intravenous (5 $\mu g\ kg^{-1}$) administration 10 min before the beginning of gastric distension or the initiation of the perfusion with

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pentagastrin. Values of acid production during stimulation were calculated for each animal as the difference (Δ) between the total production of acid in each 30 min period after stimulation and the basal level of acid output over a similar period of time and expressed as $\Delta\mu$Eq $H^+$ 100 $g^{-1}$ 30 $min^{-1}$ (when referring to the acid output of a particular period of 30 min) or $\Delta\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$ (when referring to the total acid production over the 120 min period examined after the stimulation of acid production). The level of gastric distension and the doses of pentagastrin and endotoxin were obtained from preliminary dose-response studies.

To analyse the role of endogenous nitric oxide in the acid inhibitory effects of endotoxin, further experiments were performed with rats treated with L-NAME either by i.c.v. (800 $\mu g\ kg^{-1}$) or i.v. (10 $mg\ kg^{-1}$), 15 min before administration of endotoxin. Both doses of L-NAME were selected as being near-maximal from preliminary dose-response studies. To evaluate the specificity of the effects of L-NAME on acid secretion, L-arginine (12 $mg\ kg^{-1}$, i.c.v. or 100 $mg\ kg^{-1}$, i.v.) or D-arginine (12 $mg\ kg^{-1}$, i.c.v. or 100 $mg\ kg^{-1}$, i.v.) was administered 5 min before treatment with L-NAME, to additional groups of rats.

To determine the importance of the systemic hypertension induced by L-NAME, control studies were undertaken in which phenylephrine (15 $\mu g\ kg^{-1}\ min^{-1}$, i.v.) was co-infused with pentagastrin for the duration of the experiment at a dose that produced an increase in blood pressure (Δ mmHg) similar to that induced by L-NAME (10 $mg\ kg^{-1}$, i.v.).

### *Drugs*

Endotoxin (lipopolysaccharide from *E. coli*, serotype 0111: B4), L-NAME, L-arginine, D-arginine and phenylephrine hydrochloride were purchased from Sigma Chemical Co. (U.S.A.). Pentagastrin was used as the clinically available preparation (Peptavlon®, ICI). All drugs were dissolved in saline immediately before use and administered in a volume of 10 $\mu l\ rat^{-1}$ (i.c.v.) or 1 $ml\ kg^{-1}$ (i.v.).

### *Statistical analysis*

All data are expressed as mean ± s.e.mean. Comparisons between groups were made by Student's *t* test for unpaired data, except for blood pressure studies in which Student's *t* test for paired data was used. A probability of $P<0.05$ or less was considered as statistically significant.

## Results

### *Effects of endotoxin on gastric acid secretion*

Distension of the rat stomach with an intragastric pressure of 20 cm water produced a progressive increase in acid secretion which reached a maximum value after 120 min with a $H^+$ production of 25 ± 4 $\mu$Eq $H^+$ 100 $g^{-1}$ 30 $min^{-1}$ (Figure 1). The total acid output during these experiments was Δ 76 ± 13 $\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$ ($n=11$). The i.c.v. injection of endotoxin (800 $ng\ kg^{-1}$, $n=8$) inhibited distension-induced acid secretion within 30 min of administration, which was maintained over the 120 min experimental period (Figure 1). Thus, total acid output induced by distension was reduced by 83 ± 6% ($P<0.01$) following administration of i.c.v. endotoxin. Intravenous administration of this low dose of endotoxin (800 $ng\ kg^{-1}$) did not reduce $H^+$ output stimulated by distension (Δ 76 ± 16 $\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$, $n=4$). Intravenous administration of a higher dose of endotoxin (5 $\mu g\ kg^{-1}$, $n=15$) did however, significantly ($P<0.001$) reduce total acid production by 76 ± 4%. Significant inhibition of acid output by i.v. endotoxin was observed within the first 30 min of distension, and was maintained for the whole 120 min experimental period (Figure 1).

The i.v. infusion of pentagastrin (8 $\mu g\ kg^{-1}\ h^{-1}$) induced a submaximal rate of acid secretion for the duration of the experimental period. The total acid output during these experiments was Δ 46 ± 8 $\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$ ($n=10$). The i.c.v. injection of endotoxin (800 $ng\ kg^{-1}$) did not significantly modify pentagastrin-stimulated acid secretion (Δ 57 ± 18 $\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$, $n=4$).

### *Effects of NO synthesis inhibition*

The i.c.v. administration of L-NAME (800 $\mu g\ kg^{-1}$) restored acid production to control distension levels (Δ 86 ± 14 $\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$, $n=7$, $P<0.001$) in rats receiving i.c.v. endotoxin (Figure 2). Prior i.c.v. injection of L-NAME (800 $\mu g\ kg^{-1}$) also restored acid responses in animals treated i.v. with endotoxin (5 $\mu g\ kg^{-1}$; $P<0.001$, $n=7$) as shown in Figure 2. This latter dose of L-NAME (800 $\mu g\ kg^{-1}$), when administered i.v., did not modify the acid inhibitory effects of endotoxin administered i.v. (Δ 30 ± 9 $\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$, $n=3$). However, prior i.v. administration of L-NAME (10 $mg\ kg^{-1}$, $n=15$) significantly ($P<0.01$) prevented the inhibitory effect of i.v. endotoxin on distension-stimulated $H^+$ secretion (Figure 2). In control studies the secretory response to distension was not modified by pretreatment with L-NAME administered either i.c.v. (800 $\mu g\ kg^{-1}$, Δ 57 ± 14 $\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$, $n=8$) or i.v. (10 $mg\ kg^{-1}$, Δ 62 ± 20 $\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$, $n=8$).

### *Reversal of effects of L-NAME by L-arginine*

In animals pretreated with L-NAME (10 $mg\ kg^{-1}$, i.v.) the i.c.v. administration of L-arginine (12 $mg\ kg^{-1}$) re-established the inhibitory effects of i.v. endotoxin on distension-stimulated gastric acid secretion (Figure 3). However, the same dose of L-arginine (12 $mg\ kg^{-1}$) when administered i.v., did not influence the reversal induced by i.v. L-NAME in endotoxin-treated rats (Δ 56 ± 26 $\mu$Eq $H^+$ 100 $g^{-1}$ 120 $min^{-1}$, $n=3$). The reversal by L-NAME (10 $mg\ kg^{-1}$, i.v.) of the inhibition of distension-stimulated acid secretion by i.v. endotoxin (5 $\mu g\ kg^{-1}$, i.v.) was however, prevented by i.v. administration of higher doses of L-arginine (100 $mg\ kg^{-1}$), as shown in Figure 3. Pretreatment with D-arginine (100 $mg\ kg^{-1}$, i.v. or 12 $mg\ kg^{-1}$, i.c.v.) did not modify the acid recovery induced by L-NAME (10 $mg\ kg^{-1}$, i.v.) in i.v. endotoxin-treated rats, as shown in Figure 3.

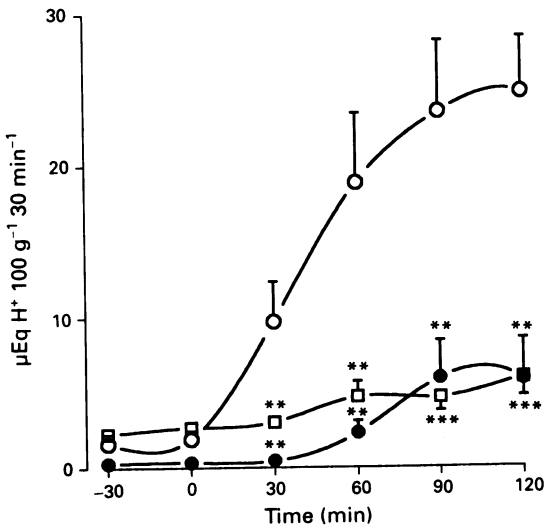


**Figure 1** Acid secretion elicited by gastric distension (intragastric pressure 20 $cmH_2O$) in the perfused stomach of anaesthetized rats after endotoxin administered either i.c.v. (800 $ng\ kg^{-1}$, ●) or i.v. (5 $\mu g\ kg^{-1}$, □). Each point represents mean ± s.e.mean of at least eight animals. Significance of difference from control, vehicle treated (○) animals is shown as $^{**}P<0.01$ and $^{***}P<0.001$.

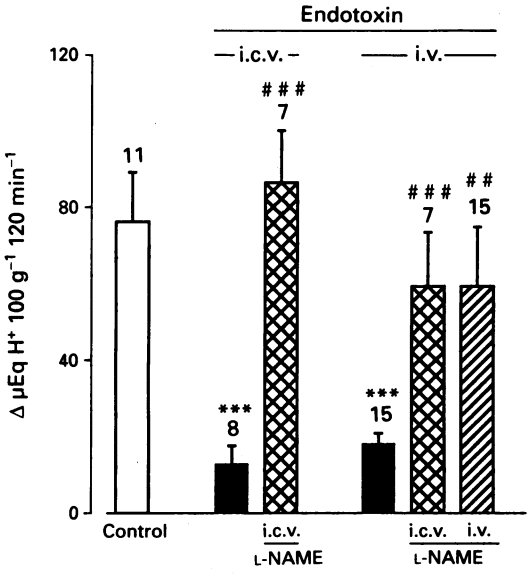


**Figure 2** Inhibition of acid secretion, induced by gastric distension (20 $cmH_2O$) by endotoxin (solid columns), administered i.c.v. (800 ng $kg^{-1}$) or i.v. (5 $\mu g\ kg^{-1}$) in the rat. Pretreatment with $N^G$-nitro-L-arginine methyl ester (L-NAME) (800 $\mu g\ kg^{-1}$, i.c.v. cross hatched columns or 10 mg $kg^{-1}$, i.v. hatched column) reversed the endotoxin-induced inhibition of the acid secretory responses elicited by gastric distension. Results, expressed as the difference between basal and stimulated acid output, are means ± s.e.mean and the number above columns is the number of animals used. The level of statistical difference from the control group (open column) is shown by $^{***}P<0.001$ and from the equivalent endotoxin-treated group as $^{**}P<0.01$ and $^{***}P<0.001$.

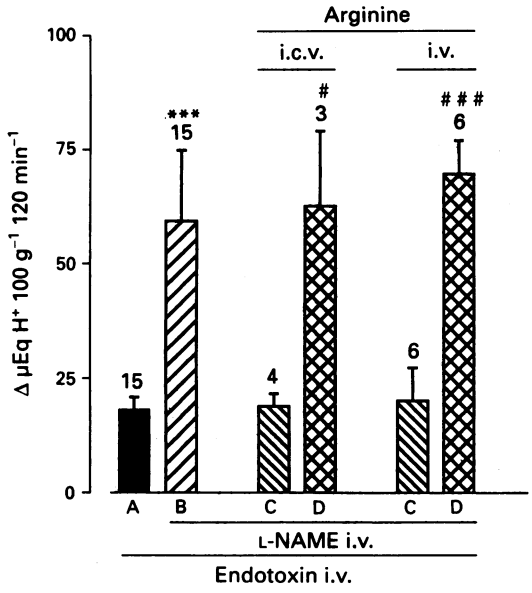


**Figure 3** Effects of the pretreatment with L-arginine (100 mg $kg^{-1}$, i.v. or 12 mg $kg^{-1}$, i.c.v., hatched columns, C), but not with D-arginine (100 mg $kg^{-1}$, i.v. or 12 mg $kg^{-1}$, i.c.v., cross-hatched columns, D), on the reversal by $N^G$-nitro-L-arginine methyl ester (L-NAME) (10 mg $kg^{-1}$, i.v., hatched column, B) of the inhibitory effects of endotoxin on acid secretion stimulated by gastric distension. Results, expressed as the differences between basal and stimulated acid output, are means ± s.e.mean and the number above columns is the number of animals used. The level of statistical difference from the endotoxin-treated group (A) is shown by $^{***}P<0.001$ and from the equivalent L-arginine-treated group as $^{*}P<0.05$ and $^{***}P<0.001$.

### *Effects on systemic blood pressure*

The administration of endotoxin, either i.c.v. or i.v., did not modify systemic arterial blood pressure, which remained between 95 and 105 mmHg ($n = 6$ for both groups) during the 120 min evaluation period. Administration of L-NAME (800 $\mu g\ kg^{-1}$, i.c.v.) did not significantly modify systemic arterial blood pressure (114 ± 7 and 125 ± 10 mmHg, $n = 5$, before and after administration). However, the initial resting blood pressure was significantly ($P<0.01$) increased from 100 ± 6 to 140 ± 5 mmHg by L-NAME (10 mg $kg^{-1}$, i.v., $n = 5$). Subsequent i.v. administration of endotoxin ($n = 6$) did not modify the hypertensive effect of L-NAME (145 ± 5 mmHg).

The i.v. infusion of phenylephrine (15 $\mu g\ kg^{-1}\ min^{-1}$, $n = 7$) produced a sustained increase in blood pressure (from 105 ± 4 to 142 ± 7 mmHg, $P<0.01$). Phenylephrine did not, however, prevent the inhibition by i.v. endotoxin of distension-stimulated acid output (88 ± 4% inhibition, $n = 7$).

## Discussion

In the present study the i.v. administration of a low dose of endotoxin (5 $\mu g\ kg^{-1}$) induced an acute and long-lasting inhibition of the acid secretory responses to gastric distension. This finding extends our previous observation showing the inhibitory effect of much higher doses of endotoxin on acid secretion stimulated by i.v. bolus administration of pentagastrin, an action mediated by the acute generation of endogenous NO (Martinez-Cuesta *et al.*, 1992; 1994). The involvement of NO was confirmed by the finding that the i.v. bolus administration of L-NAME completely reversed the inhibitory effects of endotoxin on distension-stimulated acid secretion. The failure of phenylephrine, infused at a dose producing similar increases in blood pressure as the i.v. injection of L-NAME, to restore acid responses to distension in endotoxin-treated rats, corroborates the earlier suggestions (Martinez-Cuesta *et al.*, 1992; Esplugues *et al.*, 1993a) that the actions of bolus i.v. administration of L-NAME on gastric acid output are independent of its systemic pressor actions.

In the current study, i.c.v. administration of endotoxin abolished the distension-stimulated acid responses, at doses that had no such effect when administered i.v. and which did not affect systemic blood pressure. Such findings suggest that the inhibition of acid production by endotoxin results from activation of a neuronal pathway involving the brain. This observation extends our preliminary finding that *in vivo* inhibition of acid production by systemic administration of higher doses of endotoxin requires the integrity of the peripheral nervous system and involves the acute activation of a neural pathway, as shown by the finding that local gastric serosal application of the neurotoxin, tetrodotoxin, completely restored acid responses to pentagastrin in rats treated with high doses of endotoxin (Martinez-Cuesta *et al.*, 1994). However, in the present study, i.c.v. administration of endotoxin in doses that abolished distension-induced acid secretion failed to affect the secretory response to pentagastrin. Such findings could suggest that whereas endotoxin may act both peripherally and centrally to inhibit gastric acid secretion, perhaps involving effects on the same neural pathway, neuronally mediated acid secretion is more sensitive to the endotoxin-provoked processes originating in the brain.

The nature of the inhibitory actions of endotoxin appear to involve local generation of NO in brain tissue since i.c.v. injection of L-NAME reversed the actions of endotoxin, administered either peripherally or centrally. The actions of L-NAME administered i.v. were prevented by the i.v. administration of L-arginine, but not by its enantiomer D-arginine. Furthermore, i.c.v. administration of low doses of L-arginine restored the acid inhibitory effects of i.v. administered endotoxin in rats treated with a bolus i.v. injection of L-NAME indicating penetration of the central nervous system

by L-NAME. The L-arginine: NO pathway has been identified in a number of central and peripheral neuronal tissues (Garthwaite *et al.*, 1989; Knowles *et al.*, 1989; Bredt *et al.*, 1990; Mitchell *et al.*, 1991; Nishida *et al.*, 1992; Nichols *et al.*, 1993). Although the biological functions of NO in the nervous system have not yet been fully clarified, there is substantial evidence that NO is a transmitter in at least some NANC nerves in the peripheral nervous system, and may act as a neuromodulator in others. The mechanism by which endotoxin can activate neuronal processes and the potential for multiple influences of NO thus requires careful consideration. Moreover, the selective inhibition of neuronally evoked acid secretion by i.c.v. endotoxin through a NO-dependent process is consistent with the preferential inhibition of distension-induced acid output by administration of NO donors (Barrachina *et al.*, 1994).

It had been shown that the excessive production of NO by vascular and inflammatory cells which follows the exposure to endotoxin is triggered by the induction of a NO synthase, a process which needs several hours (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Busse & Mülsch, 1990; Radomski *et al.*, 1990; Smith *et al.*, 1992). Our results make it unlikely that such an inducible enzyme is involved in the inhibition by endotoxin of acid secretion, since this response occurs within a few minutes and such induction in neuronal tissue is not readily apparent. However, it has been proposed that endotoxin can induce the acute release of NO from neutrophils (Salvemini *et al.*, 1990) and of constitutively formed NO in the cardiovascular system (Thiemermann & Vane, 1990; Julou-Schaeffer *et al.*, 1990; Kilbourn *et al.*, 1990), although the acute effects of endotoxin on the activity of neuronal NO synthase are unknown.

The present results imply the existence of an acute response to endotoxin when administered peripherally or centrally, involving NO synthesis in the brain. Such NO formation may be involved in intracellular communication by acting as a neuromodulator or neurotransmitter in a nervous reflex that leads to the inhibition of gastric acid secretion. The inhibitory mechanisms so evoked may also involve other neurotransmitters and the release of inhibitory hormones or local mediators in the vicinity of the parietal cells, that prevent the secretion of hydrogen ions.

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# Significance of nitric oxide in the stimulation of intestinal fluid absorption in the rat jejunum *in vivo*

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**1** The effects of inhibiting nitric oxide (NO)-synthase on fluid transport, mucosal cyclic GMP and cyclic AMP levels and intraluminal prostaglandin $E_2$ ($PGE_2$)-release were studied in a model of ligated jejunal loops of anaesthetized rats *in vivo*. Experiments were performed under basal conditions as well as under conditions, when net fluid secretion was induced by *Escherichia coli* heat stable enterotoxin a (*E. coli* STa) or $PGE_2$.

**2** Intravenous infusion of the NO-synthase inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 0.25–50 mg $kg^{-1}$, 45 min) dose-dependently reversed net fluid absorption to net secretion, whereas infusion of D-NAME, the inactive enantiomer of L-NAME, in corresponding doses did not influence net fluid transport. $N^{\omega}$-nitro-L-arginine (L-NOARG, 25 mg $kg^{-1}$), another NO-synthase inhibitor, also elicited net secretion of fluid.

**3** L-NAME (25 mg $kg^{-1}$)-induced net fluid secretion was reversed to net absorption by infusion of L-arginine (400 mg $kg^{-1}$) or sodium nitroprusside (1 mg $kg^{-1}$) and s.c. administration of indomethacin (10 mg $kg^{-1}$). Hexamethonium (1 mg $kg^{-1}$, s.c.), a ganglionic blocker and granisetron (100 μg $kg^{-1}$, s.c.), a 5-$HT_3$-receptor antagonist, did not influence L-NAME-induced net secretion.

**4** Net fluid secretion induced by intraluminal instillation of *E. coli* STa (10 units $ml^{-1}$) was enhanced by infusion of L-NAME (25 mg $kg^{-1}$) and was inhibited by infusion of L-arginine (400 mg $kg^{-1}$) and sodium nitroprusside (1 mg $kg^{-1}$). D-Arginine (400 mg $kg^{-1}$) did not influence *E. coli* STa-induced fluid secretion. Likewise, net fluid secretion induced by i.a. infusion of $PGE_2$ (79 ng $ml^{-1}$, 30 min) was enhanced by infusion of L-NAME and was inhibited by L-arginine and sodium nitroprusside. D-Arginine (400 mg $kg^{-1}$) did not influence $PGE_2$-induced fluid secretion.

**5** $PGE_2$ levels in intraluminal fluid were not elevated after infusion of L-NAME (25 mg $kg^{-1}$) compared to controls.

**6** Mucosal cyclic GMP and cyclic AMP levels after L-NAME-treatment were not different from control values.

**7** These results indicate that nitric oxide plays an important role in the regulation of intestinal fluid transport. The data suggest a nitric oxide-dependent proabsorptive tone in the intestine, which possibly involves the enteric nervous system and suppression of prostaglandin formation. This proabsorptive tone also may downregulate fluid secretion induced by *E. coli* STa or $PGE_2$.

**Keywords:** Intestinal fluid transport; nitric oxide; *Escherichia coli* enterotoxin-induced secretion; prostaglandin $E_2$; cyclic GMP; $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME); L-arginine; sodium nitroprusside; indomethacin

## Introduction

Nitric oxide (NO) recently has been established as an important mediator of various physiological and pathophysiological processes in the gastrointestinal tract (Stark & Szurszewski, 1992). Endogenous NO derives from enzymatic conversion of L-arginine through NO-synthase, a family of isoenzymes, the NO-generating activity of which can be inhibited stereospecifically by L- but not by D-arginine analogues (Ignarro, 1991). In the gut, NO-synthase activity has been found throughout the intestinal wall in enteric neurones (Aimi *et al.*, 1993), enteric blood vessels (Nichols *et al.*, 1993) and enterocytes (M'Rabet-Touil *et al.*, 1993). Intestinal NO can furthermore be produced by neutrophils (McCall *et al.*, 1989), macrophages (Hibbs *et al.*, 1988) and mast cells (Salvemini *et al.*, 1990). This extensive localization of potentially NO-releasing sites supports the view that NO plays a multifaceted role in intestinal physiology and disease.

Gastrointestinal functions which have been shown to be regulated and influenced by NO include gut motility (Calignano *et al.*, 1992), where NO is suggested to mediate smooth muscle relaxation as the inhibitory non-adrenergic non-cholinergic enteric neurotransmitter (Bult *et al.*, 1990), gastric mucosal blood flow (Pique *et al.*, 1989), protection of gastric (MacNaughton *et al.*, 1989) and intestinal (Hutcheson *et al.*, 1990) mucosa, gastric mucus secretion (Brown *et al.*, 1993) and intestinal electrolyte and fluid transport.

The role of NO in intestinal fluid and electrolyte transport appears ambiguous when taking into consideration the studies concerning this matter: *in vitro* studies, applying Ussing chamber technique, have shown that serosal administration of NO or NO-donating compounds, like sodium nitroprusside, to stripped intestinal tissue increases short circuit current, thus indicating a prosecretory and/or antiabsorptive role of NO (Wilson *et al.*, 1993; MacNaughton, 1993; Tamai & Gaginella, 1993). Corresponding with these results, castor-oil-induced diarrhoea was prevented by administration of NO-synthase-inhibitors in an *in vivo* study (Mascolo *et al.*, 1993). Conversely, inhibition of NO-synthase *in vivo* resulted in gastric and duodenal $HCO_3^-$ (Takeuchi *et al.*, 1993) and intestinal fluid secretion (Mourad *et al.*, 1993; Miller *et al.*, 1993a,b) and in an enhancement of intestinal epithelial permeability (Kubes, 1992; Miller *et al.*, 1993c), thus pointing towards an antisecretory and/or proabsorptive role of NO in the intestine.

The mechanisms of action underlying any effect of NO on intestinal fluid and electrolyte transport remain unclear.

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Activation of guanylate cyclase and formation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) is a commonly accepted transduction mechanism of NO (Ignarro, 1991), and some authors suggest that this mechanism is responsible also for the observed intestinal effects (Wilson *et al.*, 1993; MacNaughton, 1993; Tamai & Gaginella, 1993). Moreover, the participation of enteric nerves and arachidonic acid metabolites in the mediation of NO effects is proposed (Wilson *et al.*, 1993; Tamai & Gaginella, 1993; Takeuchi *et al.*, 1993). Similar mechanisms of signal transduction have been shown to be involved in the mediation of fluid secretion induced by *Escherichia coli* heat stable enterotoxin a (*E. coli* STa): the enterotoxin elevates intestinal cyclic GMP levels (Hughes *et al.*, 1978) and involves prostaglandins (Thomas & Knoop, 1982) and the enteric nervous system (Eklund *et al.*, 1985) in its mechanism of action. In addition, 5-hydroxytryptamine (5-HT) has also been demonstrated to participate in the mediation of *E. coli* STa-induced fluid secretion (Beubler *et al.*, 1992).

The aim of the present study was to elucidate the significance of NO in the regulation of intestinal fluid transport *in vivo* under basal as well as under secretory conditions induced by the secretagogues *E. coli* STa and prostaglandin $E_2$ ($PGE_2$).

## Methods

### *Preparation of animals and experimental design*

Female Sprague-Dawley rats (180 ± 20 g body wt.) were used in this study. They were maintained on a standard laboratory diet and were deprived of food for 18 h before the experiments but had free access to water. The rats were anaesthetized with sodium pentobarbitone (60 mg $kg^{-1}$, i.p.), the abdomen was opened via a midline incision and a polyethylene catheter (PE60) was placed in the jejunum ~5 cm distal to the flexura duodenojejunalis and fixed by ligation. The second ligation was made ~20 cm distal to the first ligation. The loop was carefully rinsed with 20 ml of body-warm saline and the distal ligation was tied off. The jejunal loop was then returned to the abdominal cavity and the whole preparation was allowed to rest for 1 h under a heating lamp to preserve body temperature. Anaesthesia was maintained by s.c. injection of sodium pentobarbitone (20 mg $kg^{-1}$).

The experiments were started by i.v. infusion of saline or the following substances into the jugular vein, using a perfusion pump (Braun-Diessel, Melsungen, Germany) at a flow rate of 0.949 ml $h^{-1}$: $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME), $N^{\omega}$-nitro-L-arginine (L-NOARG), inhibitors of NO-synthase; $N^{\omega}$-nitro-D-arginine methyl ester (D-NAME), the inactive enantiomer of L-NAME, L-arginine, the substrate of NO synthase, that can overcome the inhibitory actions of L-arginine analogues, D-arginine, the inactive enantiomer of L-arginine, and sodium nitroprusside, a spontaneously NO-releasing compound. Infusion was maintained for 45 min; 15 min after the start of i.v. infusion, the jejunal loop was slowly filled with 2.0 ml of body warm Tyrode solution via the catheter, which was subsequently closed with a stopper. In experiments with *E. coli* STa, the same procedure was carried out except that the enterotoxin was added to the Tyrode solution to give a final concentration of 10 units $ml^{-1}$.

$PGE_2$ (79 ng $min^{-1}$) was infused close i.a. into a branch of the superior mesenteric artery for 30 min, the infusion starting 15 min after the start of i.v. infusion of saline, L-NAME, L-arginine, D-arginine or sodium nitroprusside. Tyrode solution was instilled intraluminally at the same time as the $PGE_2$ infusion was started. Indomethacin (10 mg $kg^{-1}$), a cyclooxygenase blocker, hexamethonium (1 mg $kg^{-1}$), a ganglionic blocker, and granisetron (100 μg $kg^{-1}$), an antagonist at 5-$HT_3$-receptors, were administered s.c. 10 min before i.v. infusion was started.

At the end of the experiments the animals were killed by injection of a lethal dose of the narcotic.

### *Determination of net fluid transport*

Net fluid transfer rates were determined gravimetrically 30 min after instillation of Tyrode solution (with or without *E. coli* STa). The catheter was removed, the proximal ligation tied off and the jejunal loop quickly withdrawn and weighed. Net fluid transport is expressed as millilitres per gram wet weight (ml $g^{-1}$ wet wt.) of jejunum. Net absorption is indicated by a negative value and net secretion by a positive value.

### *Determination of cyclic nucleotides*

Isobutyl methyl xanthine, a phosphodiesterase inhibitor, was added to Tyrode solution (1 mmol $l^{-1}$) in experiments for determination of cyclic nucleotide levels. The cyclic GMP and cyclic AMP contents were measured at the end of the experiment. The jejunal loop was removed, opened, and the mucosa scraped off and immediately frozen in liquid nitrogen. After extraction, mucosal cyclic GMP was measured with the cyclic GMP-radioimmunoassay kit (TRK 500, Amersham International, UK), cyclic AMP was measured with the cyclic AMP assay kit (TRK 432, Amersham). Cyclic nucleotide levels are expressed as pmol $mg^{-1}$ of protein (Protein Assay Bio-Rad Laboratories, Munich, Germany).

### *Determination of $PGE_2$*

The jejunal wall was punctured with a hypodermic needle at the end of the experiment, and a small sample of fluid (~0.6 ml) was withdrawn into a polyethylene tube and immediately frozen in solid $CO_2$. The samples were stored at −70°C and analysed within 3 weeks.

After thawing and centrifuging the samples (800 *g*, 3 min), $PGE_2$ was determined in 400 μl aliquots of the supernatant by radioimmunoassay (Bukhave & Rask-Madsen, 1981), using rabbit anti-prostaglandin E-BSA serum (BioMakor, Rehovot, Israel) as antibody.

### *Chemicals*

The following chemicals and reagents were used: $N^{\omega}$-nitro-L-arginine methyl ester, $N^{\omega}$-nitro-D-arginine methyl ester, $N^{\omega}$-nitro-L-arginine, L-arginine, D-arginine, sodium nitroprusside, *Escherichia coli* heat stable enterotoxin a, isobutyl methyl xanthine, hexamethonium, prostaglandin $E_2$ (Sigma Chemical Company, Munich, Germany), indomethacin (Merck, Sharp & Dohme, Rahway, N.J., U.S.A.), granisetron (SmithKline Beecham Pharmaceuticals, Crawley, UK), sodium pentobarbitone (Sanofi, Libourne, France). All other chemicals were of analytic grade (E. Merck AG, Darmstadt, Germany).

### *Statistics*

Results are given as mean ± s.e.mean, and the data were analysed by the two-sample Student's *t* test or by analysis of variance (ANOVA) and Dunnett's test or the Student-Newman-Keuls test. Probability values $<0.05$ were considered significant.

## Results

### *Effects on basal fluid transport*

Infusion of saline resulted in net absorption of luminal fluid (Figure 1). L-NAME dose-dependently (0.25–50 mg $kg^{-1}$; 5.55 μg $kg^{-1}$ $min^{-1}$–1.11 mg $kg^{-1}$ $min^{-1}$) reversed net absorp-

tion to net fluid secretion, whereas infusion of D-NAME in corresponding doses did not influence control net absorption significantly (Figure 1). Further experiments with L-NAME were performed at the dose of 25 mg kg$^{-1}$ (= 0.55 mg kg$^{-1}$ min$^{-1}$).

L-NOARG (25 mg kg$^{-1}$) also elicited net fluid secretion of + 0.14 ± 0.04 ml g$^{-1}$ wet weight ($n$ = 6), secretion which corresponds to the value obtained with an equivalent dose of L-NAME (data not shown).

Infusion of L-arginine (400 mg kg$^{-1}$ = 8.88 mg kg$^{-1}$ min$^{-1}$) slightly but not significantly enhanced net fluid absorption compared to controls (Figure 2). L-Arginine reversed L-NAME-induced fluid secretion to net absorption (Figure 2).

Infusion of sodium nitroprusside (1 mg kg$^{-1}$ = 22.2 μg kg$^{-1}$ min$^{-1}$) showed no effect on control net absorption and also inhibited the effect of L-NAME (Figure 2). Administration of indomethacin (10 mg kg$^{-1}$, s.c.) did not influence control net fluid absorption (Figure 2). L-NAME-induced fluid secretion was significantly reduced by indomethacin-pretreatment (Figure 2).

Neither s.c. pretreatment with hexamethonium (1 mg kg$^{-1}$) nor granisetron (100 μg kg$^{-1}$) influenced basal fluid absorption or L-NAME-induced fluid secretion (Figure 2).

### *Effects on* E. coli *STa-induced fluid secretion*

Intraluminal instillation of *E. coli* STa (10 units ml$^{-1}$) reversed net fluid absorption to net secretion (Figure 3). Infusion of L-NAME resulted in a pronounced enhancement of *E. coli* STa-induced secretion (Figure 3). Infusion of L-arginine (400 mg kg$^{-1}$) as well as infusion of sodium nitroprusside (1 mg kg$^{-1}$) significantly inhibited *E. coli* STa-induced net fluid secretion, but control levels of net absorption were not reached (Figure 3). Infusion of D-arginine (400 mg kg$^{-1}$) did not significantly influence *E. coli* STa-induced net fluid secretion (+ 0.10 ± 0.06 ml g$^{-1}$ wet weight, $n$ = 10).

### *Effects on $PGE_2$-induced fluid secretion*

Close i.a. infusion of $PGE_2$ (79 ng ml$^{-1}$) reversed net fluid absorption to net secretion (Figure 4). Infusion of L-NAME markedly enhanced $PGE_2$-induced fluid secretion (Figure 4). Administration of L-arginine (400 mg kg$^{-1}$, i.v.) as well as administration of sodium nitroprusside (1 mg kg$^{-1}$, i.v.) significantly reduced $PGE_2$-induced fluid secretion (Figure 4). Infusion of D-arginine (400 mg kg$^{-1}$) did not significantly influence $PGE_2$-induced fluid secretion (+ 0.21 ± 0.04 ml g$^{-1}$ wet weight, $n$ = 9).

### *Effects on cyclic nucleotide-levels*

L-NAME (25 mg kg$^{-1}$, i.v.), D-NAME and L-NAME in combination with L-arginine (400 mg kg$^{-1}$, i.v.) did not influence

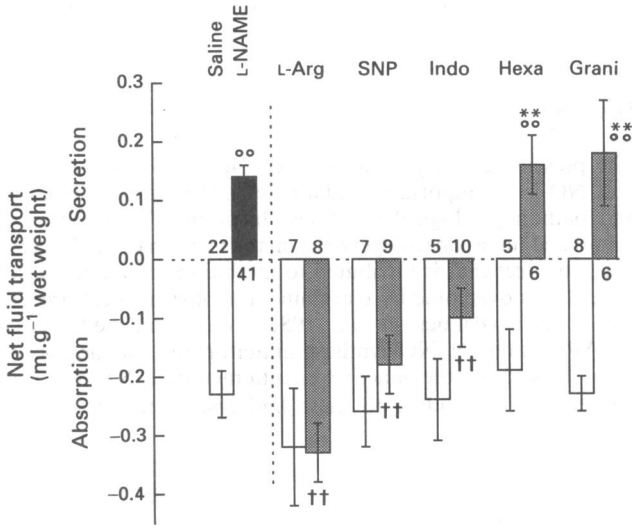


**Figure 2** Effects of L-arginine (L-Arg) (400 mg kg$^{-1}$, i.v.), sodium nitroprusside (SNP) (1 mg kg$^{-1}$, i.v.), indomethacin (Indo) (10 mg kg$^{-1}$, s.c.), hexamethonium (Hexa) (1 mg kg$^{-1}$, s.c.) and granisetron (Grani) (100 μg kg$^{-1}$, s.c.) on basal (open columns) and on L-NAME (25 mg kg$^{-1}$)-influenced net fluid transport (crosshatched columns) in the rat jejunum *in vivo* in comparison with the influence of saline and L-NAME (solid columns) on basal net fluid transport. Each column represents the mean ± s.e.mean. The numerals indicate the number of experiments; °°$P$ < 0.01 compared to saline; **$P$ < 0.01 compared to the respective control, ††$P$ < 0.01 compared to L-NAME (Student's $t$ test).

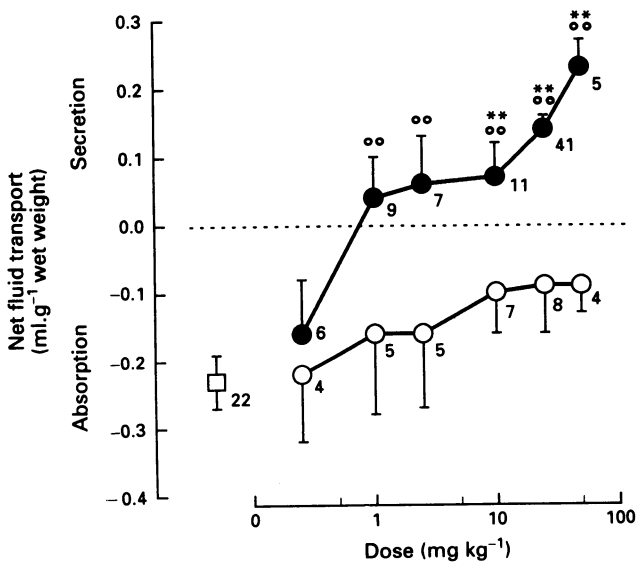


**Figure 1** Effects of infusion of saline (□) N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, ●) and D-NAME (○) (0.25–50 mg kg$^{-1}$) on net fluid transport in the rat jejunum *in vivo*. Each point represents the mean ± s.e.mean. The numerals indicate the number of experiments; °°$P$ < 0.01 compared to saline (Dunnett's test), **$P$ < 0.01 compared to D-NAME (Student's $t$ test).

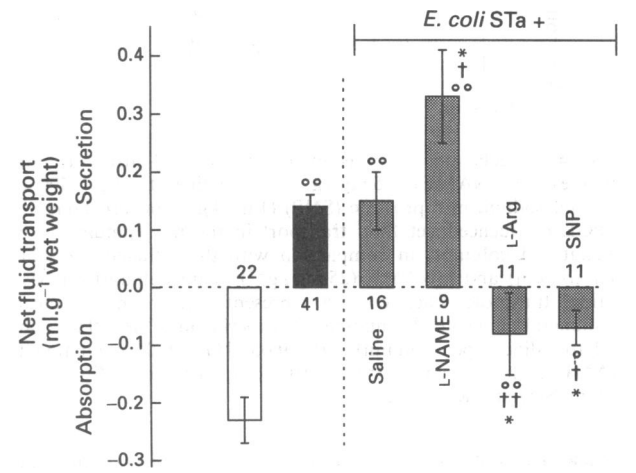


**Figure 3** Effects of i.v.-infusion of saline, N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) (25 mg kg$^{-1}$), L-arginine (L-Arg), (400 mg kg$^{-1}$) and sodium nitroprusside (SNP) (1 mg kg$^{-1}$) on *E. coli* STa (10 units ml$^{-1}$)-influenced net fluid transport in the rat jejunum *in vivo* (crosshatched columns) in comparison with the influence of saline (open column) and L-NAME (25 mg kg$^{-1}$) (solid column) on basal net fluid transport. Each column represents the mean ± s.e.mean. The numerals indicate the number of experiments; °$P$ < 0.05, °°$P$ < 0.01 compared to saline (open column); †$P$ < 0.05, ††$P$ < 0.01 compared to L-NAME (solid column); *$P$ < 0.05 compared to *E. coli* STa (saline) (Student-Newman-Keuls' test).

cyclic GMP-levels (Table 1). L-NAME furthermore did not influence mucosal cyclic AMP levels compared to control (Table 1).

### *Effects on $PGE_2$-levels*

Infusion of L-NAME (25 mg $kg^{-1}$), without and with indomethacin-pretreatment (10 mg $kg^{-1}$, s.c.), did not influence $PGE_2$-contents in intraluminal fluid compared to controls. The luminal $PGE_2$-contents were $0.12 \pm 0.03$ ng $g^{-1}$ wet weight of jejunum (saline, $n = 8$), $0.13 \pm 0.05$ (L-NAME, 25 mg $kg^{-1}$, $n = 12$), $0.03 \pm 0.02$ (saline + indomethacin, 10 mg $kg^{-1}$, $n = 6$), $0.12 \pm 0.05$ (L-NAME + indomethacin, $n = 6$).

## Discussion

The present results provide further support for the concept that NO is an important mediator of intestinal physiological and pathophysiological functions. Infusion of L-NAME i.v. stimulated in a dose-dependent manner jejunal fluid secretion, an effect that is attributed to inhibition of NO-synthase, since it was overcome by L-arginine, the physiological precursor of NO (Palmer *et al.*, 1988), was mimicked by L-NOARG, another NO-synthase-inhibitor and did not occur when D-NAME, the inactive enantiomer of L-NAME, was infused. Corresponding to these findings, sodium nitroprusside, a compound that spontaneously releases NO, did not influence basal fluid transport but completely blocked L-NAME-induced fluid accumulation. These results indicate a proabsorptive role of NO in the regulation of jejunal fluid transport.

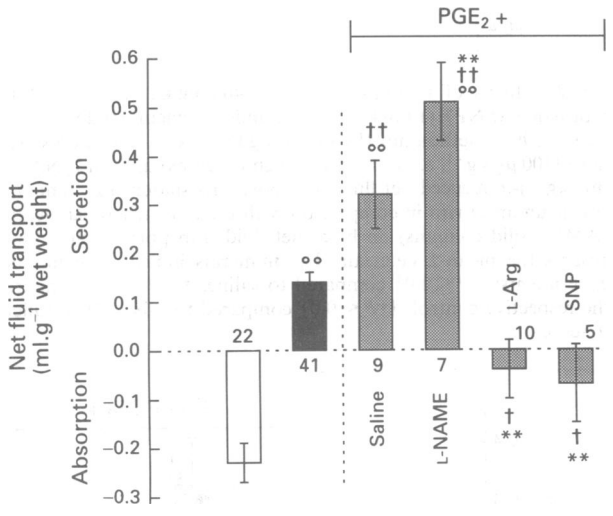


**Figure 4** Effects of i.v.-infusion of saline, $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) (25 mg $kg^{-1}$), L-arginine (L-Arg), (400 mg $kg^{-1}$) and sodium nitroprusside (SNP) (1 mg $kg^{-1}$) on prostaglandin $E_2$ ($PGE_2$)-influenced net fluid transport in the rat jejunum *in vivo* (crosshatched columns) in comparison with the influence of saline (open column) and L-NAME (25 mg $kg^{-1}$) (solid column) on basal net fluid transport. Each column represents the mean ± s.e.mean. The numerals indicate the number of experiments; $^{oo}P < 0.01$ compared to saline (open column); $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ compared to L-NAME (solid column); $^{**}P < 0.01$ compared to $PGE_2$ (saline) (Student-Newman-Keuls' test).

Assuming that a proabsorptive effect is synonymous with a beneficial action, the data are in accordance with several studies, which have demonstrated a beneficial role of NO in intestine: inhibition of NO-synthase *in vivo* enhanced *E. coli* lipopolysaccharide-induced jejunal damage and plasma leakage, whereas administration of a NO-generating compound attenuated these effects (Hutcheson *et al.*, 1990; Boughton-Smith *et al.*, 1990). NO has furthermore been demonstrated to have an important role in maintaining the integrity of the mucosal barrier, derived from the observation, that i.a. infusion of L-NAME resulted in an increase in epithelial permeability of the feline ileum. This effect was reversed by sodium nitroprusside and L-arginine and appeared to involve neither tissue injury nor alterations in blood flow (Kubes, 1992). In another study, oral administration of L-NAME to naive guinea-pigs resulted in ileal fluid secretion, whereas the same treatment ameliorated chronic ileitis (Miller *et al.*, 1993a,b). The authors proposed that NO may be protective under basal and acute pathological conditions, but deleterious under chronic pathological conditions and suggested this phenomenon might be due solely to the presence of the constitutive form of NO-synthase under basal and acute conditions and the expression of the inducible form under chronic conditions. It cannot be ruled out, that the ability of NO to participate in inflammatory processes could play a role in the observed secretory response to NO-synthase-inhibition. NO has been demonstrated to mediate inflammation (Ialenti *et al.*, 1992; Middleton *et al.*, 1993) by its cytostatic and cytotoxic properties, but its role in inflammation seems to be a dual one (Moncada & Higgs, 1993; Miller *et al.*, 1993a,b,c). We did not measure parameters of inflammation but macroscopically we did not observe reddening of the jejunum or intraluminal haemorrhage after L-NAME-treatment as an indicator of inflammation. Accordingly, Hutcheson and coworkers (1990) also did not observe jejunal damage after i.v. administration of an NO-synthase inhibitor.

In contrast to a beneficial role of NO are *in vivo* experiments, where NO-synthase inhibition prevented castor-oil-induced diarrhoea (Mascolo *et al.*, 1993). This finding seems unexplainable in the light of the present results, but it should be considered, that L-NAME was administered i.p. in the study of Mascolo *et al.* (1993) and i.v. in the present experiments. In most studies, which have demonstrated a beneficial role of NO in intestine, the route of administration of the substances was via the blood, either i.v. or i.a. (Hutcheson *et al.*, 1990; Boughton-Smith *et al.*, 1990; Kubes, 1992). When considering this fact, the question arises, whether the observed secretory effects of NO inhibitors may just reflect their influence on blood flow i.e. be the result of their vasoconstrictor effects. Conclusive evidence for a relationship between any vasoactive and secretagogue action has, however, not been demonstrated. Some secretagogues are vasodilators (Granger *et al.*, 1987) (e.g. $PGE_2$), whereas others are vasoconstrictors (e.g. 5-HT).

**Table 1** Influence of i.v. infusion of $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 25 mg $kg^{-1}$ = 0.55 mg $kg^{-1}$ $min^{-1}$), D-NAME (25 mg $kg^{-1}$) and L-NAME (25 mg $kg^{-1}$) + L- arginine (400 mg $kg^{-1}$ = 8.88 mg $kg^{-1}$ $min^{-1}$) on mucosal levels of cyclic GMP and cyclic AMP in the rat jejunum

| | *Cyclic GMP level* (pmol $mg^{-1}$ protein) | *n* | *Cyclic AMP level* (pmol $mg^{-1}$ protein) | *n* |
|---|---|---|---|---|
| Saline | 0.22 ± 0.03 | 17 | 6.36 ± 0.41 | 9 |
| L-NAME | 0.16 ± 0.01 | 17 | 5.77 ± 0.16 | 9 |
| D-NAME | 0.24 ± 0.04 | 11 | Not tested | |
| L-NAME + L-arginine | 0.26 ± 0.04 | 11 | Not tested | |

Values are mean ± s.e.mean; *n* refers to the number of animals studied.

Sodium nitroprusside or exogenous NO, administered to stripped colonic (Wilson *et al.*, 1993; Tamai & Gaginella, 1993) or ileal (MacNaughton, 1993) tissue, induced electrolyte secretion *in vitro*, whereas application of NO-synthase inhibitors did not alter the basal response in short circuit current (Wilson *et al.*, 1993; MacNaughton, 1993). These studies also seem to be in conflict with the present results. The most probable explanation of the differences of *in vivo* and *in vitro* findings may be the preparation of the tissue, which was dissected from muscle layers and neuronal influences in the Ussing chamber studies. The observed effects on fluid transport *in vivo* may involve an influence of NO-synthase inhibition on nervous transmission, comparable to the effect of NO-synthase inhibition on gut motility. Application of NO-synthase inhibitors induces intestinal contractions and increases the basal tension due to inhibition of non-adrenergic non-cholinergic neurotransmission (Boeckxstaens *et al.*, 1990; Calignano *et al.*, 1992; Rand, 1992). It can be speculated, that a NO-dependent proabsorptive tone exists in the intestine, similar to the proposed intestinal NO-dependent dilator tone (Moncada & Higgs, 1993). Hexamethonium, however, did not influence L-NAME-induced secretion in the present experiments thus excluding the participation of nicotinic receptors in the secretory response.

5-$HT_3$ receptors, that are involved in the mediation of *E. coli* STa- (Beubler *et al.*, 1992) and cholera toxin-induced secretion (Beubler & Horina, 1990; Bhardan *et al.*, 1993), are obviously not involved in the secretory response to L-NAME, since granisetron, an antagonist at these receptors, did not influence L-NAME-induced secretion, indicating that the involvement of 5-HT seems unlikely.

Indomethacin significantly reduced L-NAME-induced fluid secretion, suggesting the involvement of prostaglandins in the secretory response to NO-synthase-inhibition. Comparable findings have been reported by Takeuchi and coworkers (1993), who demonstrated that indomethacin-pretreatment inhibited L-NAME-induced duodenal $HCO_3$-secretion. The authors proposed, that NO plays a suppressive role in the regulation of $HCO_3$-secretion and that the action of L-NAME involves both prostaglandin-sensitive and neuronal pathways. In the present experiments, however, enhanced levels of $PGE_2$ in the jejunal lumen after infusion of L-NAME could not be detected. This negative result could also be due to a transient release of small amounts of $PGE_2$ and/or a fast degradation, so that a spill-over of the prostaglandin into the lumen is prevented.

Infusion of L-NAME exerted an additive effect on both *E. coli* STa and $PGE_2$-induced secretion in the present experiments. These results again argue for a proabsorptive tone, which is present also when the intestine secretes fluid due to the action of these secretagogues, and which is prevented by NO-synthase inhibition. Interestingly, infusion of L-arginine as well as sodium nitroprusside inhibited both *E. coli* STa and $PGE_2$-induced secretion. Thus, reinforcement of the NO-dependent proabsorptive action by exogenous NO may reverse active secretion of fluid to absorption.

The existence of an endogenously regulated proabsorptive tone, dependent on endogenous opiates, was also demonstrated previously by Beubler & Lembeck (1980). The authors reported, that blockade of morphine receptors by naloxone enhanced $PGE_1$-induced intestinal fluid secretion.

The mechanism of action of NO in the intestine has not yet been elucidated. MacNaughton (1993) found a 44 fold increase in mucosal cyclic GMP levels after application of sodium nitroprusside to stripped ileal tissue. This result seems to be questionable, since it has been reported that NO activates the soluble, but not the particulate form of guanylate cyclase (DeJonge, 1984) and that in the intestine predominantly the particulate form of the enzyme is present (DeJonge, 1975). Accordingly, in a previous study, soluble guanylate cyclase could not be detected in a preparation of scraped jejunal mucosa, and the particulate guanylate cyclase, which was present, could not be activated by sodium nitroprusside (Beubler *et al.*, 1993). However, elevation of mucosal cyclic GMP levels occurred in the same preparation after challenge with *E. coli* STa (Beubler *et al.*, 1992). In the present experiments we did not find alterations of mucosal cyclic GMP levels after administration of L-NAME *in vivo*.

In conclusion we suggest that there exists an endogenous proabsorptive tone in the intestine, which is dependent on NO and possibly involves the enteric nervous system and suppression of prostaglandin formation. This proabsorptive tone also seems to be present when the gut secretes fluid into the lumen after challenge with secretagogues like *E. coli* STa or $PGE_2$. Provision of exogenous NO may be a possible tool to reverse net fluid secretion to net absorption.

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# Electrophysiological effects of Ro 22-9194, a new antiarrhythmic agent, on guinea-pig ventricular cells

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**1** Cardiac effects of Ro 22-9194 were examined in papillary muscles and single ventricular myocytes isolated from guinea-pigs and compared with those of moricizine.

**2** In papillary muscles, both Ro 22-9194 ($\geqslant 10\,\mu M$) and moricizine ($\geqslant 1\,\mu M$) caused a significant dose-dependent decrease in the maximum upstroke velocity ($\dot{V}_{max}$) and a shortening of the action potential duration.

**3** In the presence of either drug, trains of stimuli at rates $\geqslant 0.2$ Hz led to an exponential decline in $\dot{V}_{max}$. This use-dependent block was enhanced at higher stimulation frequencies. A time constant ($\tau R$) for $\dot{V}_{max}$ recovery from the use-dependent block was 9.3 s for Ro 22-9194 and 26.4 s for moricizine.

**4** The curves relating membrane potential and $\dot{V}_{max}$ in single myocytes were shifted by Ro 22-9194 ($30\,\mu M$) or by moricizine ($3\,\mu M$) in a hyperpolarizing direction by 8.4 mV and 8.0 mV respectively.

**5** In myocytes treated with Ro 22-9194 ($30\,\mu M$), a 10 ms conditioning clamp to 0 mV caused a significant decrease in $\dot{V}_{max}$ of the subsequent test action potential; further prolongation of the clamp pulse duration resulted in a modest enhancement of the $\dot{V}_{max}$ inhibition. In the presence of moricizine ($3\,\mu M$), a similar conditioning clamp $>200$ ms caused a significant $\dot{V}_{max}$ reduction; the longer the clamp pulse duration, the greater the $\dot{V}_{max}$ reduction.

**6** Ro 22-9194 $\geqslant 30\,\mu M$ caused a slight decrease of calcium inward current ($I_{Ca}$) of myocytes without affecting the delayed rectifier potassium current ($I_K$).

**7** These findings suggest that the primary electrophysiological effect of Ro 22-9194 as an antiarrhythmic agent is, like moricizine, a use- and voltage-dependent inhibition of sodium channels. From the onset and offset kinetics of the use-dependent block, Ro 22-9194 belongs to the intermediate kinetic Class I drugs, while moricizine is a slow kinetic drug. From the state-dependence of sodium channel block, Ro 22-9194 may belong to activated channel blockers, while moricizine belongs to inactivated channel blockers.

**Keywords:** Ro 22-9194; moricizine; ventricular cell; action potential; $\dot{V}_{max}$; use-dependent block

## Introduction

Ro 22-9194, (2**R**)-2-amino-*N*-(2,6-dimethylphenyl)-*N*-[3-(3-pyridyl) propyl] propionamide D-tartrate, is a newly synthesized antiarrhythmic compound related to the chemical structure of lignocaine (Murakami *et al.*, 1990; 1992). *In vivo* experiments in dogs have shown that Ro 22-9194, given as an oral or parenteral dose, has a potent inhibitory action against ventricular and supraventricular tachyarrhythmias induced by coronary artery occlusion, reperfusion, aconitine or ouabain. Ro 22-9194 was, unlike most antiarrhythmic drugs currently available, shown to inhibit the aggregation and thromboxane $A_2$ ($TXA_2$) synthetase activity of rabbit platelets at concentrations comparable to those producing antiarrhythmic action (unpublished observations). Based on such properties, Ro 22-9194 is expected to have a potential usefulness especially in the treatment of ischaemic arrhythmias.

In experiments on dog isolated atrial and ventricular preparations which were cross-perfused with blood from another donor dog, Murakami *et al.* (1990, 1992) demonstrated that both **R**- and S-enantiomers of Ro 22-9194 caused a similar decrease in the spontaneous sinus rate (negative chronotropic effect) and in the contractile force (negative inotropic effect). They also observed in anaesthetized, open chest dogs that these compounds decreased heart rate, and prolonged the atrio-ventricular conduction time with a greater prolongation of His bundle-ventricular (HV) intervals than atrio-His (AH) intervals. In preliminary experiments on dog Purkinje fibres, Ro 22-9194 ($\geqslant 10\,\mu M$) was shown to decrease the maximum upstroke velocity ($\dot{V}_{max}$) and duration of the action potential in a concentration-dependent manner. These findings suggest that the antiarrhythmic activity of Ro 22-9194 is mediated primarily by inhibition of the excitatory sodium current, as with local anaesthetic-type (Class I) antiarrhythmic agents. Ro 22-9194 could also inhibit calcium inward current ($I_{Ca}$) since it possesses negative chronotropic and negative inotropic effects. The present study was designed to clarify these points.

We investigated the effects of Ro 22-9194 on the transmembrane action potential in right ventricular papillary muscles, as well as in single ventricular myocytes isolated from guinea-pig hearts in comparison with moricizine, a potent Class I antiarrhythmic drug. The modulation of drug-induced $\dot{V}_{max}$ inhibition by stimulation frequencies or by membrane potential level was studied extensively in order to elucidate the characteristics of the sodium channel blocking actions. In single ventricular myocytes, we also examined the effects of Ro 22-9194 on calcium inward current ($I_{Ca}$) and delayed rectifier potassium current ($I_K$).

## Methods

### *Papillary muscle*

Guinea-pigs weighing 200 to 250 g were killed by a blow on the head under pentobarbitone anaesthesia, and their hearts were quickly removed. Papillary muscles, 2 to 3 mm in length and 0.3 to 0.4 mm in diameter, were dissected from the right

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ventricle. The preparation was mounted in a tissue bath and superfused continuously at 34°C with modified Krebs solution gassed with 95% $O_2$ and 5% $CO_2$. The composition of the solution was as follows (in mM): NaCl 120.3, KCl 4.0, $CaCl_2$ 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 25.2 and glucose 5.5 (pH 7.4). The preparation was stimulated by a pair of 1.0 mm platinum wire electrodes placed 1.0 mm apart on both sides of the muscle. Pulses used for stimulation were 0.5–1.0 ms in duration and 20% higher in intensity than the diastolic threshold unless otherwise specified. Equipment used for recording the transmembrane potential was the same as that described previously (Kodama *et al.*, 1985; Toyama *et al.*, 1987).

To study the use-dependent effects of Ro 22-9194 and moricizine on the maximum upstroke velocity ($\dot{V}_{max}$) of action potentials, the preparation was stimulated repeatedly at varying rates ranging from 0.2 to 2.5 Hz. Rest periods of 60–90 s, which were sufficient to ensure full recovery from the rate-dependent decrease in $\dot{V}_{max}$, were interposed between the trains of stimuli. The experimental protocol is able to detect the existence of two types of drug-induced $\dot{V}_{max}$ inhibition, tonic and use-dependent block. The former is defined as the decrease of $\dot{V}_{max}$ of the first action potential after the rest period, and the latter is the decrease of $\dot{V}_{max}$ during the train (from the value of first action potential to the new steady state level).

The recovery of $\dot{V}_{max}$ from the use-dependent block was studied by applying a single test stimulus at various coupling intervals following a stimulation for 60–90 s at 1 Hz. The intensity of the test stimuli was adjusted to obtain a constant latency from the stimulus artifact to the initiation of the action potential upstroke.

### *Single ventricular myocytes*

Single ventricular myocytes were isolated enzymatically from guinea-pig hearts by the same procedure as described by Kodama *et al.* (1990). A few drops of cell suspension were placed in a recording chamber attached to an inverted microscope. The chamber was perfused at a rate of 2 ml $min^{-1}$ with normal Tyrode solution of the following composition (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $NaH_2PO_4$ 0.33, HEPES 5.0 and glucose 5.0; pH was adjusted to 7.4 by adding NaOH, and the solution was equilibrated with 100% $O_2$. The temperature was maintained at 36°C. Following the increase in the calcium concentration of the medium to 1.8 mM (normal Tyrode solution), 30 to 40% of myocytes deteriorated into round-shaped cells due to irreversible contracture. The remaining cells were tolerant to calcium; their intact rod-shape was maintained without spontaneous beating. The experiments were carried out on these myocytes.

In experiments to investigate the voltage-dependence of $\dot{V}_{max}$ inhibition by Ro 22-9194 and moricizine, the whole-cell clamp technique was used with a patch pipette (2–3 Mohm) containing a solution of (mM): KCl 120.0, $NaH_2PO_4$ 10.0, EGTA 1.0, MgATP 5.0 and HEPES 10.0; pH was adjusted to 7.3 by addition of KOH. Single cells were voltage-clamped by Axoclamp-2A (Axon Instruments, Burlingame, California, U.S.A.) in a single-electrode discontinuous mode (sampling frequency; 8–11 kHz). To elicit action potentials, the voltage-clamp was switched to the current-clamp mode by computer-operated TTL signals, during which a square current pulse (2 ms) for stimulation was applied. The upstroke velocity ($\dot{V}_{max}$) was sampled at 70 kHz by an on-line analysis system (Anno *et al.*, 1990).

Three different conditioning clamp protocols were employed. The first one examined the relationship between $\dot{V}_{max}$ and the resting membrane potential from which the action potential originates. Following a rest period of 90 s at − 80 mV, which is long enough to eliminate the use-dependent depression of $\dot{V}_{max}$ by the drugs, the membrane potential was clamped for 10 s to various levels (from − 100 mV to − 55 mV). The voltage-clamp was then released and a stimulus was applied to elicit the test action potential.

The second protocol is to determine whether the use-dependent $\dot{V}_{max}$ inhibition by the drugs is due to the blockade of an activated or inactivated sodium channel. Following a rest period of 90 s at − 80 mV, the membrane potential was clamped up from the resting level to 0 mV for 10 to 5,000 ms. At the end of step depolarization, the membrane potential was clamped back to − 80 mV for 100 ms, which is long enough for a drug-free channel to reactivate fully (Carmeliet & Vereeke, 1979; Ebihara & Johnson, 1980), but short enough for only partial dissociation of the drug from the blocked channel to occur (Grant *et al.*, 1984). The voltage clamp was then released and a stimulus was applied to elicit the test action potential.

The third protocol is to compare the amount of use-dependent block during trains of a short or a long depolarization pulse. Following a rest period of 90 s to allow full recovery of $\dot{V}_{max}$ at − 80 mV, a train of 10 ms or 200 ms depolarization to 0 mV was applied with an interpulse interval of 500 ms. Each depolarizing pulse was preceded by a 4 ms window of a current clamp, during which the action potential upstroke to measure $\dot{V}_{max}$ was elicited (the membrane potential was voltage-clamped for the remainder of the period).

Calcium inward current ($I_{Ca}$) and delayed rectifier potassium current ($I_K$) were also measured in the whole-cell voltage clamp experiments. For $I_{Ca}$ measurements, KCl in the external and pipette solutions was replaced by equimolar CsCl in order to eliminate the influence of potassium currents. Various depolarization steps up to + 40 mV with a duration of 200 ms were applied at a frequency of 0.2 Hz from the holding potential − 40 mV.

In experiments to measure $I_K$, the normal Tyrode external solution was replaced by a sodium-free and potassium-free solution including nisoldipine in order to isolate the current from other current components. The composition of the solution was as follows (mM): N-methyl-glucamine 149, $MgCl_2$ 5.0, $CaCl_2$ 1.8, HEPES 5.0, nisoldipine 0.003. Various depolarization steps up to + 50 mV with a duration of 3 s were applied at a frequency of 0.03 Hz from the holding potential at − 40 mV.

### *Drugs and data analysis*

Ro 22-9194 (Kissei Co. Ltd., Nagano, Japan) and moricizine (Sankyo Co. Ltd., Tokyo, Japan) were dissolved in deionized water and diluted with superfusate (Krebs solution or Tyrode solution) to achieve the final concentration required. In our pilot study on guinea-pig papillary muscles, we have confirmed that the electrophysiological effects of **R**- and **S**-enantiomers of Ro 22-9194 are virtually identical. We, therefore, used only the **R**-enantiomer of Ro 22-9194 to obtain all the data described below. An equilibration period of 60–70 min in papillary muscles and of 10–20 min in single cells was allowed before data were collected.

Values are presented as means ± s.e.mean unless otherwise stated. Analysis of variance (ANOVA) with Scheffe's test was used for critical differences among multiple means, and Student's *t* test was used for comparison between the two means. Differences were considered significant at $P<0.05$. All fitting of the exponential and Boltzmann curves to the data was accomplished with a nonlinear least-squares algorithm.

## Results

### *Effects on membrane action potential*

Effects of Ro 22-9194 (3–100 μM) and moricizine (0.3–10 μM) on the membrane action potential configuration were examined in each of the five papillary muscles con-

**Table 1** Effects of Ro 22-9194 on action potential characteristics

| | *RP* (mV) | $\dot{V}_{max}$ ($V\,s^{-1}$) | $APD_{30}$ (ms) | $APD_{80}$ (ms) |
|---|---|---|---|---|
| Control | −92.0 ± 0.3 | 206 ± 7 | 237 ± 5 | 311 ± 6 |
| Ro 22-9194 | | | | |
| 3 μM | −91.4 ± 0.5 | 197 ± 4 | 235 ± 7 | 307 ± 7 |
| 10 μM | −90.8 ± 0.4 | 166 ± 10* | 212 ± 13* | 284 ± 11* |
| 30 μM | −90.6 ± 0.4 | 123 ± 11* | 174 ± 9* | 246 ± 8* |
| 100 μM | −90.6 ± 0.9 | 81 ± 10* | 126 ± 10* | 196 ± 10* |

Values are means ± s.e.mean of five experiments. The preparation were constantly stimulated at 1.0 Hz. Data were obtained before (control) and 60 min after application of Ro 22-9194 at a given concentration. RP: resting membrane potential; $\dot{V}_{max}$: maximum upstroke velocity of action potential; $APD_{30}$ and $APD_{80}$: action potential duration from the upstroke to 30% and 80% repolarization. *Significantly different from control at $P<0.05$.

**Table 2** Tonic and use-dependent block of $\dot{V}_{max}$ by Ro 22-9194 and moricizine

| | n | *Tonic block* (%) | *Onset rate of use-dependent block* 1.0 Hz ($AP^{-1}$) | 2.0 Hz ($AP^{-1}$) |
|---|---|---|---|---|
| Ro 22-9194 | | | | |
| 10 μM | (5) | 2.0 ± 0.9 | 0.13 ± 0.02 | 0.08 ± 0.01 |
| 30 μM | (5) | 4.4 ± 1.8 | 0.16 ± 0.01 | 0.12 ± 0.01 |
| Moricizine | | | | |
| 1 μM | (5) | 1.4 ± 0.5 | 0.05 ± 0.01 | 0.03 ± 0.01 |
| 3 μM | (5) | 3.0 ± 5.4 | 0.06 ± 0.01 | 0.05 ± 0.01 |

Values are mean ± s.e.mean. $n$ = numbers of preparations. $AP^{-1}$: per action potential.

stantly stimulated at 1.0 Hz. Treatment with Ro 22-9194 at 3 μM for 60 min resulted in no significant changes in the action potential configuration. Ro 22-9194 ⩾ 10 μM caused a significant decrease in the maximum upstroke velocity ($\dot{V}_{max}$) and in the action potential duration at 30% and 80% repolarization ($APD_{30}$, $APD_{80}$) in a concentration-dependent manner, whereas the resting potential (RP) was unaffected even at the highest concentration (100 μM) tested (Table 1).

After exposure to moricizine at 0.3 μM for 60 min, no significant change in the action potential configuration was observed. Moricizine ⩾ 1 μM caused a dose-dependent $\dot{V}_{max}$ reduction without affecting RP. $APD_{30}$ and $APD_{80}$ were shortened as well at concentrations ⩾ 3 μM (data not shown). At 10 μM moricizine, $\dot{V}_{max}$ was decreased by 61 ± 9% from control; $APD_{30}$ and $APD_{80}$ were shortened by 47 ± 8% and 37 ± 5% respectively ($n = 5$). The $IC_{20}$ of $\dot{V}_{max}$ inhibition by Ro 22-9194 and moricizine, which were obtained by interpolation in a graph of log molar drug concentration versus response, were 11 ± 1 μM and 1.3 ± 0.2 μM respectively.

### *Use-dependence of* $\dot{V}_{max}$ *inhibition*

The effects of Ro 22-9194 and moricizine on $\dot{V}_{max}$ were examined with trains of different rates of stimulation separated from each other by a 60–90 s rest period. In control preparations, the value of $\dot{V}_{max}$ was almost unchanged with stimulation trains at rates of from 0.2 to 2.5 Hz. After treatment with Ro 22-9194 or moricizine, $\dot{V}_{max}$ of the first action potential in each train had decreased a little, indicating a minimal tonic block (Table 2). Further decline of $\dot{V}_{max}$ during the repetitive activity (use-dependent block) was dependent on the stimulation frequency; the higher the frequency, the greater the block.

Figure 1 summarizes the percentage decrease of $\dot{V}_{max}$ from the first action potential to the new steady-state level, which was attained at around the 15th to 20th action potentials for Ro 22-9194 and the 45th to 50th action potentials for moricizine. The use-dependent block was larger at higher drug concentrations.

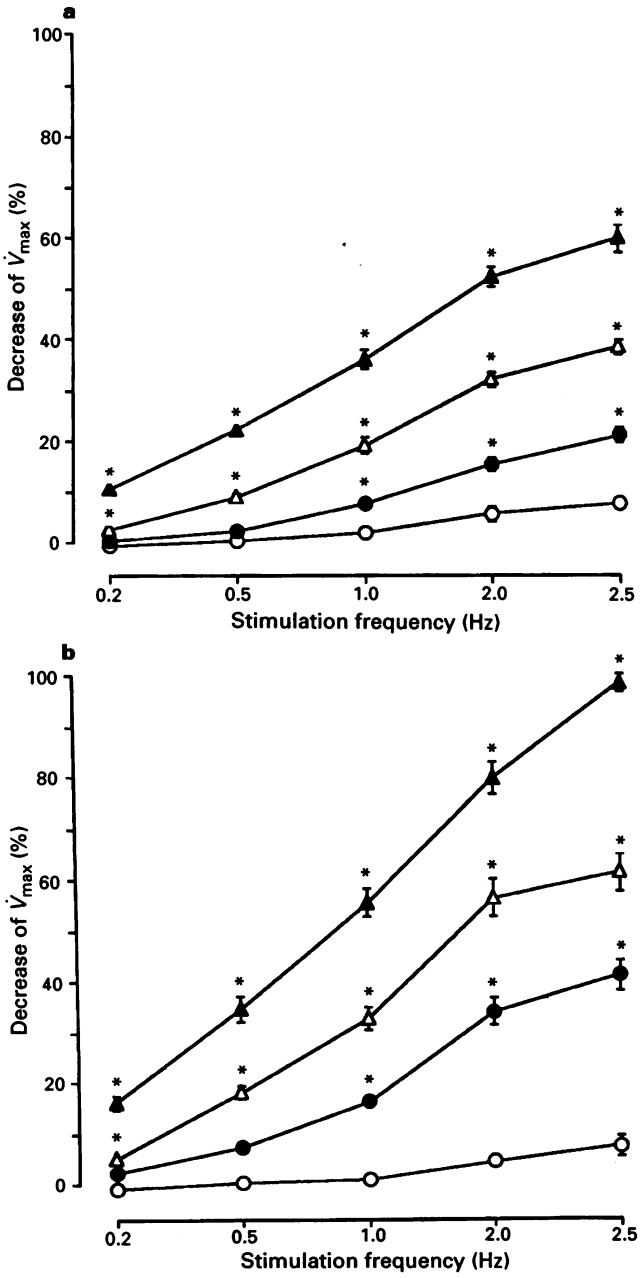


**Figure 1** Relationship between stimulation frequency and intensity of the use-dependent block. Ordinate scales: % decrease of maximum upstroke velocity of action potential ($\dot{V}_{max}$) from first action potential of stimulation trains to new steady-state level. Abscissa scales: stimulation frequency. Data were obtained before (control ○) and 60–70 min after application of (a) Ro 22-9194 at 3 μM (●), 10 μM (△), and 30 μM (▲), or (b) moricizine at 1 μM (●), 3 μM (△), and 10 μM (▲). Values are means with s.e.means of the five preparations. *Changes were statistically significant from control at $P<0.05$.

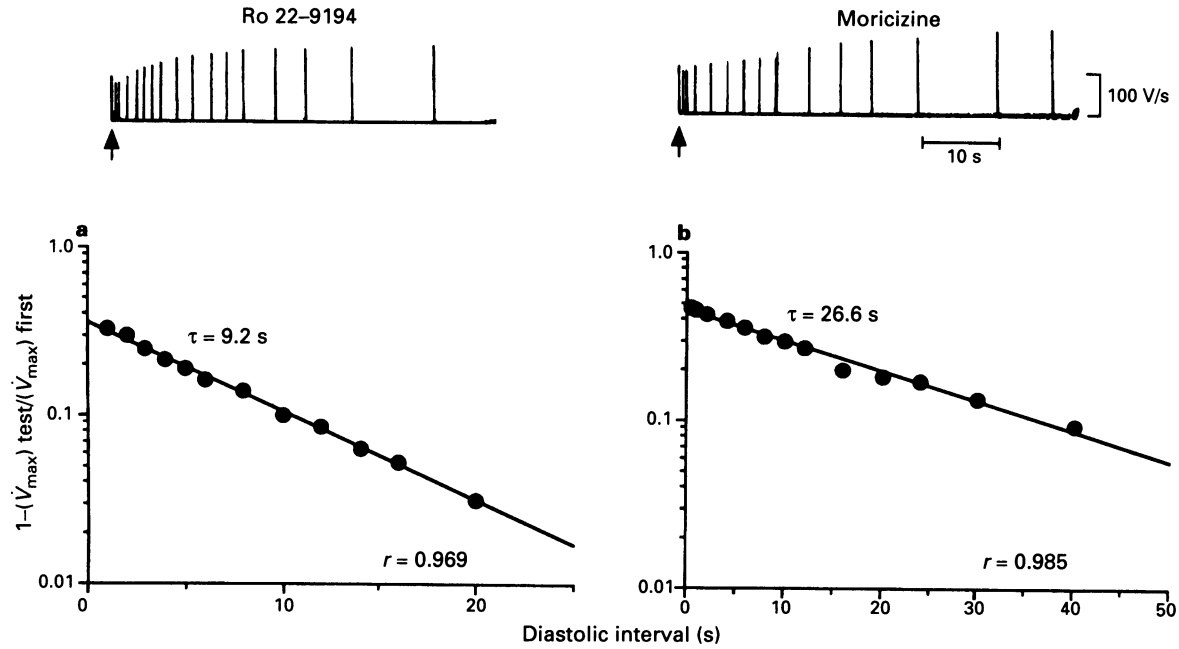


**Figure 2** Offset of the use-dependent block of $\dot{V}_{max}$ by Ro 22-9194 (a) and by moricizine (b) in papillary muscles. Top panels show records of differentiated upstroke spikes of action potentials 60–70 min after application of (a) Ro 22-9194 (30 μM) or (b) moricizine (3 μM). The preparation was stimulated at 1.0 Hz for 60 s following a rest period. Arrows indicate the last action potential by the stimulation train. After cessation of the train, a single test stimulation was applied with various coupling intervals. The lower graph indicate recovery process of $\dot{V}_{max}$ of the test action potential. Ordinate scales: fractional $\dot{V}_{max}$ reduction of the test action potential as compared with $\dot{V}_{max}$ of the first action potential by the train. Abscissa scales: diastolic interval, which was measured from the end (at 95% repolarization) of the last action potential. The time course was approximated by single exponential function with a time constant (τR) of 9.2 s for (a) Ro 22-9194 and 26.6 s for (b) moricizine.

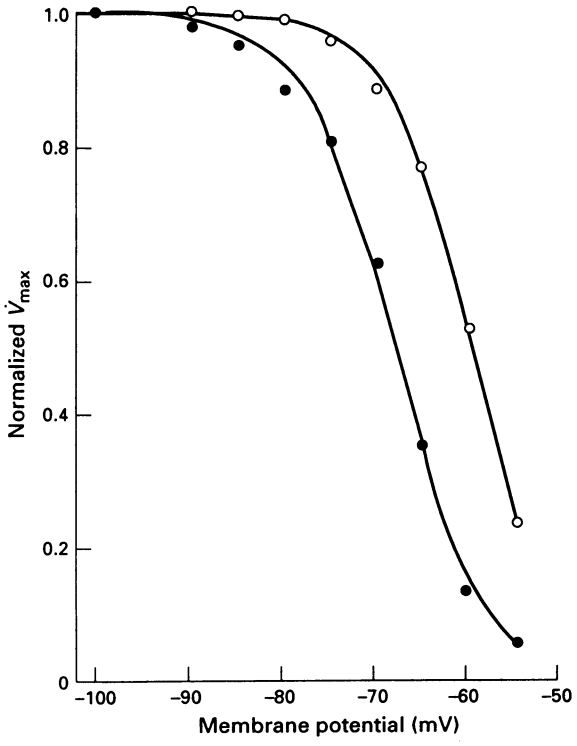


**Figure 3** Voltage-dependence of $\dot{V}_{max}$ inhibition by Ro 22-9194 in single ventricular myocytes. Ordinate scales: $\dot{V}_{max}$ of test action potential normalized by the value at conditioning membrane potential of −100 mV. Abscissa scales: conditioning membrane potential from which a test action potential was elicited. Following a rest period of 90 s at a holding potential of −80 mV, the membrane potential was clamped for 10 s to various conditioning level (from −100 mV to −55 mV). At the end of the conditiong clamp, the voltage-clamp was released, and a test action potential was elicited to measure $\dot{V}_{max}$. Data were obtained before (○) and 10–20 min after application of Ro 22-9194 (30 μM) (●).

The beat-to-beat decline of $\dot{V}_{max}$ during each stimulation train fitted well a single exponential curve, so the onset rate per action potential ($AP^{-1}$) at which $\dot{V}_{max}$ fell to the new steady-state level could be calculated (Table 2). The rate of onset with Ro 22-9194 was faster than that with moricizine when compared at the same stimulation frequency.

The recovery of $\dot{V}_{max}$ from use-dependent block was studied by applying a single test stimulus at various coupling intervals following a stimulation train for 60–90 s at 1.0 Hz. Before the application of the drugs, $\dot{V}_{max}$ of the test action potential recovered almost completely within 100 ms of the diastolic interval. After treatment with Ro 22-9194 (30 μM) or with moricizine (3 μM), a much slower $\dot{V}_{max}$ recovery was observed. Representative results are shown in Figure 2, where fractional reduction $\dot{V}_{max}$ of the test action potential was plotted against the diastolic interval in a semilogarithmic graph. In the presence of Ro 22-9194 (30 μM), the recovery time course of $\dot{V}_{max}$ with a diastolic interval longer than 100 ms was approximated by a single exponential function. The average time constant (τR) was 9.3 ± 0.5 s ($n = 5$). Similar exponential recovery of $\dot{V}_{max}$ was also observed in papillary muscles treated with moricizine (3 μM) at τR of 26.4 ± 1.7 s ($n = 4$). The τR value for Ro 22-9194 is appreciably shorter than that for moricizine (approximately one third).

### *Voltage-dependence of $\dot{V}_{max}$ inhibition*

The baseline characteristics of the action potential elicited in single ventricular cell at a long interstimulus interval (90 s) were as follows; RP, −80.2 ± 0.2 mV; $\dot{V}_{max}$, 630 ± 19 $Vs^{-1}$; $APD_{80}$, 158 ± 8 ms ($n = 20$). There was no significant difference in any of these parameters between the subgroups of cells exposed to Ro 22-9194 and moricizine.

First, the relationship between $\dot{V}_{max}$ and the resting membrane potential from which the action potential originates were examined. Test action potentials to measure $\dot{V}_{max}$ were elicited from a conditioning potential ranging between

− 100 mV and − 55 mV. Ro 22-9194 at 30 μM caused a greater $\dot{V}_{max}$ reduction at less negative conditioning membrane potentials. A fraction of $\dot{V}_{max}$ was calculated in each experiment before (control) and after application of the drug by normalizing the data with the value at − 100 mV (Figure 3). The data points fitted the Boltzman equation;

$$y = (1 + \exp\ (Vm - Vh)^{-1} \quad (1)$$

where $V$m is the conditioning clamp potential, $V_h$ is the membrane potential showing a half maximal $\dot{V}_{max}$. $V_h$ was changed from − 57.1 ± 1.5 mV in control to − 65.4 ± 2.0 mV after Ro 22-9194, indicating a hyperpolarizing shift of 8.4 ± 0.7 mV ($n = 5$). The $k$ values were almost identical before (0.228 ± 0.02 control), and after Ro 22-9194 (0.209 ± 0.02).

Moricizine (3 μM) caused a similar hyperpolarizing shift of the normalized $\dot{V}_{max}$-membrane potential curves by 8.0 ± 0.4 mV ($n = 5$, data not shown).

In the second protocol, a single conditioning 0 mV clamp of varying duration was applied from the resting level (− 80 mV), and a test action potential to measure $\dot{V}_{max}$ was elicited 100 ms after returning to the resting level (Figure 4). In untreated control myocytes, such a clamp pulse with a duration less than 1,000 ms had no significant effect on $\dot{V}_{max}$ of the test action potential. However, further prolongation of 0 mV clamp resulted in a slight but significant $\dot{V}_{max}$ reduction probably due to slow inactivation of sodium channels (Saikawa & Carmeliet, 1982; Clarkson *et al.*, 1984). A clamp pulse of 5,000 ms in duration decreased $\dot{V}_{max}$ by 12.9 ± 1.2% ($n = 5$) from the value of the action potential without the conditioning clamp (reference level). Treatment of the myocytes with Ro 22-9194 (30 μM) or moricizine (3 μM) for 10 min did not affect the resting potential (RP). $\dot{V}_{max}$ of the reference action potential was decreased by 7.5 ± 3.1% with Ro 22-9194 ($n = 5$) and by 2.7 ± 2.1% with moricizine ($n = 5$) from the value before the drug application. In such myocytes, a conditioning 0 mV clamp caused much greater $\dot{V}_{max}$ reduction of the test action potential. In the presence of Ro 22-9194, the shortest clamp pulse (10 ms) caused a significant $\dot{V}_{max}$ reduction by 10.7 ± 1.2% ($n = 5$) from the reference level. When the clamp pulse duration was prolonged, the $\dot{V}_{max}$ reduction was enhanced, and reached 63.1 ± 2.8% ($n = 5$) at 5,000 ms. In the myocytes treated with moricizine, the shortest (10 ms) clamp pulse had no significant effect on $\dot{V}_{max}$. However, 0 mV clamp pulses of longer than 200 ms caused a significant $\dot{V}_{max}$ reduction and it reached 55.6 ± 4.2% ($n = 5$) at 5,000 ms.

In the third protocol, a train of a short (10 ms) or a long (200 ms) depolarization was applied from the resting level (− 80 mV) to 0 mV with an interpulse interval of 500 ms (Figure 5). Such trains of depolarization caused a significant decrease in $\dot{V}_{max}$ only after treatment by either drug. In the

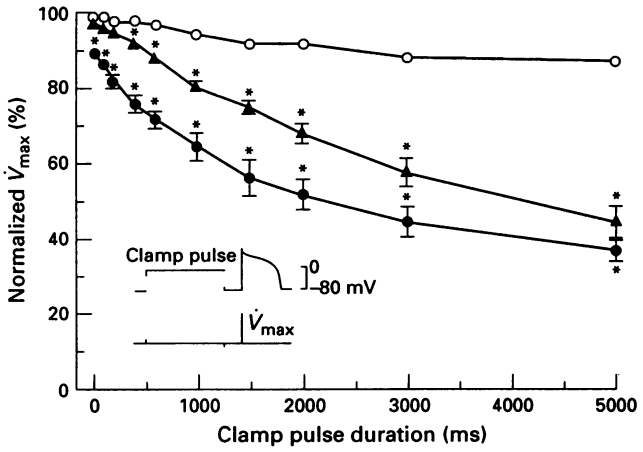


**Figure 4** Effects of a single 0 mV conditioning clamp pulse on the $\dot{V}_{max}$ inhibition induced by Ro 22-9194 and moricizine. Ordinate scale: $\dot{V}_{max}$ of test action potential normalized by the value of action potential without clamp pulse (referenced at the tonic block substracted value). Abscissa scale: duration of the conditioning 0 mV clamp. Values are presented as means ± s.e.mean of 10 cells for control and each 5 cells after the drug application. Data were obtained before (control ○) and 10–20 min after application of Ro 22-9194 at 30 μM (●) or moricizine at 3 μM (▲). *Significantly different from control at $P < 0.05$.

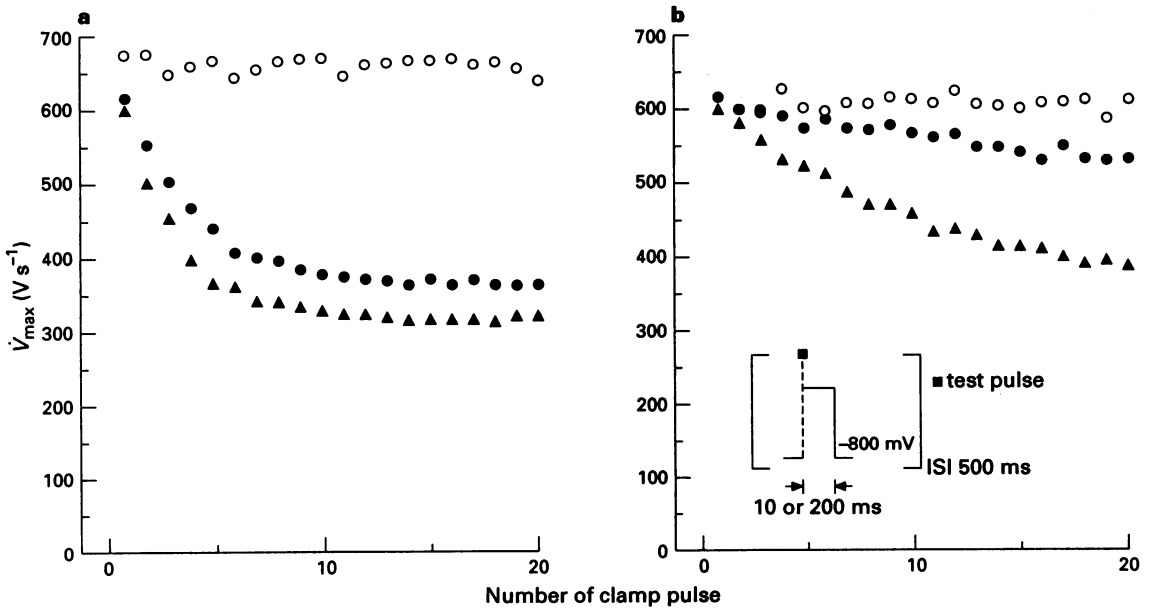


**Figure 5** Use-dependent decrease of the maximum upstroke velocity ($\dot{V}_{max}$) of action potential in single ventricular cells. Following a rest period of 90 s, a train of short (10 ms) or long (200 ms) depolarization from the resting potential level (− 80 mV) to 0 mV was applied in voltage-clamp mode with an interpulse interval (ISI) of 500 ms. Each depolarizing pulses was preceded by 4 ms window of current clamp mode, during which action potential upstroke to measure $\dot{V}_{max}$ was elicited. Ordinate scales: $\dot{V}_{max}$. Abscissa scales: number of pulses from the initiation of the train. Data were obtained before (control ○, 10 ms pulses), and 10–20 min after application of (a) Ro 22-9194 at 30 μM (●, 10 ms pulses; ▲, 200 ms pulses) or (b) moricizine at 3 μM (●, 10 ms pulses; ▲, 200 ms pulses).

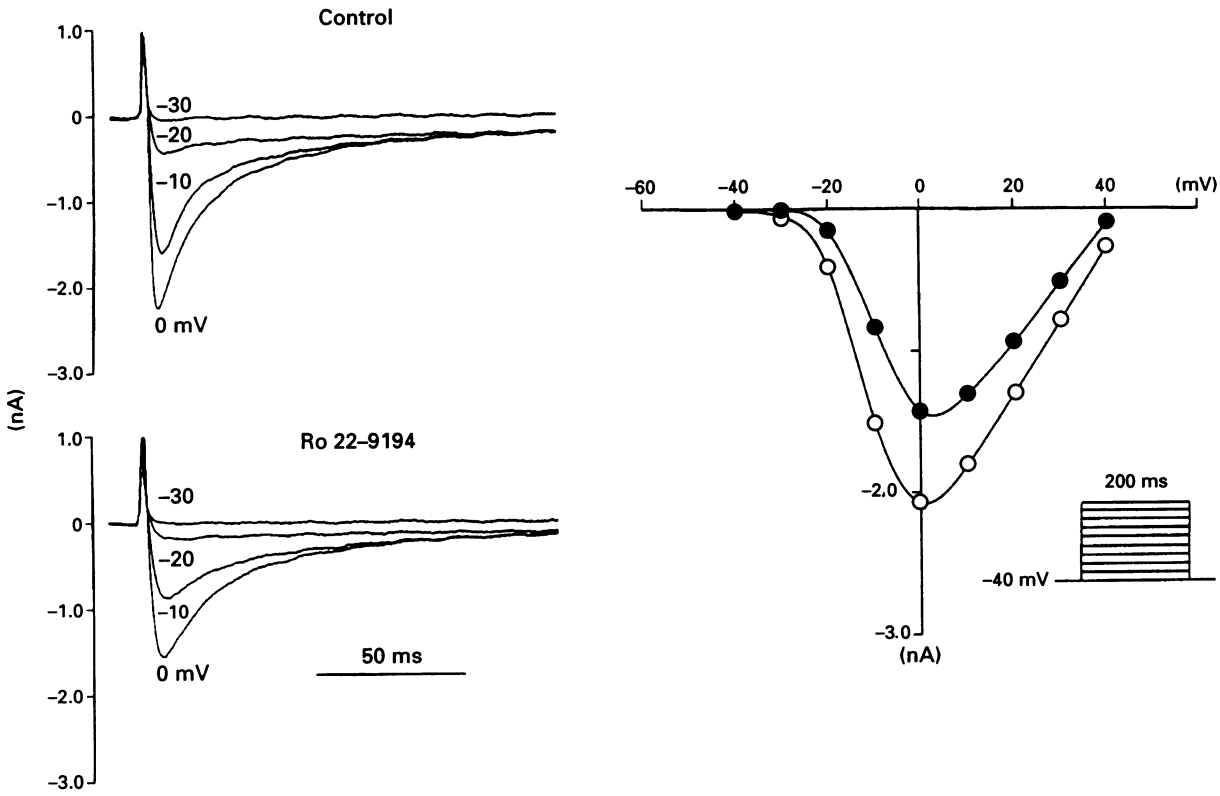


**Figure 6** Effects of Ro 22-9194 on the calcium inward current ($I_{Ca}$). $I_{Ca}$ was elicited by depolarizing pulses of 200 ms duration to various potentials at 0.2 Hz from a holding potential of −40 mV. Current traces are the records from a ventricular cell before (a) and 10–20 min after application of Ro 22-9194 at 100 μM (b). Current-voltage relationship of peak $I_{Ca}$ before (○) and after Ro 22-9194 (●) are also shown.

presence of Ro 22-9194 (30 μM), both short and long pulse trains caused a progressive $\dot{V}_{max}$ reduction in a pulse number-dependent manner (Figure 5a). The use-dependent $\dot{V}_{max}$ reduction by 20 pulses were 40.7 ± 1.0% ($n = 5$) for short pulse trains and 48.6 ± 2.2% ($n = 5$) for long pulse trains. Thus, the use-dependent block was enhanced only slightly by the prolongation of each clamp pulse duration. In the myocytes treated with moricizine (3 μM), similar prolongation of the clamp pulse duration resulted in much greater enhancement of the use-dependent block (Figure 5b); $\dot{V}_{max}$ reduction induced by a train of 20 pulses were 9.0 ± 0.4% ($n = 4$) with short (10 ms) and 40.0 ± 5.2% ($n = 4$) with long (200 ms) pulses.

### *Calcium inward current and delayed rectifier potassium current*

The effects of Ro 22-9194 (10–100 μM) on the calcium inward current ($I_{Ca}$) were examined in ten myocytes. A contact time of the drug was limited to 20 min because of spontaneous run-down of $I_{Ca}$. We, therefore, applied only a single concentration of the drug in each experiment. Ro 22-9194 ⩾ 30 μM caused a significant and concentration-dependent decrease of $I_{Ca}$ (Figure 6). The average decrease of $I_{Ca}$ at 0 mV was 15.5 ± 1.2% at 30 μM ($n = 4$, $P < 0.05$) and 27.0 ± 1.2% at 100 μM ($n = 3$, $P < 0.05$). $IC_{20}$ of $I_{Ca}$ inhibition was calculated to be 55 ± 2 μM. The effect of Ro 22-9194 on $I_{Ca}$ was reversible when the drug was washed out.

Effects of Ro 22-9194 on the delayed rectifier potassium current were measured by applying various depolarization steps for 3 s from the holding potential at −40 mV. Each depolarizing step elicited a gradually increasing outward current ($I_K$). When the membrane potential was returned to −40 mV, an outward current tail ($I_K$ tail) was recorded. Ro 22-9194 at 30 μM did not affect the amplitude of $I_K$ and $I_K$ tail (data not shown).

## Discussion

The present results have revealed that both Ro 22-9194 ⩾ 10 μM and moricizine ⩾ 1 μM cause a concentration-dependent decrease in $\dot{V}_{max}$ and APD of papillary muscles. The data for moricizine are more or less in agreement with those obtained by previous investigators in experiments on dog Purkinje fibres (Danilo *et al.*, 1977; Ruffy *et al.*, 1979; Tsuji *et al.*, 1983; Lee & Rosen, 1991; Case *et al.*, 1992) and guinea-pig papillary muscles (Jurevicius *et al.*, 1991).

Since the entire tissue was excited simultaneously and there was no conduction within the preparation under the present experimental conditions, the decrease in $\dot{V}_{max}$ by Ro 22-9194 or moricizine without any accompanying change in RP reflects an inhibitory effect of these drugs on the fast sodium inward current, $I_{Na}$ (Gintant *et al.*, 1983; Grant *et al.*, 1984). The potency of $\dot{V}_{max}$ inhibition by Ro 22-9194 in guinea-pig papillary muscles constantly driven at 1.0 Hz ($IC_{20} = 11$ μM) is approximately one tenth of moricizine ($IC_{20} = 1.3$ μM) but is still comparable to those of quinidine, disopyramide or mexiletine (Campbell, 1983).

The inhibition of $\dot{V}_{max}$ by Ro 22-9194 was, like moricizine, enhanced at the higher stimulation frequency. Thus, in normally polarized papillary muscles, Ro 22-9194 (3–30 μM) and moricizine (1–10 μM) caused negligible or minimal tonic $\dot{V}_{max}$ inhibition despite their marked use-dependent $\dot{V}_{max}$ inhibition. Such frequency-dependent or use-dependent effects can be interpreted within the framework of the 'modulated receptor' hypotheses (Hondeghem & Katzung, 1977) or 'guarded receptor' hypothesis (Starmer *et al.*, 1984) to explain the interaction between local anaesthetic-type (Class I) antiarrhythmic drugs and cardiac sodium channels. According to these hypothesis, the reduction of $I_{Na}$ is due to the accumulation of drug-associated non-conducting channels (blocked channels). Ro 22-9194 and moricizine, like most other Class I antiarrhythmic drugs, may bind to the sodium channel receptors

mainly during activated and/or inactivated states. These characteristics would lead to an accumulation of blocked channels during stimulation trains above certain rates.

The use-dependent block of $\dot{V}_{max}$ by Ro 22-9194 and moricizine was observed during stimulation trains at rates $\geqslant 0.2$ Hz. The present data for the onset rate of use-dependent block with moricizine (Table 2) is similar to that in the previous report by Jurevicius *et al.* (1991) in guinea-pig papillary muscle (0.5 $AP^{-1}$ at 2 $\mu$M, 2.0 Hz). Our experiments have revealed that the onset kinetics of use-dependent block by Ro 22-9194 are faster than moricizine (Table 2) and comparable to those for quinidine, procainamide (Campbell, 1983), aprindine (Toyama *et al.*, 1987) and propafenone (Honjo *et al.*, 1989; Kodama *et al.*, 1992).

The average time constant ($\tau$R) of $\dot{V}_{max}$ recovery from the use-dependent block by Ro 22-9194 was 9.2 s. This is also comparable to quinidine and procainamide (Campbell, 1983), aprindine (Toyama *et al.*, 1987) and propafenone (Honjo *et al.*, 1989; Kodama *et al.*, 1992). The $\tau$R value for Ro 22-9194 is considerably shorter than those for moricizine in the present experiments (26.6 s) as well as in previous studies by Lee & Rosen (1991) (17 s) and by Jurevicius *et al.* (1991) (44 s). These facts may indicate that Ro 22-9194 belongs to intermediate kinetic Class I drugs such as quinidine, procainamide, aprindine and propafenone (Campbell, 1989; Kodama *et al.*, 1991), whereas moricizine belongs to slow kinetics Class I drugs.

The relationship between $\dot{V}_{max}$ and the membrane potential was examined in single ventricular cells following a rest period of 90 s (Figure 3). Under such experimental condition, a decrease in $\dot{V}_{max}$ may reflect only the tonic block of the sodium channel by the drugs, while the use-dependent block may be negligible. The present results have revealed that the decrease in $\dot{V}_{max}$ as a result of the tonic block by Ro 22-9194 and moricizine is more pronounced at less negative membrane potentials. This effect is most likely explained by a much higher affinity of these drugs for inactivated sodium channels than for resting ones (Grant *et al.*, 1984). In voltage-clamp experiments using rat ventricular cells, Schubert *et al.* (1986) demonstrated that moricizine (10 $\mu$M) shifted the inactivation curve of $I_{Na}$ in a hyperpolarizing direction by 6 mV. Our data for moricizine are in agreement with their findings.

We further investigated the state-dependence of the sodium channel block by Ro 22-9194 and moricizine in single cell experiments by using single and multiple 0 mV conditioning clamp pulses. The single pulse protocol was essentially the same as in our previous studies (Kodama *et al.*, 1990). In the presence of Ro 22-9194 (30 $\mu$M), the shortest (10 ms) conditioning clamp caused a substantial $\dot{V}_{max}$ reduction of the test action potential. The $\dot{V}_{max}$ reduction was enhanced modestly by further prolongation of the conditioning clamp duration. In the myocytes treated with moricizine (3 $\mu$M), a simlar conditioning clamp caused a significant $\dot{V}_{max}$ reduction only when the clamp pulse duration was longer than 200 ms; the longer the clamp pulse duration, the greater the $\dot{V}_{max}$ inhibition. These findings may indicate that Ro 22-9194 blocks the sodium channel in both the activated and inactivated states, whereas the sodium channel block by moricizine occurs primarily during the inactivated states. The approximate extent of the activated channel block (ACB), which was defined by a percentage of $\dot{V}_{max}$ reduction by a 10 ms clamp pulse from the reference level (tonic block substracted value), was estimated to be on average 10.7% for Ro 22-9194 (30 $\mu$M) and 2.0% for moricizine (3 $\mu$M). The approximate extent of the inactivated channel block (ICB), which was defined by an additional percentage decrease in $\dot{V}_{max}$ when the clamp pulse duration was prolonged from 10 ms to 500 ms, was 15.3% for Ro 22-9194 and 8.2% for moricizine. The ratio of ICB/ACB was 1.5 with Ro 22-9194 and 5.5 with moricizine. The value for Ro 22-9194 is comparable to that for quinidine (1.6) and disopyramide (1.5), whereas that for moricizine is comparable to mexiletine (6.4), lignocaine (5.9) and aprindine (9.5) (Kodama *et al.*, 1990). The high contribution of the inactivation block for the moricizine action is consistent with a previous study by Makielski *et al.* (1990) in dog single Purkinje cells, where use-dependent $I_{Na}$ block by moricizine was shown to occur predominantly during the maintained depolarization (inactivated state).

In our single cell experiments using multiple 0 mV conditioning clamp, both the short (10 ms) and the long (200 ms) pulse trains applied to the myocytes treated with Ro 22-9194 caused a marked use-dependent $\dot{V}_{max}$ reduction, and the enhancement of the $\dot{V}_{max}$ reduction by the prolongation of the clamp pusle duration was minimal. In contrast, similar prolongation of the clamp pulse duration resulted in a much greater enhancement of the use-dependent $\dot{V}_{max}$ reduction by moricizine (Figure 5). This can also be interpreted by the different state-dependences of their sodium channel block. Only repetition of the activated state would be sufficient for the development of the use-dependent block by Ro 22-9194, whereas the use-dependent block by moricizine may require repetition of the inactivated state.

The *in vitro* concentrations of Ro 22-9194 used in the present study to decrease $\dot{V}_{max}$ of action potential were within a range of the effective i.v. dose in dog experiments of arrhythmias (unpublished data). Accordingly, it seems reasonable to conclude that the primary electrophysiological effects of Ro 22-9194 as an antiarrhythmic agent are, like those of moricizine, a use- and voltage-dependent inhibition of fast sodium channels (Class I action). From the onset and offset kinetics of use-dependent $\dot{V}_{max}$ inhibition, Ro 22-9194 belongs to intermediate kinetic drugs, while moricizine belongs to slow kinetics drugs. From the state-dependency of the sodium channel block, Ro 22-9194 may belong to the activated channel blockers, and moricizine to the inactivated channel blockers.

We also investigated the effects of Ro 22-9194 on the calcium inward current ($I_{Ca}$) and the delayed recitifier potassium current ($I_K$) in single cell experiments. Ro 22-9194 $\geqslant 30$ $\mu$M caused a significant concentration-dependent decrease of $I_{Ca}$ without affecting $I_K$. $IC_{20}$ of $I_{Ca}$ suppression was calculated to be 55 $\mu$M. Since this value is several times higher than the $IC_{20}$ of $\dot{V}_{max}$ inhibition (11 $\mu$M), the relative potency of this compound for inhibition of calcium channels is considerably less than that for inhibition of sodium channels. However, we cannot rule out a possibility that $I_{Ca}$ inhibition by Ro 22-9194 could be enhanced in a use- and frequency-dependent manner. Further experimental studies will be required to claify this point. The APD shortening effect of Ro 22-9194 might be attributed in part to the $I_{Ca}$ inhibition.

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# The role of nitric oxide in cardiac depression induced by interleukin-1β and tumour necrosis factor-α

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1 Myocardial dysfunction during septic shock is associated with enhanced production of cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α). These cytokines depress cardiac mechanical function by a mechanism which is not well defined.

2 Bacterial endotoxin or cytokines cause the expression of $Ca^{2+}$-independent nitric oxide (NO) synthase in cardiac myocytes, vascular endothelial cells and endocardial endothelial cells, causing enhanced production of NO. As NO has negative inotropic actions on cardiac muscle, we tested the sum effects of IL-1β plus TNF-α in the intact heart to determine whether enhanced expression of NO synthase activity in the cells that comprise the heart is involved in cardiac depression associated with cytokine stimulation.

3 Rat isolated working hearts perfused with IL-1β plus TNF-α showed a markedly greater depression in contractile function, measured as cardiac work, after 2 h of perfusion compared with time-matched control hearts. The depressant action of IL-1β plus TNF-α was first apparent after 1 h of perfusion; no early (15 min) cardiac depressant actions were seen.

4 The competitive inhibitor of $Ca^{2+}$-dependent and $Ca^{2+}$-independent NO synthases, $N^G$-nitro-L-arginine methyl ester (L-NAME, 3 μM) when given concurrently with IL-1β plus TNF-α prevented the loss in contractile function such that these hearts after 2 h of perfusion had similar function to time-matched controls. L-NAME did not acutely reverse the loss of contractile function in hearts exposed for 2 h to IL-1β plus TNF-α. The protective action of L-NAME in the presence of cytokines was concentration-dependent and was not seen at a higher concentration (10 μM) due to the significant reduction in coronary flow observed at this concentration.

5 In contrast, when L-NAME (3 μM) was given in the absence of IL-1β plus TNF-α it depressed contractile function over the 2 h perfusion period by significantly reducing coronary flow.

6 Inhibition of protein synthesis with cycloheximide (Cx) abolished the loss in function that occurred over 2 h in both control and IL-1β plus TNF-α-treated hearts.

7 Inducible, $Ca^{2+}$-independent NO synthase activity was not observed in freshly isolated hearts but was observed in control hearts perfused for 2 h *in vitro* and was doubled in hearts perfused with IL-1β plus TNF-α. Cx prevented the expression of $Ca^{2+}$-independent NO synthase in both control and cytokine-treated hearts.

8 In summary, these results suggest that the depression of myocardial function by IL-1β plus TNF-α is mediated, at least in part, by induction of $Ca^{2+}$-independent NO synthase activity in the heart.

**Keywords:** Nitric oxide; nitric oxide synthase; myocardial contraction; interleukin-1β; tumour necrosis factor-α; working heart; $N^G$-nitro-L-arginine methyl ester (L-NAME); cycloheximide; shock

## Introduction

During endotoxaemia and septic shock a depression of myocardial contractile function occurs which is distinct from vascular haemodynamic effects (Solis & Downing, 1966; Kadowitz & Yard, 1970; Parker & Adams, 1985; Suffredini *et al.*, 1989; Cunnion & Parrillo, 1989; Abel, 1989). This is not mediated directly by bacterial products themselves but is due to the release of endogenous substances, including cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon, all of which are mediators of the immune response (Zentella *et al.*, 1991; Dinarello, 1991). Indeed, one of the limiting side effects of anti-cancer therapy with interleukin-2, which causes the release of the aforementioned cytokines *in vivo* (Kassid *et al.*, 1989; Boccoli *et al.*, 1990), is the depression of myocardial function which occurs several hours after initiating treatment (Ognibene *et al.*, 1988; Deyton *et al.*, 1989; Nora *et al.*, 1989). In experimental studies, exposure of isolated heart preparations to these cytokines will also cause a severe myocardial depression (Hosenpud *et al.*, 1989; Sobotka *et al.*, 1990); however, these studies did not elucidate the mechanism by which this occurs.

Nitric oxide (NO) synthesized by a $Ca^{2+}$-dependent NO synthase in the vascular endothelial cell is an endogenous mediator of vasodilatation. These effects are brought about by the action of NO on its intracellular receptor, the soluble guanylate cyclase, in vascular smooth muscle cells. A $Ca^{2+}$-independent enzyme releasing large quantities of NO over several hours is expressed *de novo* in a variety of cell types in response to inflammatory cytokines (for review see Moncada *et al.*, 1991). First characterized in endotoxin-activated macrophages, the production of NO by this enzyme is one of their cytotoxic defence mechanisms against tumour cells and microorganisms (Hibbs *et al.*, 1990). During sepsis, induction of $Ca^{2+}$-independent NO synthase in vascular smooth muscle and the resultant enhanced production of NO is largely responsible for the associated hypotension and hyporeactivity to vasoconstrictor agonists, an effect which can be blocked

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by inhibitors of NO synthase (Kilbourn *et al.*, 1990; Rees *et al.*, 1990a; Nava *et al.*, 1991; Petros *et al.*, 1991).

Recent evidence suggests that NO modulates cardiac muscle function (for review see Schulz & Triggle, 1994). $Ca^{2+}$-dependent NO synthase activity has been characterized in cells of the heart, including cardiac myocytes (Schulz *et al.*, 1992) and endocardium (Schulz *et al.*, 1991). These cells, along with NO released from coronary endothelium, account for a significant basal production of NO from the heart which is estimated to be 161 pmol $min^{-1}$ from the guinea-pig isolated heart (Kelm & Schrader, 1990). Nitric oxide and the NO donor, sodium nitroprusside, exert a negative inotropic effect in isolated papillary muscles and cardiac myocytes (Smith *et al.*, 1991; Shah & Lewis, 1993; Brady *et al.*, 1993).

Immunological stimulation of the heart may increase NO production to levels which inhibit cardiac contractile function. We have shown that endotoxin treatment of rats or exposure of isolated cardiac myocytes from untreated animals to the combination of IL-1β plus TNF-α induces the expression of $Ca^{2+}$-independent NO synthase activity in the myocardium and cardiac myocytes, respectively. This was prevented by cycloheximide (Cx), an inhibitor of protein synthesis, as well as by the glucocorticoid, dexamethasone (Schulz *et al.*, 1992). As induction of $Ca^{2+}$-independent NO synthase may cause an overproduction of NO in the myocardium, and the time course of induction is similar to the depression of cardiac contractile function which appears during endotoxaemia (Solis & Downing, 1966; Parker & Adams, 1985), we postulated that excess NO synthesis could be involved in the myocardial depression seen in septic shock or anti-cancer therapy with cytokines (Schulz *et al.*, 1992). Cardiac myocytes isolated from endotoxin-treated guinea-pigs (Brady *et al.*, 1992), and those from untreated rats incubated with culture medium from endotoxin-activated macrophages (Balligand *et al.*, 1993a) show depressed contractile function which is attenuated by NO synthase inhibitors. These studies did not identify whether cytokines mediate the response, nor was NO synthase activity measured directly in the cells. Moreover, isolated papillary muscle preparations treated with IL-1β (Evans *et al.*, 1993) or with IL-2, IL-6 or TNF-α (Finkel *et al.*, 1992) undergo negative inotropic changes which are attenuated by inhibitors of NO synthase. These observed functional changes in isolated myocyte and papillary muscle preparations cannot be directly compared with the isolated heart with an intact coronary circulation in which a variety of cells are capable of expressing inducible NO synthase activity following exposure to endotoxin and/or cytokines, including cardiac myocytes (Schulz *et al.*, 1992), coronary vascular endothelium (Balligand *et al.*, 1993b), smooth muscle (Rees *et al.*, 1990a; Busse & Mülsch, 1990) and endocardial endothelium (Smith *et al.*, 1993).

We have, therefore, investigated the mechanisms by which the combination of IL-1β plus TNF-α depresses function in the isolated working heart of the rat by measuring the changes in cardiac function, coronary flow and the activities of both $Ca^{2+}$-dependent and $Ca^{2+}$-independent NO synthases. Furthermore, we have determined whether Cx or the NO synthase inhibitor $N^G$-nitro-L-arginine methyl ester (L-NAME) can prevent cytokine-mediated cardiac depression.

## Methods

### *Heart perfusions*

The procedures for heart isolation were approved by the University of Alberta Health Sciences Animal Welfare Committee. The isolated working heart model of Neely *et al.* (1967) was used, as previously described (Davies *et al.*, 1992). This was modified by the use of ultrasonic flow probes (Transonic Systems) placed in the left atrial preload and aortic outflow lines for the measurement of cardiac output and coronary flow. Hearts from male Sprague-Dawley rats (250–300 g, University of Alberta Biosciences) were perfused with recirculating Krebs-Henseleit buffer at 37°C (100 ml volume), oxygenated with 5% $CO_2$ in 95% $O_2$ and containing 11 mM glucose, 5 mM sodium pyruvate, 1.75 mM $CaCl_2$, 0.5 mM EDTA, and 0.2% bovine serum albumin. Following a 10 min period of retrograde perfusion via the aorta, the preparation was switched to the working mode by allowing buffer to enter the left atrium at a preload of 9.5 mmHg and subsequently to be pumped out of the left ventrile into an afterload column equivalent to 70 mmHg in height. Hearts were paced at 300 b.p.m. throughout the experiment with a Grass SD9 stimulator (regular stimuli; duration 0.6 ms; delay 0.4 ms) with leads placed on the aortic and atrial cannulas. Heart rate and peak systolic pressure were measured with a Gould P21 pressure transducer in the aortic outflow line.

After 20 min equilibration at 9.5 mmHg preload, the preload was altered between 5.5 and 15.5 mmHg in increments of 2 mmHg. Cardiac output, aortic pressure and coronary flow were measured at each interval in order to obtain a baseline ventricular function curve (referred to as t = 0 h). Cardiac work, the product of cardiac output (ml $min^{-1}$) × peak systolic pressure (mmHg), was used as an index of mechanical function. Thereafter, the preload was reset at 9.5 mmHg and a combination of IL-1β (5 ng $ml^{-1}$) and TNF-α (20 ng $ml^{-1}$), with or without L-NAME (1, 3 or 10 μM), was added to some hearts. These concentrations of L-NAME are well below that reported to antagonize muscarinic receptor binding (>100 μM) (Buxton *et al.*, 1993). We had previously observed that these concentrations of IL-1β and TNF-α induced $Ca^{2+}$-independent NO synthase activity in isolated cardiac myocytes (Schulz *et al.*, 1992). The hearts were perfused for two more hours and preload function curves were repeated at $t = 1$ h and $t = 2$ h. Some hearts were treated with Cx (10 μM) at the beginning of perfusion in the working mode. A further group of hearts was perfused without addition of cytokines in the presence of Cx or L-NAME (3 μM). A separate group of IL-1β plus TNF-α-treated hearts was challenged with L-NAME (3 μM) after 2 h perfusion. At the end of the perfusion protocol, heart ventricles were rapidly frozen with Wollenberger clamps cooled to the temperature of liquid nitrogen and then stored at −80°C for later processing.

### *Preparation of cytosolic fraction from frozen ventricle*

The frozen ventricular tissue was powdered in a pestle and mortar cooled to the temperature of liquid nitrogen. A portion of this was placed in five volumes of ice-cold homogenization buffer (composition given in Schulz *et al.*, 1992) and homogenized with an Ultra-Turrex disperser using three strokes of 3 s duration each. The homogenate was centrifuged (100,000 *g* for 35 min) at 4°C and the cytosolic (supernatant) fraction was kept on ice for immediate assay of NO synthase activity.

### *Determination of NO synthase activity*

NO formation in the cytosolic fraction was measured by the formation of [$^{14}$C]-citrulline from [$^{14}$C]-L-arginine as described by Schulz *et al.* (1992). Duplicate incubations for 12 min at 37°C were performed for each sample in the absence or presence of either EGTA (1 mM), or EGTA plus L-NMMA (1 mM), to determine the level of $Ca^{2+}$-dependent and $Ca^{2+}$-independent NO synthase activities, respectively. The only modifications included the addition of 10 μM (6**R**,**S**)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride in the assay buffer and the termination of incubation by addition of 1.5 ml of a slurry of AG 50W-X8 cation exchange resin ($Na^+$ form, suspended in three volumes of water) to separate [$^{14}$C]-L-arginine from [$^{14}$C]-citrulline. After allowing the resin suspension to settle, 4.0 ml of water were added to dilute the sample and 3.5 ml of the supernatant were taken for liquid scintillation counting.

*Protein determination*

Protein content of the cytosolic fraction was determined by the method of Bradford (1976) with bovine serum albumin used as a standard.

*Determination of perfusate endotoxin level*

In one representative experiment, samples of perfusate were measured for endotoxin content with an E-toxate kit (Sigma) which is based on the *Limulus* amoebocyte lysate assay.

*Materials*

Recombinant human IL-1β and TNF-α (Upjohn, Kalamazoo, MI, U.S.A.) and $N^G$-monomethyl-L-arginine (L-NMMA, Wellcome, Beckenham, U.K.) were kind gifts from the sources indicated. The following reagents were purchased as follows: (6R,S)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride (Dr B. Schircks Laboratories, Jona, Switzerland); L-[U-$^{14}$C]-arginine monohydrochloride, 305 mCi mmol$^{-1}$ (Amersham); NADPH tetrasodium salt and bovine serum albumin (Boehringer Mannheim); AG50W-X8 cation exchange resin (200–400 mesh, Bio-Rad); all other reagents were obtained from Sigma.

*Statistics*

Results are expressed as the mean ± s.e.mean for $n$ experiments. As appropriate, Student's paired or unpaired $t$ test or one-way ANOVA, followed by Fisher's least significant difference test to compare individual means, were used for statistical comparisons. $P<0.05$ was used as the criterion for statistical significance.

## Results

*Acute effects of IL-1β plus TNF-α in the rat isolated working heart*

Table 1 gives values of peak systolic pressure, cardiac output, cardiac work and coronary flow for the control, IL-1β plus TNF-α, IL-1β plus TNF-α plus L-NAME and L-NAME-treated hearts before (0 min) and 15 min after addition of drugs. Following addition of IL-1β plus TNF-α, there was a small increase in peak systolic pressure and cardiac output, which resulted in a 12% increase in cardiac work ($P<0.05$). No statistically significant changes in any functional parameters occurred if L-NAME was added together with IL-1β plus TNF-α. In contrast, L-NAME (3 μM) given alone caused an 18% reduction of coronary flow ($P<0.05$).

*Time course of cardiac depressant action of IL-1β plus TNF-α*

Figure 1 shows the time course of changes in cardiac function, measured as the change in cardiac work during stepwise increments in preload from 5.5 to 15.5 mmHg, before addition of drugs ($t=0$ h) and then 1 and 2 h later. All heart groups showed equivalent cardiac function at the start of the experiment (Figure 1a). Control hearts did not show any loss of function over the first hour of perfusion (Figure 1b), but by 2 h there was a significant reduction (Figure 1c). Measured at 9.5 mmHg preload, cardiac work was 45.0 ± 5.3 and 28.0 ± 5.0 ml min$^{-1}$ × mmHg × 10$^{-2}$ ($n=8$) at 0 h and 2 h, respectively ($P<0.05$). In contrast to control hearts, hearts treated with IL-1β plus TNF-α showed a significant reduction in cardiac function after 1 h of perfusion at preload values >9.5 mmHg (Figure 1b). Two hours exposure of hearts to IL-1β plus TNF-α resulted in a markedly greater depression of cardiac function when compared with time-matched controls (Figure 1c). In the IL-1β plus TNF-α-treated hearts, cardiac work measured at a preload of 9.5 mmHg was 48.2 ± 5.6 and 12.5 ± 2.7 ml min$^{-1}$ × mmHg × 10$^{-2}$ ($n=8$) at 0 h and 2 h, respectively ($P<0.05$) (Figure 1c). Over the 2 h perfusion period the loss of cardiac work was 40.4 ± 7.7% in control hearts and was nearly doubled in IL-1β plus TNF-α-treated hearts (75.4 ± 3.5%, $P<0.05$).

*Effects of L-NAME and Cx on the cardiac depressant action of IL-1β plus TNF-α*

The actions of either L-NAME (3 μM) or Cx (10 μM) were tested on cytokine-mediated depression of cardiac function. After 1 h of treatment with IL-1β plus TNF-α plus L-NAME, significantly greater cardiac function at preload values >9.5 mmHg were seen compared to hearts treated with only IL-1β plus TNF-α alone ($P<0.05$, Figure 1b). Moreover, after 2 h perfusion, the enhanced depression of cardiac function seen in IL-1β plus TNF-α-treated hearts was prevented if L-NAME was present, such that the cardiac function curve of this group is superimposable on that of the control hearts (Figure 1c). Remarkably, pretreatment with Cx prevented all loss of function in cytokine-treated hearts over the 2 h perfusion period. At 2 h, cardiac function in these hearts was significantly greater than time-matched controls (Figure 1c). No significant loss of function was seen in the Cx plus IL-1β plus TNF-α group comparing cardiac work values at any preload at 0 and 2 h. This suggests a requirement for protein synthesis for the depression of cardiac function to occur.

*Effects of L-NAME and Cx on hearts perfused without IL-1β and TNF-α*

As both L-NAME and Cx reduced the loss of function in cytokine-treated hearts, we assessed their actions on hearts perfused without addition of IL-1β plus TNF-α over a 2 h perfusion period (Figure 2). In marked contrast to the protective action of L-NAME in cytokine-treated hearts (Figure 1b and c), perfusion of untreated hearts with L-NAME (3 μM) alone caused a tendency (not significant) by 1 h to depress cardiac function compared with controls (Figure 2b). The depression in cardiac function progressed and was significantly enhanced after 2 h of perfusion (Figure 2c). Hearts perfused with Cx alone showed no deterioration of cardiac function over the 2 h perfusion period.

*Effects of treatments on coronary flow*

Table 2 summarizes the coronary flow data for the heart groups at 0, 1 and 2 h of perfusion. Compared with control hearts, which showed no significant loss of coronary flow over the 2 h perfusion period, IL-1β plus TNF-α-treated hearts show no changes at 1 h but by 2 h show a 31.0 ± 8.9% decrease in coronary flow ($P<0.05$). In the presence of L-NAME, a similar reduction in coronary flow of 33.6 ± 7.6% ($P<0.05$) was also seen in cytokine-treated hearts at $t=2$ h. A marked loss in coronary flow occurred, however, in hearts treated with L-NAME alone, which was reduced by 18%, 35% and 57% after 15 min, 1 h and 2 h perfusion, respectively. Note that this is the only group which showed a reduction in coronary flow within the first hour of perfusion. In Cx-treated hearts, both with and without IL-1β plus TNF-α, there was no change in coronary flow over the perfusion period. Thus, it is apparent that treatment groups which show impairment in coronary flow (IL-1β plus TNF-α with or without L-NAME, or L-NAME alone) also have reduced cardiac function. In the IL-1β plus TNF-α hearts, this might be due to the stimulation by cytokines of the release of vasoconstrictor mediators (Tracey & Cerami, 1993) or due to a down-regulation of $Ca^{2+}$-dependent NO synthase in the coronary vascular endothelium, similar to the effect of TNF-α observed in cultured endothelial cells (Yoshizumi *et al.*, 1993). The effect of L-NAME in the absence of added cytokines is probably due to its unopposed coronary vasocon-

**Table 1** The acute effects of cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) and $N^G$-nitro-L-arginine methyl ester (L-NAME) after 15 min exposure in rat isolated working hearts

| | *Peak systolic pressure* (mmHg) | | *Cardiac output* (ml $min^{-1}$) | |
|---|---|---|---|---|
| | *0 min* | *15 min* | *0 min* | *15 min* |
| Control ($n = 8$) | 95.1 ± 1.6 | 96.1 ± 2.2 | 47.9 ± 4.9 | 48.5 ± 4.4 |
| IL-1β + TNF-α ($n = 8$) | 93.3 ± 2.8 | 96.3 ± 2.7* | 50.9 ± 4.6 | 55.4 ± 3.6* |
| IL-1β + TNF-α + L-NAME ($n = 7$) | 92.6 ± 3.3 | 95.7 ± 4.4 | 55.6 ± 2.3 | 60.7 ± 2.7 |
| L-NAME ($n = 11$) | 99.1 ± 3.1 | 96.7 ± 3.1 | 55.5 ± 3.2 | 51.0 ± 3.5 |
| | *Cardiac work* (ml $min^{-1}$ × mmHg × $10^{-2}$) | | *Coronary flow* (ml $min^{-1}$) | |
| | *0 min* | *15 min* | *0 min* | *15 min* |
| Control ($n = 8$) | 45.0 ± 5.3 | 45.4 ± 4.9 | 25.9 ± 1.9 | 26.1 ± 1.8 |
| IL-1β + TNF-α ($n = 8$) | 48.2 ± 5.6 | 53.8 ± 4.7* | 24.3 ± 1.5 | 24.3 ± 1.6 |
| IL-1β + TNF-α + L-NAME ($n = 7$) | 51.1 ± 1.7 | 58.2 ± 4.1 | 23.7 ± 1.2 | 22.1 ± 2.0 |
| L-NAME ($n = 11$) | 53.8 ± 4.8 | 51.0 ± 3.5 | 23.3 ± 1.2 | 19.2 ± 1.3* |

Following equilibration and establishment of the baseline ventricular function curve and resetting of the preload to 9.5 mmHg (see Methods), functional parameters were measured before (0 min) and 15 min after addition of agents. Concentration of agents used are: IL-1β (5 ng $ml^{-1}$), TNF-α (20 ng $ml^{-1}$) and L-NAME (3 μM).
*Significantly different at 0 min value (Student's paired *t* test)

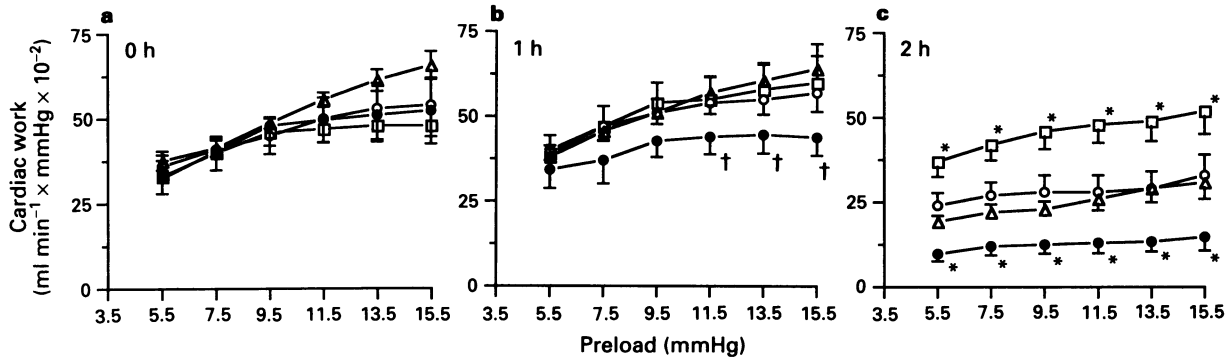


**Figure 1** Time course of changes in cardiac work (peak systolic pressure × cardiac output) as a function of preload (varied between 5.5 and 15.5 mmHg) in rat isolated working hearts perfused against a constant afterload of 70 mmHg. (a) Initial function (*t* = 0 h) before addition of drugs except for cycloheximide (Cx) which was added at the start of perfusion. Cardiac function curves were repeated after 1 h (b) and 2 h (c) of perfusion without (Control ○, $n = 8$) or with interleukin-1β (IL-1β) + tumour necrosis factor-α (TNF-α) (●, $n = 8$); IL-1β + TNF-α + $N^G$-nitro-L-arginine methyl ester (L-NAME) (△, $n = 7$) or cycloheximide (Cx) + IL-1β + TNF-α (□, $n = 8$). Each point represents the mean ± s.e.mean. *Significantly different versus control or IL-1β + TNF-α + L-NAME at same preload (Student's unpaired *t* test). †Significantly different versus IL-1β + TNF-α + L-NAME at same preload (Student's unpaired *t* test).

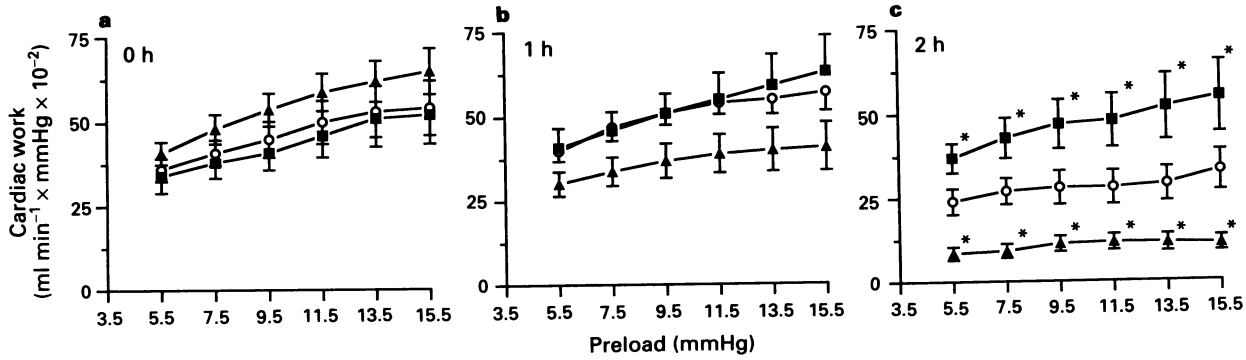


**Figure 2** Time course of changes in cardiac work as a function of preload (varied between 5.5 and 15.5 mmHg) in isolated working hearts perfused without addition of cytokines against a constant afterload of 70 mmHg. (a) Initial function (*t* = 0 h) before addition of drugs except for cycloheximide (Cx) which was added at the start of perfusion. Cardiac function curves were repeated after 1 h (b) and 2 h (c) of perfusion. Control (○) $n = 8$; Cx (10 μM) (■) $n = 6$; or $N^G$-nitro-L-arginine methyl ester (L-NAME) (3 μM) (▲) $n = 11$. Each point represents the mean ± s.e.mean. *Significantly different versus control at same preload (Student's unpaired *t* test).

**Table 2** Effect of treatment protocols on coronary flow

| | Coronary flow (ml min$^{-1}$) | | |
|---|---|---|---|
| | *0 h* | *1 h* | *2 h* |
| Control ($n = 8$) | 25.9 ± 1.9 | 24.8 ± 2.4 | 23.7 ± 3.5 |
| IL-1β+TNF-α ($n = 8$) | 24.3 ± 1.5 | 22.9 ± 1.9 | 17.3 ± 3.0* |
| IL-1β+TNF-α+L-NAME ($n = 7$) | 23.7 ± 1.2 | 22.3 ± 3.9 | 17.7 ± 2.6* |
| Cx + IL-β+TNF-α ($n = 8$) | 23.8 ± 1.5 | 26.5 ± 2.6 | 24.9 ± 2.4 |
| Cx ($n = 6$) | 23.2 ± 2.1 | 22.8 ± 2.6 | 23.0 ± 2.3 |
| L-NAME ($n = 11$) | 23.3 ± 1.2 | 15.1 ± 1.3† | 10.4 ± 1.1* |

Coronary flow was measured, following equilibration and establishment of the baseline ventricular function curve and resetting of the preload to 9.5 mmHg (see Methods), before (0 h) and 1 and 2 h after addition of agents (with the exception of cycloheximide (Cx) which was added to the perfusate at the start of perfusion in working mode). Concentration of agents used were: interleukin-1β (IL-1β) (5 ng ml$^{-1}$), tumour necrosis factor-α (TNF-α) (20 ng ml$^{-1}$), N$^G$-nitro-L-arginine methyl ester (L-NAME) (3 μM), Cx (10 μM).
*Significantly different from 0 and 1 h values (Student's paired $t$ test).
†Significantly different from 0 h value (Student's paired $t$ test).

strictor action by inhibition of basal release of NO from coronary vascular endothelium (Smith *et al.*, 1992). That coronary flow in the IL-1β plus TNF-α plus L-NAME group is not further diminished compared with cytokine treatment alone suggests that the excess release of NO by induction of $Ca^{2+}$-independent NO synthase in the heart is not completely abolished by L-NAME and that some portion of this NO may compensate for the L-NAME-induced inhibition of $Ca^{2+}$-dependent NO synthase in endothelial cells of the coronary circulation.

### *Concentration-dependent effect of L-NAME in preventing cytokine-induced myocardial depression*

The concentration-dependence of L-NAME in preventing the enhanced loss of cardiac function in IL-1β plus TNF-α-treated hearts was tested. Figure 3a shows the cardiac work which remains at the end of 2 h perfusion with IL-1β plus TNF-α (measured at 9.5 mmHg preload) in the absence or presence of L-NAME at 1, 3 or 10 μM. These concentrations approximate the $EC_{15}$, $EC_{25}$ and $EC_{50}$ values, respectively, in raising the resting coronary perfusion pressure in the isolated Langendorff-perfused heart of the rabbit (Smith *et al.*, 1992). L-NAME (3 μM) was significantly protective in reducing loss of function in cytokine-treated hearts, whereas concentrations of 1 and 10 μM were without benefit. Cardiac work at 2 h, expressed as a percentage of function at $t = 0$ h was 24.6 ± 3.5% ($n = 8$) and 45.1 ± 5.5% ($n = 7$) in the absence and presence of 3 μM L-NAME ($P < 0.05$). The protective action of L-NAME in preventing the enhanced loss of cardiac function by IL-1β plus TNF-α may be limited by its concentration-dependent coronary vasoconstrictor action (Smith *et al.*, 1992). Figure 3b shows that at $t = 2$ h, coronary flow in hearts treated with IL-1β plus TNF-α was 69.1 ± 8.9% of that measured at $t = 0$ h ($n = 8$). L-NAME at 1 or 3 μM had no further effect on coronary flow over the 2 h exposure period to cytokines, however, 10 μM L-NAME caused a significantly greater reduction in coronary flow, which at $t = 2$ h was 43.2 ± 3.7% of that seen at $t = 0$ h ($n = 5$, $P < 0.05$).

### *NO synthase activity*

$Ca^{2+}$-dependent and $Ca^{2+}$-independent NO synthase activities were measured in the cytosolic fraction prepared from ventricular tissue frozen at the end of the 2 h perfusion period (Table 3). For comparison, a group of hearts perfused for only 10 min with Krebs-Henseleit buffer in the Langendorff

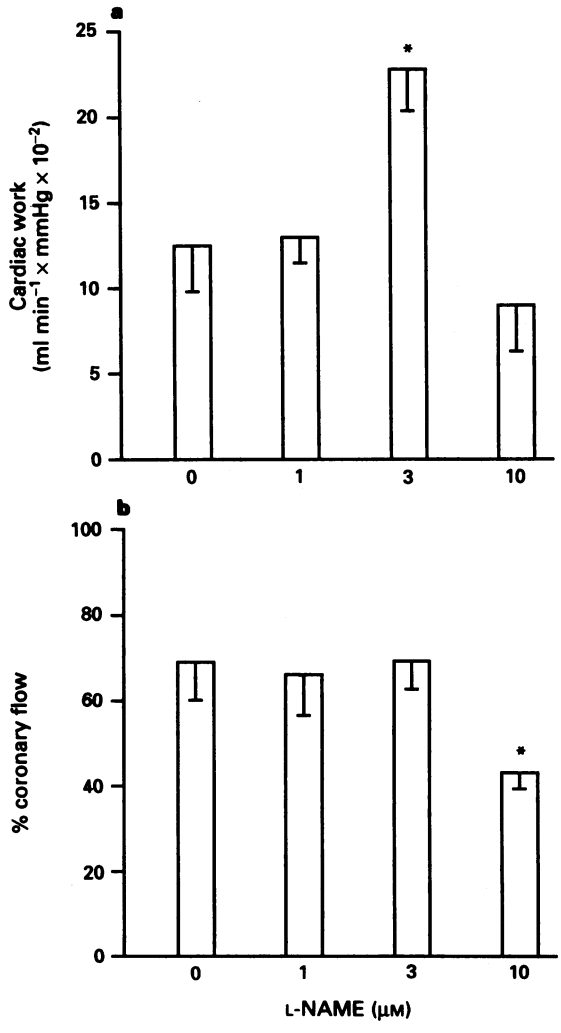


**Figure 3** Concentration-dependence of N$^G$-nitro-L-arginine methyl ester (L-NAME) on cardiac work and coronary flow in interleukin-1β plus tumour necrosis factor-α-treated hearts measured at $t = 2$ h at 9.5 mmHg preload and 70 mmHg afterload. All drugs were added at $t = 0$ h. (a) Cardiac work of hearts perfused in the absence or presence of L-NAME (1, 3 or 10 μM). (b) Coronary flow for the same hearts measured as a percentage of flow at $t = 0$ h. $n = 5$–8 hearts per group. *Significantly different versus 0 μM L-NAME group (Student's unpaired $t$ test).

**Table 3** NO synthase activity in ventricles of rat isolated hearts

| *Treatment* | *NO synthase activity* (pmol $min^{-1} \times mg^{-1}$ protein) | |
|---|---|---|
| | *$Ca^{2+}$-dependent* | *$Ca^{2+}$-independent* |
| *After 10 min perfusion* | 4.96 ± 1.30* | ND |
| *After 2 h perfusion* | | |
| Control | 1.91 ± 0.34 | 1.17 ± 0.20 |
| Cx | 1.46 ± 0.16 | 0.21 ± 0.14* |
| IL-1β + TNF-α | 2.56 ± 0.19 | 2.31 ± 0.38* |
| Cx + IL-1β + TNF-α | 1.59 ± 0.12 | ND |

NO synthase activity following *in vitro* perfusion for 10 min or 2 h was determined in cytosolic extracts from heart ventricles by conversion of [$^{14}$C]arginine to [$^{14}$C]citrulline (see Methods). For concentrations of agents see legend to Table 2. ND: not detected. *Significantly different from control value ($P<0.05$) in same column using ANOVA followed by Fisher's least significant difference test to compare individual means. ($n = 4-5$ hearts per group).

mode before freeze clamping were also examined. In agreement with our previous observation, all heart groups express $Ca^{2+}$-dependent NO synthase activity (Schulz *et al.*, 1992). Hearts perfused for 10 min show significantly higher $Ca^{2+}$-dependent NO synthase activity than all other hearts perfused for 2 h. The former also did not express any $Ca^{2+}$-independent NO synthase activity, in accordance with our previous results with freshly excised rat hearts (Schulz *et al.*, 1992). In contrast, control and IL-1β plus TNF-α-treated hearts perfused for 2 h express inducible, $Ca^{2+}$-independent NO synthase activity; however, the level of $Ca^{2+}$-independent activity in the latter was double that of the control hearts ($P<0.05$). Treatment with Cx prevented the expression of $Ca^{2+}$-independent NO synthase in hearts perfused in the absence or presence of IL-1β plus TNF-α.

### *Acute actions of L-NAME in IL-1β plus TNF-α-treated hearts at t = 2 h*

In a separate series of experiments, the response of IL-1β plus TNF-α-treated hearts at $t = 2$ h to acute exposure to L-NAME (3 μM) was examined. Measured at a preload of 9.5 mmHg, 10 min after addition of L-NAME ($n = 6$) there was a 44.8 ± 3.7% decrease in cardiac work ($P<0.05$) which was accompanied by a significant decrease in coronary flow of 31.0 ± 5.7% ($P<0.05$). This suggests that the depression in cardiac function following 2 h exposure to IL-1β plus TNF-α cannot be acutely reversed with L-NAME. As coronary flow is reduced in IL-1β plus TNF-α-treated hearts at $t = 2$ h (Table 2), addition of L-NAME at this time only further exacerbates an already compromised coronary circulation. Thus, the protective action of L-NAME in preventing the delayed depression of function in IL-1β plus TNF-α-treated hearts requires its presence during the course of exposure to cytokines.

### *Perfusate endotoxin level*

The complete perfusate contained 0.8 ng $ml^{-1}$ endotoxin before recirculation through the perfusion apparatus and 1.6 ng $ml^{-1}$ following 10 min of recirculation through the apparatus without a heart in the circuit. After perfusion for 2 h with an isolated heart in the circuit, the endotoxin level of the perfusate was 6.4 ng $ml^{-1}$.

## Discussion

We have investigated the actions of IL-1β and TNF-α on contractile function and coronary flow in the isolated working heart and have shown that their cardiac depressant effect is due in part to the induction of $Ca^{2+}$-independent NO synthase activity. The enhanced depression of contractile function by IL-1β plus TNF-α is prevented by the NO synthase inhibitor L-NAME. Pretreatment of the hearts with Cx, an inhibitor of protein synthesis, prevents the loss of function over time in both control and IL-1β plus TNF-α-treated hearts, suggesting that *de novo* protein synthesis is necessary for the development of cardiac depression. We have also shown that the protective actions of L-NAME are potentially limited at higher concentrations since these concentrations impair coronary flow, probably by inhibiting the basal production of NO (Smith *et al.*, 1992).

Isolated working hearts perfused over a 2 h period show a depression in contractile function which is delayed in onset. The spontaneously beating, isolated working heart of the rat has been shown to lose approximately 30% of its original cardiac output by 2 h and 60% by 2.5 h (Taegtmeyer *et al.*, 1980). The mechanism by which this occurs *in vitro* is not well understood and may be partially dependent upon work load, extracellular $Ca^{2+}$ concentration, temperature, as well as the choice of energy substrate used to support mechanical work (Taegtmeyer *et al.*, 1980). As control hearts perfused for 2 h *in vitro* (but not freshly isolated hearts) express $Ca^{2+}$-independent NO synthase activity it is possible that some of the contractile dysfunction is mediated through excess NO formation under these conditions. The induction of NO synthase in control hearts might be due to inadvertant exposure to hearts to bacterial endotoxin during the perfusion itself, which was found in detectable quantities in the perfusate, or possibly due to a spontaneous release of cytokines in the isolated heart.

Exposure of hearts to IL-1β plus TNF-α results in a greatly enhanced depression of ventricular function over 2 h of perfusion compared to control hearts. This was accompanied by a doubling of $Ca^{2+}$-independent NO synthase activity. Our preliminary experiments show that in hearts perfused for 2 h with IL-1β plus TNF-α, a mRNA species is induced that hybridizes to the cDNA probe for macrophage type NO synthase (Jakiwczyk, Bateson and Schulz, unpublished observations). In contrast to the delayed effects of IL-1β plus TNF-α, which did not occur for at least 1 h, we observed a small increase in cardiac contractile function following 15 min exposure to the cytokines.

Our results show that L-NAME or Cx prevent the enhanced depression in myocardial function in response to IL-1β plus TNF-α. The fact that Cx produces a complete prevention not only of the enhanced depression due to cytokines, but also of that which occurs in control hearts, together with the fact that there is some induction of the $Ca^{2+}$-independent enzyme at the end of 2 h of prefusion in control hearts, suggests that the effect of Cx might be primarily related to inhibition of induction of NO synthase.

L-NAME is non-selective in its inhibition of both the $Ca^{2+}$-dependent NO synthase, such as that found in the vascular endothelium, and the inducible $Ca^{2+}$-independent enzyme. A fine balance must be achieved in using a concentration of L-NAME low enough to affect minimally the basal

release of NO necessary for the maintenance of coronary perfusion and high enough to provide sufficient inhibition of the excess release of NO by the inducible, $Ca^{2+}$-independent enzyme. L-NAME, in contrast to L-NMMA, has a steep inhibitory concentration-response curve in preventing endothelium-dependent relaxation due to NO formation in rat aorta (Rees *et al.*, 1990b). We observe a marked concentration-dependence of L-NAME in preventing cytokine-mediated injury (Figure 3). Indeed, that L-NAME at 10 $\mu$M is not beneficial in preventing cytokine-induced depression of cardiac function may be related to its significant impairment of coronary flow (Figure 3). Furthermore, major differences in the actions of L-NAME (3 $\mu$M) are seen in the presence and absence of IL-1$\beta$ plus TNF-$\alpha$ (cf. Figures 1 and 2). L-NAME alone causes a significant reduction in coronary flow in control hearts occurring within 15 min and increasing in magnitude throughout the perfusion period (Tables 1 and 2). This action of L-NAME thus began earlier and was of greater magnitude than when it was administered with IL-1$\beta$ plus TNF-$\alpha$. The direct and early impairment in coronary flow by L-NAME may result in the depression of cardiac function within 2 h of perfusion of hearts not exposed to cytokines (Table 2 and Figure 2). This does not preclude the possibility that a selective inhibitor of $Ca^{2+}$-independent NO synthase or a reduced concentration of the non-selective inhibitor L-NAME may have been protective in the non-cytokine treated hearts. Thus, it seems that the induction of excess NO synthesis by IL-1$\beta$ plus TNF-$\alpha$ counteracts to some degree the vasopressor action of L-NAME. This may be due to induction of NO synthase in coronary blood vessels, as has recently been shown in cytokine-treated coronary microvascular endothelial cells (Balligand *et al.*, 1993b). Similar concentration-dependent effects of NO synthase inhibitors have been observed in models of septic shock (Nava *et al.*, 1991; Wright *et al.*, 1992).

The protective action of L-NAME in preventing the enhanced depression of cardiac function requires its presence during the course of cytokine exposure, as its addition after 2 h exposure to IL-1$\beta$ plus TNF-$\alpha$ did not acutely reverse the cardiac depression. As coronary flow in the cytokine-treated hearts was at that time significantly compromised, the further reduction of coronary flow following acute exposure to L-NAME probably precluded any immediate protective action. Alternatively, the detrimental action of IL-1$\beta$ plus TNF-$\alpha$ may be by some degree of irreversible alteration in cardiac function due, in part, to the cumulative actions of exposure to enhanced levels of NO over the time course of the experiment. Whether the enhanced production of NO in the heart mediates cardiac depression by stimulation of soluble guanylate cyclase (Smith *et al.*, 1991; Shah & Lewis, 1993; Brady *et al.*, 1993) or by the direct inhibitory actions of NO on mitochondrial respiratory enzymes (Drapier & Hibbs, 1988; Geng *et al.*, 1992) or other actions, is unknown.

The pathophysiological consequences of an overproduction of NO in the heart may represent a novel mechanism in the development of cardiac depression associated with anti-cancer cytokine therapy (Ognibene *et al.*, 1988; Deyton *et al.*, 1989; Nora *et al.*, 1989) and in immune activated states associated with an enhanced release of cytokines, such as septic shock (Solis & Downing, 1966; Kadowitz & Yard, 1970; Parker & Adams, 1985; Suffredini *et al.*, 1989), burn trauma (Suzuki *et al.*, 1991), myocarditis, endocarditis, allograft rejection, and idiopathic dilated cardiomyopathy. Indeed, $Ca^{2+}$-independent NO synthase activity has been observed in right ventricular biopsies obtained from patients with dilated cardiomyopathy of non-ischaemic origin (de Belder *et al.*, 1993). In man, IL-2 chemotherapy stimulates endogenous IL-1 and TNF-$\alpha$ production and is associated with enhanced release of NO metabolites in urine and serum (Hibbs *et al.*, 1992). During acute cardiac rejection in man, an enhanced expression of TNF-$\alpha$ in the myocardium has been shown (Arbustini *et al.*, 1991). Furthermore, in rat heart allografts undergoing rejection, an iron-nitrosyl electron paramagnetic resonance signal indicative of NO-mediated nitrosylation of proteins (Lancaster *et al.*, 1992), as well as expression of $Ca^{2+}$-independent NO synthase in the myocardium has been observed (Michler *et al.*, 1993). The possible contribution of excess NO production to the decline in cardiac function in these disorders raises the possibility of novel therapeutic targets and strategies for the treatment of immune-activated heart diseases.

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# Evidence for the presence of both pre- and postjunctional $P_2$-purinoceptor subtypes in human isolated urinary bladder

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**1** In order to characterize $P_2$-purinoceptor(s) in human urinary bladder the contractile effects of ATP and its slowly-hydrolyzable analogues α,β-methylene ATP (α,β-MeATP) and β,γ-methylene ATP (β,γ-MeATP) were investigated on human detrusor strips taken from patients undergoing cystectomy for bladder carcinoma.

**2** Serial concentration-response curves (SCRC) for ATP, α,β-MeATP and β,γ-MeATP were constructed with an interval of 25 min between two successive doses to avoid tachyphylaxis. ATP (10 μM–10 mM) induced a phasic contraction, which was very rapid in onset. The dose-response curve to ATP appeared not to be monophasic: at the lower concentrations (10–300 μM) the curve was shallow, whilst at high concentrations (1–10 mM) the curve was steeper. The magnitude of the response obtained at the highest concentration tested (10 mM) was only 21.1 ± 2.8% (mean ± s.e.mean; $n = 4$) of the KCl (100 mM)-induced contraction.

**3** α,β-MeATP (0.3 μM–1 mM) and β,γ-MeATP (10 μM–1 mM) elicited a phasic contraction with a time course similar to that exhibited by ATP. The magnitude of the response obtained at the highest concentration tested (1 mM) was 70.3 ± 6.3% for α,β-MeATP ($n = 10$) and 27.9 ± 4.5% for β,γ-MeATP ($n = 8$) of KCl (100 mM)-induced contraction. The rank order of potency was α,β-MeATP > β,γ-MeATP > ATP. A plateau of response could not be achieved by any of these agonists.

**4** The $P_2$-purinoceptor antagonist, suramin (10–300 μM), dose-dependently antagonized only the lower part of α,β-MeATP dose-response curve. Data were analysed in terms of dose-ratio estimated at two levels of response (10% and 35% of KCl 100 mM-induced contraction). At 10% of KCl response the Schild plot slope was 0.98 and the estimated $pK_B$ was 5.85, whereas using the dose-ratio at the 35% level of the KCl response, the Schild plot was not linear suggesting an interaction of α,β-MeATP with a heterogeneous receptor population.

**5** The putative $P_2$-purinoceptor antagonist, Coomassie Brilliant Blue G (CB-G) at 0.3 and 1 μM ($n = 5$), shifted to the left the α,β-MeATP SCRC. The response at the highest concentration of agonist was potentiated, being equal to 78.8 ± 11.7% of the KCl (100 mM) response ($n = 5$). CB-G at 0.3 μM also shifted to the left the β,γ-MeATP SCRC and significantly potentiated the response at 1 mM up to 46.3 ± 5.6% of KCl 100 mM response ($n = 4$).

**6** Pretreatment with terodotoxin (TTX) at 1 μM shifted to the left the α,β-MeATP SCRC but the response to the highest concentration of the agonist was not potentiated, being 73.6 ± 9.9% of the KCl (100 mM) response ($n = 5$). TTX (1 μM) shifted to the left the β,γ-MeATP SCRC and significantly potentiated the response at 1 mM (61.6 ± 3.1% of KCl response; $n = 4$).

**7** The NO synthase inhibitor $N^G$-nitro-L-arginine methyl ester (L-NAME) at 100 μM did not modify the SCRC to either α,β or β,γ-MeATP.

**8** We conclude that in human detrusor muscle there is a heterogeneity of purinoceptors. The complex antagonism exhibited by suramin suggests the presence not only of $P_{2x}$-purinoceptors but also of another contractile $P_2$-purinoceptor subtype insensitive to suramin. Moreover, the activity of CB-G and TTX seems to support the existence of a prejunctional $P_2$-purinoceptor subtype inducing the release of one or more inhibitor neurotransmitters.

**Keywords:** Human isolated urinary bladder; adenosine triphosphate; $P_2$-purinoceptors; contraction; suramin; coomassie brilliant blue G

## Introduction

The pharmacological effects of extracellular adenosine and adenine nucleotides are mediated through two distinct classes of receptors named $P_1$ and $P_2$. At the $P_1$-purinoceptors, the rank order of agonist potency is adenosine > ATP and methylxanthines are selective antagonists, whereas at the $P_2$-purinoceptors the rank order of potency is ATP > adenosine and methylxanthines are without effect. It is well established that $P_1$ and $P_2$ purinoceptors are heterogeneous, $P_1$ being subdivided into $A_1$ and $A_2$ subtypes and $P_2$ into $P_{2X}$, $P_{2Y}$, $P_{2T}$ and $P_{2Z}$ subtypes (Burnstock, 1991). However, this classification is not definitive because it is based only on agonist potencies and because of a lack of selective and potent antagonists for the proposed subtypes.

It was recently reported that the trypanocidal drug, suramin, behaves as a competitive antagonist on contractile responses induced by the $P_{2X}$ selective agonist, α,β-methylene ATP (α,β-MeATP) in rabbit isolated ear artery (Leff *et al.*, 1990). On the other hand, Hoyle and collaborators (1990) reported that suramin did not disciminate between the $P_{2X}$ subtype mediating contraction of the guinea-pig urinary bladder and the $P_{2Y}$ subtype mediating relaxation of guinea-pig taenia-coli. Another compound, reactive blue 2 (RB-2) was claimed to be a selective antagonist of $P_{2Y}$-mediated effects in vascular and visceral smooth muscles (Burnstock & Warland, 1987; Matharu & Hollingsworth, 1992). However,

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insufficient selectivity between muscarinic and purinoceptors in guinea-pig urinary bladder has been reported for this compound (Choo, 1981; Crema *et al.*, 1983). Furthermore, the sulphonic dye, Coomassie Brilliant Blue G (CB-G), was found to be a more potent and selective $P_{2Y}$ antagonist with respect to RB-2 in antagonizing the ATP-induced $^{45}Ca^{2+}$ entry in rat parotid acinar cells (Soltoff *et al.*, 1989).

In guinea-pig urinary bladder ATP and its slowly degradable analogue, α,β-MeATP, contract the detrusor muscle acting on $P_{2X}$-purinoceptors (Hoyle *et al.*, 1990). In human isolated urinary bladder the presence of $P_{2X}$-purinoceptors mediating contraction was proposed on the basis of the rank order of potency exhibited by ATP and α,β-MeATP (Hoyle *et al.*, 1989) but more detailed studies were not performed. Hence, we decided to evaluate the effect of some purinoceptor agonists and of two putative $P_2$ antagonists, namely suramin and CB-G, in human isolated detrusor muscle strips. Furthermore, because it was recently reported that the putative $P_{2X}$ selective purinoceptor agonist β,γ-methylene ATP (β,γ-MeATP) has a direct contractile effect on $P_1$-purinoceptor of the $A_1$ subtype (Bailey *et al.*, 1992), the effect of the potent and selective $A_1$ antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; Collis *et al.*, 1989) was also evaluated.

Some of these results were presented at the Symposium 'Purines '92: Pharmacology and Clinical Applications', Milan, June 21–24, 1992 (Palea *et al.*, 1992).

## Methods

Specimens from the anterior part of the dome of the urinary bladder were obtained from male patients undergoing radical cystectomy because of bladder malignancy. Immediately after surgical removal a bladder specimen (macroscopically normal, with no sign of tumour or inflammation) was taken from the anterior part of the dome and immediately transported to the laboratory. The serosal and mucosal layers were dissected away and muscular strips were cut off. Each bladder strip was mounted vertically in a 2 ml glass organ bath containing a modified Krebs solution, gassed with 5% $CO_2$ in $O_2$ and maintained at 37°C. The composition of the modified Krebs solution was (mM): NaCl 120, KCl 4.7, $MgSO_4 .7H_2O$ 0.6, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, glucose 10 and indomethacin 0.001. Strips were initially placed under a tension of 2 g and allowed to equilibrate for 1 h. The tension generated was measured with a Grass Instrument FT03 isometric force displacement transducer, connected to a polygraph (Linseis model 7025).

### *Concentration-response curves to purinoceptor agonists*

Serial concentration-response curves (SCRC) to α,β-MeATP, β,γ-MeATP and ATP were constructed by adding to the organ bath a single dose of the agonist and washing it out immediately after the maximal response was obtained. Twenty five minutes were allowed between two consecutive applications of the agonist and in this interval the tissue was washed every 5 min. This protocol was used to avoid desensitization of $P_{2X}$-purinoceptors, according to other authors (Hoyle *et al.*, 1989). SCRC to α,β-MeATP and β,γ-MeATP were also performed in the presence of 1 μM tetrodotoxin (TTX) using the same interval between two consecutive concentrations plus 15 min of exposure to TTX.

### *Studies with purinoceptor antagonists*

SCRC to α,β-MeATP were performed in the absence and presence of increasing concentrations of suramin. The incubation times of this antagonist were derived from the study of Leff *et al.* (1990) and were inversely related to the concentrations used. The following pairs of concentrations of suramin and time were used: 10 μM for 4 h; 30 μM for 2 h, 100 μM for 90 min and 300 μM for 45 min. SCRC to β,γ-MeATP were carried out in the absence and presence of 30 μM suramin by using 2 h as incubation time. Incubation time of 90 min and 45 min for CB-G (0.003–0.3 μM) and DPCPX (1 μM) were used, respectively. Antagonists were dissolved in the appropriate solvent (see *Drugs and solutions*) then diluted in the modified Krebs solution which was used to wash out the strips between two doses of the agonist. Because of the sensitivity to light of suramin, experiments with this antagonist were performed in the absence of a direct light source.

### *Effect of suramin and CB-G on contraction induced by exogenous acetylcholine*

In order to show nonspecific effects of suramin and CB-G on cholinergic contractility, a cumulative dose-response curve to acetylcholine (ACh) was constructed on each bladder strip to determine the dose that elicited a contraction approximately equal to half of the maximal response produced by ACh. Then, this concentration (normally 10 or 30 μM) was applied two or three times to each bladder strip in order to obtain a constant response and when it was achieved, suramin or CB-G, both at 1 mM, were incubated in the organ bath for 30 and 45 min respectively; the ACh effect was then evaluated again.

### *Drugs and solutions*

α,β-MeATP, β,γ-MeATP and ATP (Sigma, St., Louis, Missouri, U.S.A.) were dissolved in distilled water on the day of the experiment and then diluted in Krebs solution. CB-G, L-NAME (Sigma), suramin (kindly donated by Bayer, Wuppertal, Germany) and TTX were dissolved in distilled water. DPCPX (Cookson Chemicals Ltd, Nursling, Southampton, U.K.) was dissolved in DMSO at 1 mM.

### *Analysis of agonist concentration-response curves*

Since the agonists under study did not reach a maximal effect at the highest concentrations used, to measure the agonists' potency the concentration of the agonist required to produce the equivalent of 35% of the KCl (100 mM) response was estimated. This value has been referred as $-\log A_{35}$ and has been obtained by evaluating the linear part of the concentration-response curves to the agonists. $-\log A_{35}$ are expressed as single values with 95% confidence interval (c.i.).

### *Analysis of the antagonism of suramin*

The antagonism of α,β-MeATP-induced contractions by suramin (at 10–30–100–300 μM) was quantified by use of the Schild plot regression analysis (Arunlakshana & Schild, 1959): $\log(DR-1) = m\log[B] - \log K_B$.

Where, DR is the equiactive dose-ratio of the agonist in the presence and absence of the antagonist, [B] the concentrations of the antagonist and $K_B$ the equilibrium dissociation constant for the antagonist when the slope of the regression line (m) is equal to one.

However, two plots were constructed by utilising the dose-ratios between treated and control-curves estimated at two levels of KCl 100 mM response: 10 and 35% versus the concentrations of suramin. The corresponding $A_{10}$ and $A_{35}$ (concentration of the agonist that induces 10 and 35% of the KCl (100 mM) response) were obtained by linear regression analysis as previously reported and they were utilized to calculate the DR at 10 and 35% of response, respectively. When the Schild plot slope was found not to be significantly different from one ($P > 0.05$) a regression analysis was performed with the slope equal to one, to estimate the $pK_B$ ($-\log K_B$) of suramin.

The antagonism of β,γ-MeATP (where suramin was tested only at 30 μM) was quantified by using the equation:

$pK_B$ = − log ([B]/DR-1) where [B] is the suramin concentration used (30 μM) and DR is as defined before (detected by linear regression analysis at 10% of the KCl 100 mM response in the presence and absence of the antagonist).

### *Statistical analysis*

All data are expressed as means ± s.e.mean. Differences in the response induced by the highest concentration tested of α,β-MeATP and β,γ-MeATP in control conditions versus preincubation with TTX, L-NAME or CB-G were analysed by means of a Student's *t* test for unpaired data (significant level = $P<0.05$).

## Results

### *Effect of α,β-MeATP, β,γ-MeATP and ATP*

The selective $P_{2X}$ agonists, α,β-MeATP (0.3 μM−1 mM; $n=10$) and β,γ-MeATP (10 μM−1 mM; $n=8$) and the non selective purinergic agonist ATP (10 μM−10 mM; $n=4$) all contracted the detrusor muscle strip (Figure 1). A plateau of response could not be reached at the highest concentration tested. Agonist potencies are expressed as − log $A_{35}$ values and were calculated as described in the Methods. The rank order of potency obtained was as follows: α,β-MeATP > β,γ-MeATP > ATP.

SCRC to ATP seemed non-monophasic since at the lower concentrations (10−300 μM) the curve was shallow, whilst at higher concentrations (1−10 mM) the curve was steeper. In the presence of TTX (1 μM) SCRC for α,β-MeATP was shifted to the left and the − log $A_{35}$ value of the TTX-treated strips (4.96; 95% c.i. = 4.87−5.06) was significantly higher than that of controls (4.56; 95% c.i. = 4.39−4.71). However, the response at the highest concentration of agonist was unchanged (70.3% of KCl 100 mM response in controls versus 73.6% in TTX-treated; Figure 2a). The contractile response to β,γ-MeATP at the highest concentration tested was too low to calculate a − log $A_{35}$ value but, in TTX-treated strips, responses to 1 mM β,γ-MeATP were greatly potentiated being 61.6% of KCl 100 mM response versus 27.7% of controls ($P<0.01$; Figure 3). A − log $A_{35}$ value of 3.95 (c.i. = 3.75−4.13) was estimated. These results are summarized in Table 1.

### *Effect of CB-G on α,β-MeATP and α,β-MeATP SCRC*

CB-G was tested at 0.003, 0.3 and 1 μM against α,β-MeATP-induced contractions. CB-G at 0.003 μM was without effect ($n=3$), but at 0.3 and 1 μM ($n=5$) shifted to the left the SCRC to α,β-MeATP without potentiating significantly the response at the highest concentration of the agonist (Figure 2b and Table 1). The − log $A_{35}$ value for CB-G-treated strips was 5.18 (c.i. = 5.03−5.33) and it was significantly higher than the corresponding value for controls (4.56; c.i. = 4.39−4.71). Furthermore, CB-G at 0.3 μM slightly shifted the SCRC to β,γ-MeATP to the left (Figure 3) and significantly potentiated the response at 1 mM (46.3 ± 5.6%

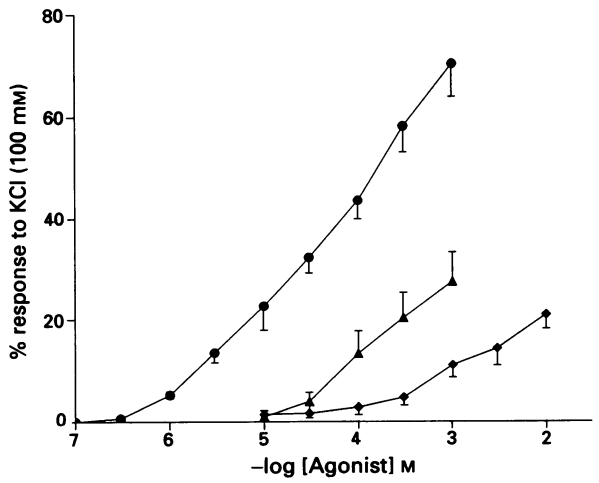


**Figure 1** Serial concentration response curves to α,β-methylene ATP (●), β,γ-methylene ATP (▲) and ATP (◆). Each point represents the mean ± s.e.mean of 4−10 replications.

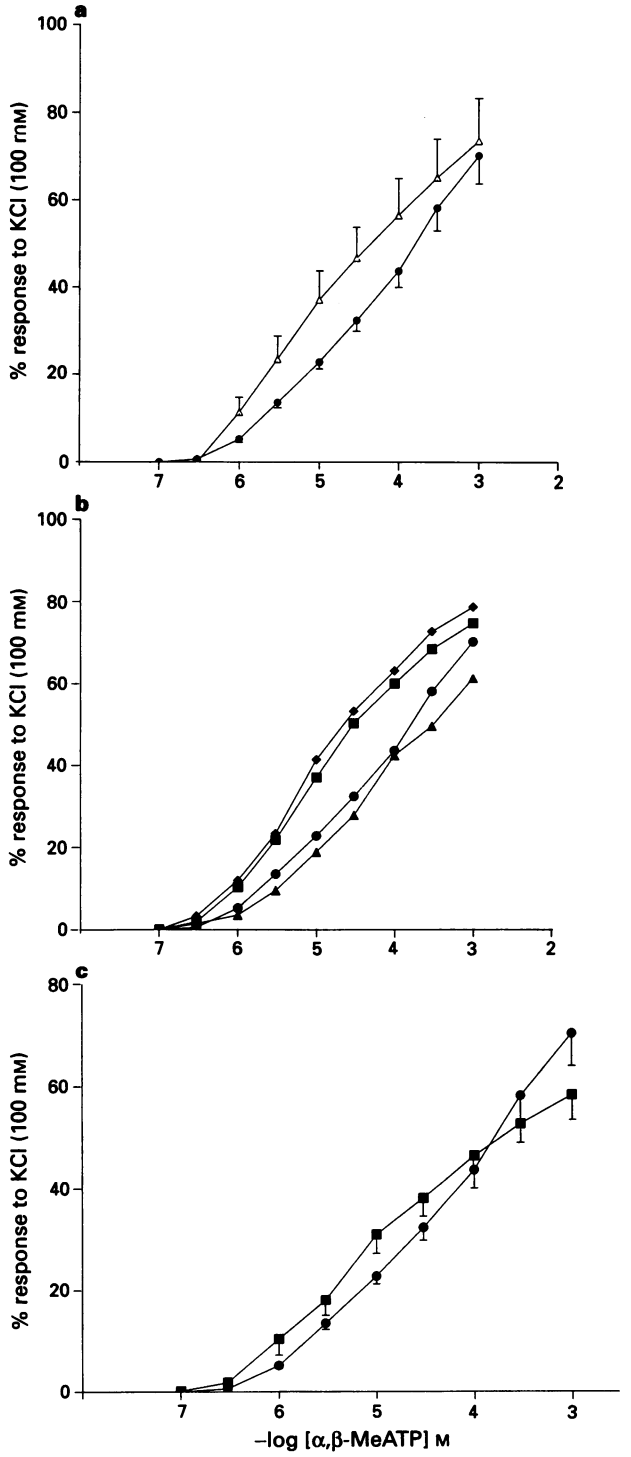


**Figure 2** Serial concentration response curves to α,β-methylene ATP alone (●) or in presence of tetrodotoxin at 1 μM (△; a), Coomassie Brilliant Blue G at 0.003 μM (▲), 0.3 μM (◆) and 1 μM (■) (b) and L-NAME at 100 μM (■; c). Each point represents the mean ± s.e.mean of 3−5 replications except for the control curve ($n=10$). In (b) error bars have been omitted for clarity.

in CB-G-treated vs 27.9 ± 4.5% in controls, $P = 0.034$). These results are summarized in Table 1.

### *Effect of L-NAME on SCRC to a α,β-MeATP and β,γ-MeATP*

The nitric oxide synthase inhibitor L-NAME at 100 μM did not modify the α,β-MeATP SCRC (Figure 2c). However, L-NAME, at 100 μM, slightly shifted the SCRC to β,γ-MeATP to the left (Figure 3) with a $-\log A_{35}$ value of 3.21 (c.i. = 3.00–3.41) and potentiated, although not significantly, the response to the highest dose of β,γ-MeATP (41.4 ± 2.4% of KCl 100 mM response in treated strips versus 27.9 ± 4.5% in controls; $P = 0.068$).

### *Antagonism by suramin*

Suramin (10–300 μM; $n = 4$–7) dose-dependently antagonized the lower part of α,β-MeATP SCRC, whereas the upper part was much less affected (Figure 4). The antagonism was analysed by using dose-ratios at two different magnitude of agonist's effect as described in Methods. By using the dose-ratio at 10% of KCl response there was a good correlation between the right shifts and doses of suramin. The Schild plot slope was 0.98 ± 0.07 and it was not significantly different from one ($P > 0.05$; Figure 5). A $pK_B$ value of 5.85 ± 0.04 (c.i. = 5.73–5.96) was estimated. On the other hand, the Schild plot using the dose-ratio at 35% of KCl 100 mM response was not linear and a lack of correlation between the concentrations of suramin tested and its effect was observed. In fact, 10 and 30 μM suramin gave a similar dose-ratio (DR = 1.88), as did 100 and 300 μM (DR = 10). This non-competitive antagonism of suramin on the upper portion of the agonist SCRC did not allow us to estimate a $pK_B$.

Suramin at 30 μM ($n = 3$) clearly antagonized β,γ-MeATP SCRC but the low efficacy of this agonist precluded the use of larger concentrations of suramin to evaluate further the

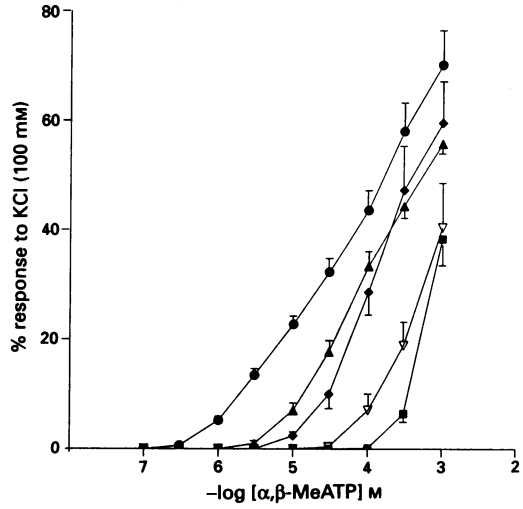


**Figure 4** Serial concentration response curves to α,β-methylene ATP alone (●) or in the presence of suramin at 10 μM (▲), 30 μM (◆), 100 μM (▽) and 300 μM (■). Each point represents the mean ± s.e.mean of 4–7 replications except for the control curve ($n = 10$).

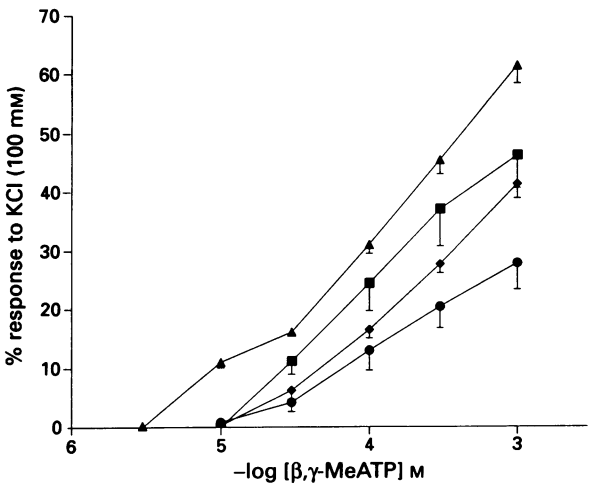


**Figure 3** Serial concentration response curves to β,γ-methylene ATP alone (●) or in the presence of tetrodotoxin at 1 μM (▲), Coomassie Brilliant Blue G at 0.3 μM (■) and L-NAME at 100 μM (◆). Each point represents the mean ± s.e.mean of 3–6 replications.

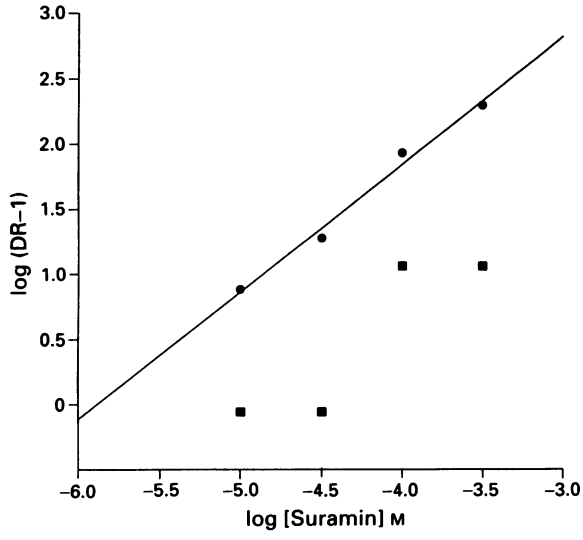


**Figure 5** Schild regression analysis for suramin versus α,β-methylene ATP-induced contractions using the dose-ratios obtained at the level of agonist corresponding to 10% (●) and 35% (■) of KCl (100 mM)-effect.

**Table 1** $-\log A_{35}$, confidence intervals (95% c.i.) and responses at the highest concentration tested (expressed as % of the contractile response to KCl 100 mM) (mean ± s.e.) induced by $P_{2X}$-purinoceptor agonists alone or in the presence of tetrodotoxin (TTX) at 1 μM or Coomassie Brilliant Blue G (CB-G) at 0.3 μM to contract human isolated urinary bladder

| | *Control* | | *TTX-treated* | | *CB-G-treated* | |
|---|---|---|---|---|---|---|
| | $-\log A_{35}$ | *Response* | $-\log A_{35}$ | *Response* | $-\log A_{35}$ | *Response* |
| α,β-MeATP | 4.56 (4.39–4.71) | 70.3 ± 6.3 | 4.96 (4.87–5.06) | 73.6 ± 9.9 | 5.18 (5.03–5.33) | 78.8 ± 11.7 |
| β,γ-MeATP | <3.0 | 27.7 ± 5.8 | 3.95 (3.75–4.13) | 61.6 ± 3.1 | 3.54 (3.43–3.63) | 46.3 ± 5.6 |
| ATP | <2.0 | 21.1 ± 2.8 | NT | | NT | |

$-\log A_{35}$ values were calculated as described in Methods.
NT = not tested.

nature of the antagonism (Figure 6). Nevertheless, a dose-ratio analysis gave an estimated $pK_B$ of 5.76, a value similar to that obtained with α,β-MeATP as agonist.

### *Effect of the selective $A_1$ antagonist DPCPX on α,β-MeATP and β,γ-MeATP SCRC*

DPCPX at 1 μM ($n = 5$) elicited a slight shift of the dose-response curve to β,γ-MeATP to the right (Figure 6) and was completely ineffective versus α,β-MeATP SCRC ($n = 3$; Figure 7).

### *Effects of suramin and CB-G on acetylcholine-induced contractions*

Suramin and CB-G, both at 1 mM, did not induce a significant variation in the magnitude of the response to ACh ($n = 3$ for both antagonists).

## Discussion

The rank order of potency for purinoceptor agonist found in this study seems to be consistent with the presence in the human urinary bladder of the $P_{2X}$-purinoceptor subtype (Burnstock, 1991). However, the results obtained with the antagonists, suramin and CB-G and with the neuroblocking agent, TTX, also reveal that the purinoceptor population in human urinary bladder is not homogeneous. In fact, the antagonism by suramin was not identical on the entire SCRC to α,β-MeATP. This implies that the system may not be constituted of a single receptor population. It was found that suramin was a competitive antagonist only of the lower portion of the α,β-MeATP SCRC, whereas the upper part was much less affected. The Schild analysis using dose-ratios at 35% of the effect allowed us to draw a plot that was flat and therefore it did not satisfy the condition of competitive interaction between agonist and antagonist (Kenakin, 1987). On the other hand the Schild plot obtained at 10% of response was linear with a slope not statistically different from one, indicating that suramin was competitive in that portion of the α,β-MeATP SCRC and that the incubation times used were adequate. Hence two receptor populations with a different sensitivity to suramin, may be activated by α,β-MeATP and on the most sensitive receptor suramin showed a $pK_B$ value of 5.85. Interestingly enough, a similar potency for suramin was found when the other agonist β,γ-MeATP was used ($pK_B = 5.76$).

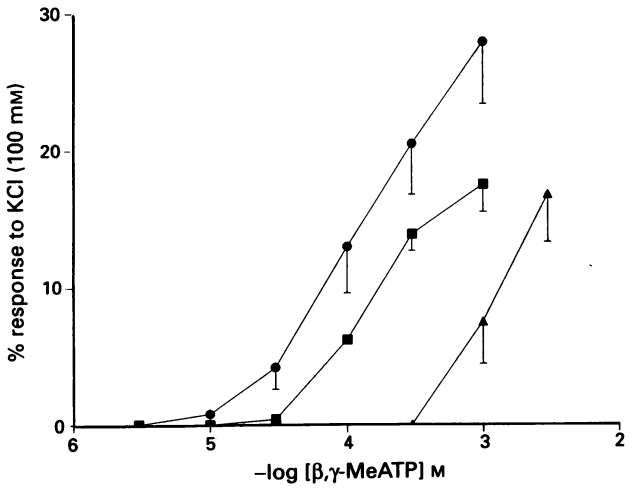


**Figure 6** Serial concentration-response curves to β,γ-methylene ATP alone (●) or in the presence of DPCPX 1 μM (■) and suramin 30 μM (▲). Each point represents the mean ± s.e.mean of 3 replications except for the control curve ($n = 8$).

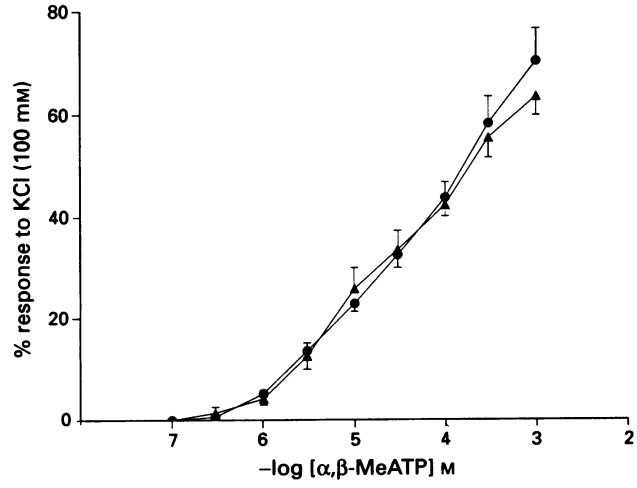


**Figure 7** Serial concentration response curves to α,β-methylene ATP alone (●) or in the presence of DPCPX 1 μM (▲). Each point represents the mean ± s.e.mean of 5 replications except for the control curve ($n = 10$).

However, such $pK_B$ values are considerably greater than those reported for the same antagonist on $P_{2X}$ receptors in rabbit isolated ear artery (Leff *et al.*, 1990). Thus we cannot exclude the existence of either $P_{2X}$ receptor subtypes or some forms of species-differences. It is interesting to point out that other investigators recently proposed the presence of a population of $P_{2X}$ purinoceptors insensitive to suramin in cat colon circular muscle (Venkova & Krier, 1993). Moreover, a non $P_{2X}$ non $P_{2Y}$ purinoceptor subtype not blocked by suramin and mediating the contraction of the rat vas deferens was postulated by Bultmann & Starke (1994). Again, it was recently shown that [$^3$H]-α,β-MeATP labelled two populations of binding sites in several peripheral organ and brain regions of the rat, thus suggesting the existence of two different $P_2$-purinoceptors both activated by α,β-MeATP (Michel & Humphrey, 1993).

The nature of the purinoceptor insensitive to suramin needs to be further elucidated. However, it may not be an $A_1$ type because DPCPX was without effect on α,β-MeATP-induced contractions. We can also exclude that this receptor is the same as that activated by ADPβS recently proposed in human detrusor (Palea *et al.*, 1994). In fact ADPβS-induced contractions, in contrast to those induced by α,β-MeATP, were antagonized by *p*-chloro-mercuribenzensulphonic, acid.

The antagonist, CB-G, and the neuroblocking agent, TTX, shifted to the left both the two agonists SCRC. This effect was not nonspecific because CB-G did not enhance the contractility to exogenous ACh. Consequently, it might be that α,β-MeATP and β,γ-MeATP interact also with a prejunctional purinoceptor which induces the release of one or more inhibitory neurotransmitter(s). This latter, acting on the smooth muscle cell, counteracts the contractile effect evoked by $P_{2X}$-purinoceptor activation. To test the hypothesis that NO could be released as the inhibitory neurotransmitter by α,β-MeATP and β,γ-MeATP, experiments were carried out by using the NO synthase inhibitor L-NAME. Unfortunately, L-NAME had only a weak potentiating effect on β,γ-MeATP and α,β-MeATP SCRC, thus suggesting a modest role in the presynaptic effect of the two purine agonists.

The existence of prejunctional purinoceptors having some characteristics of the $P_{2Y}$ subtype was proposed in the mouse isolated vas deferens (Von Kugelgen *et al.*, 1989). Furthermore, it was claimed that Reactive Blue 2 and CB-G were able to antagonize the inhibitory effect of β,γ-MeATP on urinary bladder contractions induced by pelvic nerve stimulation *in vivo* (Theobald, 1992), still suggesting the existence in the mammalian urinary bladder of a presynaptic inhibitory purinoceptor activated by β,γ-MeATP and antagonised by CB-G. Reactive Blue 2 is considered a relatively selective $P_{2Y}$

antagonist although in a small concentration-range, so by analogy we can speculate that the prejunctional purinoceptor in our preparation is a $P_{2Y}$ subtype. However, the fact that α,β-MeATP and β,γ-MeATP are reported to interact poorly with the $P_{2Y}$ purinoceptor (Ralevic *et al.*, 1991; Boland *et al.*, 1992) casts some doubt about this hypothesis.

In conclusion, in human detrusor α,β-MeATP and β,γ-MeATP both act on an excitatory and an inhibitory purinoceptor. The physiological significance of the former could be the mediation of neurogenic contraction following ATP and ACh release from parasympathetic nerve terminals. In fact, a non cholinergic neurogenic response, inhibited by $P_{2X}$ desensitization with α,β-MeATP was detected in human detrusor strips taken from patients undergoing cystectomy for an intractable form of interstitial cystitis (Palea *et al.*, 1993). Moreover it was occasionally found also in muscle strips taken from patients undergoing cystectomy for bladder malignancy or surgery for bladder reconstruction (Hoyle *et al.*, 1989). The physiological role of the inhibitory purinoceptors may be found in the hypothesis that a basal release of ATP or a related compound (Burnstock *et al.*, 1978) activates this purinoceptor which, in turn, releases some inhibitory neurotransmitters in order to prevent detrusor muscle contraction during urinary bladder filling. This effect could be physiologically important in assuring the necessary compliance of the urinary bladder in response to bladder filling, a property which is fundamental for continence.

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# Responses of the aorta of the garter snake (*Thamnophis sirtalis parietalis*) to purines

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**1** Isolated aortic rings from the garter snake (*Thamnophis sirtalis parietalis*) were investigated in order to identify and classify responses to adenosine and adenosine 5'-triphosphate (ATP) and their analogues as part of a comparative study of vertebrate purinoceptors.

**2** Adenosine, D-5'-(N-ethylcarboxamide) adenosine (NECA), **R**- and **S**-$N^6$-(2-phenylisopropyl) adenosine (**R**- and **S**-PIA) and 2-chloroadenosine (2-CA) all concentration-dependently relaxed aorta preconstricted with noradrenaline (NA). The order of potency was: NECA > **R**-PIA = 2-CA > adenosine > **S**-PIA. Individual $pD_2$ values for the analogues were: NECA $7.12 \pm 0.13$ (9), **R**-PIA $5.93 \pm 0.25$ (7), 2-CA $5.64 \pm 0.40$ (5), adenosine $5.04 \pm 0.10$ (13) and S-PIA $4.26 \pm 0.10$ (7). The order of potency has characteristics of both $A_1$ and $A_2$ receptors and cannot satisfactorily be classified according to the $P_1$-(adenosine) purinoceptor subtypes established in mammalian preparations.

**3** ATP, α,β-methylene ATP (α,β-MeATP), 2-methylthio ATP (2MeSATP), β,γ-methylene ATP (β,γ-MeATP) and uridine 5'-triphosphate (UTP) all concentration-dependently constricted the isolated aorta. The order of potency was α,β-MeATP = 2MeSATP > ATP > β,γ-MeATP > UTP. Only ATP, α,β-MeATP and 2MeSATP consistently produced a maximum response; $pD_2$ values were: ATP $3.98 \pm 0.07$ (10), α,β-MeATP $5.86 \pm 0.15$ (12) and 2MeSATP $6.06 \pm 0.23$ (9). In vessels preconstricted with NA neither ATP nor 2MeSATP caused relaxation in the presence or absence of the endothelium.

**4** Suramin (0.1 mM) inhibited vasoconstriction to ATP, α,β-MeATP, 2MeSATP and β,γ-MeATP; however, since contractions to ATP and analogues did not reach a maximum response in the presence of this and other antagonists, $pD_2$ values could not be calculated.

**5** Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS; 30 μM), a $P_{2X}$-purinoceptor antagonist, antagonized constrictions to α,β-MeATP only. Reactive blue 2 (RB2; 30 μM), a $P_{2Y}$-purinoceptor antagonist, inhibited vasoconstrictions to 2MeSATP only.

**6** Indomethacin (30 μM) inhibited vasoconstriction in response to ATP and 2MeSATP, but not α,β-MeATP, suggesting that the presence of an unaltered phosphate chain on the ATP analogue was necessary to stimulate the production of a prostanoid.

**7** Repeated administration of α,β-MeATP (3 μM) caused desensitization of the receptor responsible for the constriction due to α,β-MeATP whereas the responses to ATP and 2MeSATP were unaltered.

**8** In summary, both $P_1$-purinoceptors mediating vasodilatation and $P_2$-purinoceptors mediating vasoconstriction are present on the garter snake aorta. However, in contrast to mammalian vessels, both $P_{2X}$ and $P_{2Y}$ subtypes mediate vasoconstriction. There was no evidence for vasodilatation to ATP or analogues. Stimulation of the $P_2$-purinoceptor by ATP and 2MeSATP caused the synthesis of a prostanoid. In addition, the possibility of a receptor activated by ATP, separate from $P_{2X}$ and $P_{2Y}$ subtypes is discussed since contractions to ATP proved to be insensitive to both PPADS and RB2. A comparison is made of purinoceptors in the garter snake aorta with those in other vertebrate vessels.

**Keywords:** Garter snake aorta; adenosine; ATP; $P_{2X}$-purinoceptor; $P_{2Y}$-purinoceptor; vasodilatation; vasoconstriction

## Introduction

Since the initial observation of Drury & Szent Györgyi (1929) that adenosine compounds have potent inhibitory actions on the mammalian heart, extensive investigations have shown that both adenine nucleosides and nucleotides have important physiological actions concerning the local control of the vasculature (Su, 1985; Burnstock & Kennedy, 1986; Burnstock, 1990b; Olsson & Pearson, 1990).

Two distinct receptor classes have been recognised for adenosine and adenosine 5'-triphosphate (ATP) (Burnstock, 1978, 1990a); $P_1$- and $P_2$-purinoceptors. $P_1$-purinoceptors are identified as being preferentially activated by adenosine and adenosine 5'-monophosphate (AMP) and are blocked by methylxanthines (see Kennedy, 1990); this receptor has been further subdivided into $A_1$- and $A_2$-receptors and an $A_3$ subtype has been proposed using analogue potency orders (Van Calker *et al.*, 1979; Londos *et al.*, 1980; Ribeiro & Sebastiao, 1986; Stone, 1991); the $P_1$-purinoceptor has been subdivided further (see Stone, 1989; Hoyle, 1992). $P_2$-purinoceptors are described as being preferentially activated by ATP and adenosine 5'-diphosphate (ADP); these were initially divided into $P_{2X}$ and $P_{2Y}$ subgroups, based on potency orders of ATP analogues (Burnstock & Kennedy, 1985); additional subtypes of purinoceptors have been proposed, for example $P_{2Z}$, $P_{2T}$, and $P_{2U}$ subclasses (Gordon, 1986; Hoyle & Burnstock, 1991a; Stone, 1991; Hoyle, 1992; O'Connor, 1992). Further subclasses have been put forward including $P_{2R}$, $P_{2S}$ and $P_3$, but lack widespread support (see Hoyle & Burnstock, 1991a,b; Stone, 1991).

Both $P_1$- and $P_2$-purinoceptor agonists have widespread actions on the cardiovascular system. Typically, adenosine dilates blood vessels by activating vascular smooth muscle $A_2$-receptors (Berne *et al.*, 1983; Burnstock & Kennedy, 1986; Collis, 1989), although endothelial adenosine receptors have

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been identified (Vials & Burnstock, 1993). Activation of $A_1$-purinoceptors generally mediates prejunctional inhibition of neurotransmitter release (Verhaeghe *et al.*, 1977).

In the cardiovascular system ATP stimulates both $P_{2X}$- and $P_{2Y}$-purinoceptors (Ralevic & Burnstock, 1991). $P_{2X}$-purinoceptors are found predominantly on vascular smooth muscle cells and mediate vasoconstriction (Burnstock & Kennedy, 1986; Burnstock, 1990a,b). Activation of endothelial $P_{2Y}$-purinoceptors results in vasodilatation, via endothelium-derived relaxing factor (EDRF) known to be nitric oxide (NO) (De Mey & Vanhoutte, 1981; Houston *et al.*, 1987; Pearson & Gordon, 1989; Boeynaems & Pearson, 1990) and prostacyclin (Moncada & Vane, 1979), however, some vascular smooth muscle $P_{2Y}$-purinoceptors have been demonstrated such as in the rabbit portal vein (Mathieson & Burnstock, 1985) and hepatic artery (Brizzolara & Burnstock, 1991).

Whereas there is extensive documentation of the presence and activity of both $P_1$- and $P_2$-purinoceptors on mammalian vascular preparations, information from lower vertebrate groups is limited. $P_1$-purinoceptors have been identified in the coronary artery of the trout (Small *et al.*, 1990), gill vasculature of the cichlid (Okafor & Oduleye, 1986) and dogfish atria and aorta (Meghji & Burnstock, 1984a; Evans, 1992). In the coronary artery of the skate, adenosine constricted vascular rings whereas ADP and ATP caused vasodilatation at high concentrations (Farrell & Davie, 1991a) indicating different purinoceptor subtypes. ATP is excitatory in the atria of the frog (Hoyle & Burnstock, 1986), axolotl and toad (Meghji & Burnstock, 1983a,b). There is very little documentary evidence of the effect of purine compounds on the vasculature of reptiles or birds, although dilatation in response to ATP has been observed in the perfused foot vasculature of ducks and chickens (McGregor, 1979). The present investigation was carried out to examine the effect of adenosine and ATP on the isolated dorsal aorta of the garter snake.

## Methods

### *General Procedures*

Red-backed garter snakes (*Thamnophis sirtalis parietalis*) of either sex supplied by Blades Biological, Cowden, Edenbridge, Kent, were anaesthetized with sagatal (50 mg $kg^{-1}$, i.p.) and decapitated. The brain was destroyed with a metal seeker. The dorsal aorta was rapidly removed and placed in a physiological saline solution. Ring segments of approximately 5–6 mm were dissected free and carefully mounted so as not to damage the endothelial layer in 10 ml organ baths containing continuously gassed (95% $O_2$/5% $CO_2$) saline of the following composition (mM): NaCl 144.17, KCl 4.95, $Na_2H_2PO_4$ 1.20, $NaHCO_3$ 29.60, $MgSO_4$ 1.21, $CaCl_2$ 2.75 and glucose 5.50 (Yamanouye *et al.*, 1992). Experiments were carried out at room temperature, 23 ± 1.0°C. For some experiments the endothelium was removed by rubbing the intima with a tungsten wire. The efficiency of this technique was tested by the ability of the vessel to relax to acetylcholine (ACh) when preconstricted with noradrenaline (NA; data not shown).

Segments were mounted horizontally in the organ bath by inserting 2 tungsten wires through the lumen of the vessel and attached to a rigid support and a Grass force displacement transducer FT03C. Mechanical activity was displayed on a Grass ink-writing oscillograph. An initial load of 1 g was applied and the tissues were allowed to equilibrate for 1 h.

### *Concentration-response relationships*

Prior to the experiment, the integrity of the endothelial layer was ascertained by the response to ACh on the NA ($EC_{50}$ concentration) preconstricted aorta. Vasodilatation to ACh indicated that the endothelium was intact, whereas the absence or only a very limited degree of vasodilatation indicated that the endothelium had been disrupted during preparation.

Concentration-response curves were constructed for NA to obtain an $EC_{50}$ value for each segment, which was used to constrict the vessel in order to observe relaxations to adenosine and analogues D-5'-(N-ethylcarboxamide) adenosine (NECA), **R**- and S-$N^6$-(2-phenylisopropyl) adenosine (**R**- and S-PIA) and 2-chloroadenosine (2-CA).

Concentration-response curves were constructed for ATP and analogues α,β-methylene ATP (α,β-MeATP), β,γ-methylene ATP (β,γ-MeATP), 2-methylthio ATP (2MeSATP) and uridine triphosphate (UTP) on vessels at basal tone.

In order to characterize the purinoceptors, the concentration-response curves for adenosine and analogues were repeated in the presence of the $P_1$-purinoceptor antagonists 8-*p*-sulphophenyl-theophylline (8-PSPT) and caffeine; those of ATP, α,β-MeATP, β,γ-MeATP and 2MeSATP were repeated in the presence of the $P_2$-purinoceptor antagonist suramin, the $P_{2X}$-purinoceptor antagonist pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS), the $P_{2Y}$-purinoceptor antagonist reactive blue 2 (RB2), and finally the prostanoid inhibitor, indomethacin. All antagonists were equilibrated for 20 min.

### *Drugs used*

NA bitartrate, adenosine, NECA, **R**-PIA, S-PIA, 2CA, ATP, α,β-MeATP, β,γ-MeATP, UTP, indomethacin, RB2 and ascorbic acid were supplied by Sigma Chemical Co; 2MeSATP, cyclopentyladenosine (CPA), CGS21680 (2-[p-(2-carboxyethyl) phenethyl amino]-5'-N-ethylcarboxamido adenosine) and 8-PSPT by Research Biochemicals Inc.; suramin was the kind gift of I.C.I. and PPADS the kind gift of Dr G. Lambrecht, Johann Wolfgang Goethe University, Frankfurt, Germany. NA was dissolved in 100 μM ascorbic acid; indomethacin in 0.2 M $NaCO_3$ solution and NECA, **R**- and S-PIA, CPA and CGS21680 in dimethyl sulphoxide (DMSO) to produce a stock solution, with subsequent dilutions in distilled water. All other drugs were dissolved in distilled water.

### *Statistical analysis*

Vasodilator responses are expressed as mean % relaxation of the NA-induced contraction ± standard error (s.e.) (*n*) and vasoconstrictor responses are expressed as a percentage of the maximum response, or as a mean maximum tension developed in g. Significance was tested by analysis of variance followed by Tukey's test, or a paired Student's *t*-test. A probability of $P<0.05$ was taken as significant.

## Results

### *Vasodilator responses to adenosine and analogues*

On NA-preconstricted ($EC_{50}$ concentration) vessels, adenosine and analogues NECA, **R**- and S-PIA and 2-CA caused concentration-dependent, endothelium-independent relaxations, the maximum relaxation being between 90–100%. Individual $pD_2$ values were: NECA 7.12 ± 0.13 (9), **R**-PIA 5.93 ± 0.25 (7), 2-CA 5.64 ± 0.40 (5), adenosine 5.04 ± 0.10 (13) and S-PIA 4.26 ± 0.10 (7). Analysis of variance showed the order of potency for the compounds to be: NECA > **R**-PIA = 2-CA > adenosine > S-PIA (Figure 1).

Adenosine antagonists were examined for their ability to inhibit the dilatation to adenosine, however, 8-PSPT (up to 10 μM) and caffeine (up to 0.1 mM), inhibited constriction of the aorta to NA at all concentrations of antagonist tested and could not be used. The more selective adenosine agonists

CPA and CGS 21680, selective for $A_1$- and $A_2$-receptors respectively, were without activity on this preparation.

Neither ATP (up to 10 mM) nor 2MeSATP (up to 30 μM) caused relaxation of preconstricted vessels in either the absence or presence of the endothelium; instead, a further constriction was observed to the agonists.

### *Vasoconstrictor responses to ATP and analogues*

At basal tone, ATP, α,β-MeATP, β,γ-MeATP, 2MeSATP and UTP all concentration-dependently and endothelium-independently constricted the isolated aortic rings. ATP, α,β-MeATP and 2MeSATP reached a maximum contraction, $pD_2$ values being: ATP 3.98 ± 0.07 (10), α,β-MeATP 5.86 ± 0.15 (12) and 2MeSATP 6.06 ± 0.23 (9). There was no significant difference between the $pD_2$ values for α,β-MeATP and 2MeSATP, but both these compounds were more potent than ATP; $P<0.05$ (Figure 2).

Since concentration-response curves to β,γ-MeATP and UTP did not consistently reach a maximum response, the 5 agonists were also compared by their absolute tensions developed. Analysis of variance and Tukey's test revealed the order of potency to be: α,β-MeATP = 2MeSATP > ATP = β,γ-MeATP = UTP (data not shown).

### *Desensitization to α,β-MeATP*

Repeated applications of α,β-MeATP (3 μM) caused diminished contractions until there was no response to an additional dose of α,β-MeATP. In the presence of α,β-MeATP the responses to ATP and 2MeSATP were unchanged.

### *Effect of antagonists on constrictor responses to ATP and analogues*

Suramin (0.1 mM) significantly inhibited the constrictor responses to ATP, α,β-MeATP, β,γ-MeATP and 2MeSATP. As maximum responses to the ATP analogues were not always reached in the presence of suramin and the other antagonists, $pD_2$ values were not calculated. Student's *t*-test revealed that suramin significantly inhibited responses to all the ATP analogues; $P<0.05$ (Figures 3a -3d).

In the presence of PPADS (30 μM) the constrictor response to α,β-MeATP was significantly ($P<0.05$) inhibited (Figure 4), while having no effect on the ability of the other agonists to constrict the aorta (not shown).

RB2 (30 μM) significantly ($P<0.05$) inhibited constrictor responses to 2MeSATP only, without having an effect on the responses of the other agonists (Figure 5).

Contractile responses to ATP and 2MeSATP were significantly ($P<0.05$) inhibited by indomethacin (30 μM) (Figure 6a and 6b) whereas the responses to the other agonists were unaffected.

## Discussion

The results from this study reveal that the dorsal aorta of the garter snake possesses both $P_1$- and $P_2$-purinoceptors that are antagonistic in action and appear to be independent of the presence of an intact endothelium, the $P_1$-purinoceptor mediating vasodilatation and $P_2$-purinoceptors mediating vasoconstriction.

Identification of the $P_1$-purinoceptor subtype mediating vasodilatation of the aorta, founded on potency order alone, can be somewhat misleading. An agonist profile of R-PIA > adenosine > NECA describes an $A_1$-purinoceptor and the reverse an $A_2$-purinoceptor, according to the original definition by Van Calker *et al.* (1979). In addition, a further criterion has been suggested to aid the identification of $P_1$-purinoceptors, the stereoselectivity for the PIA isomers (Stone, 1989). A potency ratio of the R/S isomers of PIA, with R-PIA approximately 100 times more potent than S-PIA indicates an $A_1$-purinoceptor whereas a ratio of 10 or less is indicative of an $A_2$-receptor. However, the potency ratios of R- and S-PIA have proved extremely variable.

Based on these two sets of criteria, the results from this

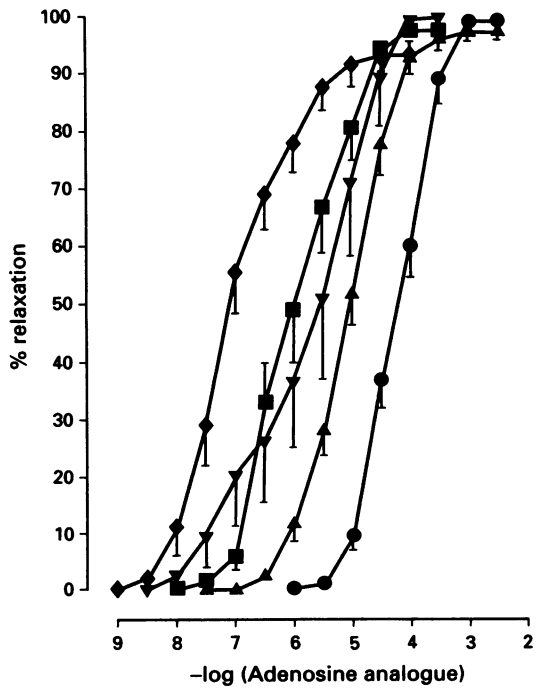


**Figure 1** Cumulative concentration-response relationships for adenosine (▲, $n=13$), NECA (◆, $n=9$), R-PIA (■, $n=7$), S-PIA (●, $n=7$) and 2-CA (▼, $n=5$) on noradrenaline ($EC_{50}$ concentration) preconstricted garter snake aorta. All symbols represent mean % relaxation ± s.e. (unless masked by the symbol). For abbreviations, see text.

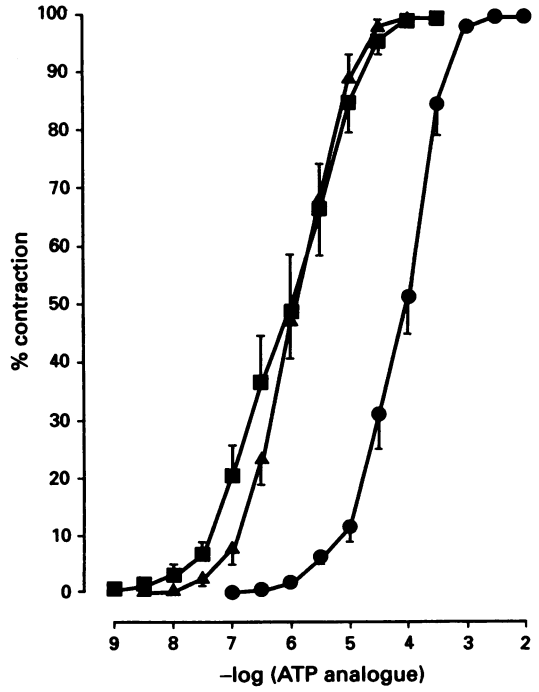


**Figure 2** Concentration-response relationships for ATP (●, $n=10$), α,β-MeATP (▲, $n=12$) and 2MeSATP (■, $n=9$) on basal tone of the dorsal aorta of the garter snake. All symbols show mean % contraction ± s.e. (unless masked by the symbol). For abbreviations, see text.

study can be interpreted in a variety of ways, and may be indicative of both an $A_1$- and an $A_2$-receptor. The order of potency for NECA and **R**-PIA suggest that the receptor is of the $A_2$ subtype, since NECA was significantly more potent than the PIA isomers (Van Calker *et al.*, 1979; Londos *et al.*, 1980). If the stereoselectivity of the isomers for PIA is considered alone, then the opposite interpretation can be concluded; i.e. the receptor might be considered of the $A_1$ subtype since **R**-PIA is approximately 100 times more potent than **S**-PIA (Ribeiro & Sebastiao, 1986; Stone, 1989). The identification of this receptor is confounded further if one takes into consideration the proposed existence of the $A_3$-receptor believed to exist on nerve endings and the heart and not coupled to adenylyl cyclase. The $A_3$-receptor has been described as having an agonist profile of cyclohexyl adenosine (CHA), **R**-PIA and NECA being the most potent, irrespective of their order, and more potent than 2-CA (Ribeiro & Sebastiao, 1985). The adenosine receptor of the snake aorta does not appear to fall into either $P_1$-purinoceptor subtype, thus it could also be argued that the receptor

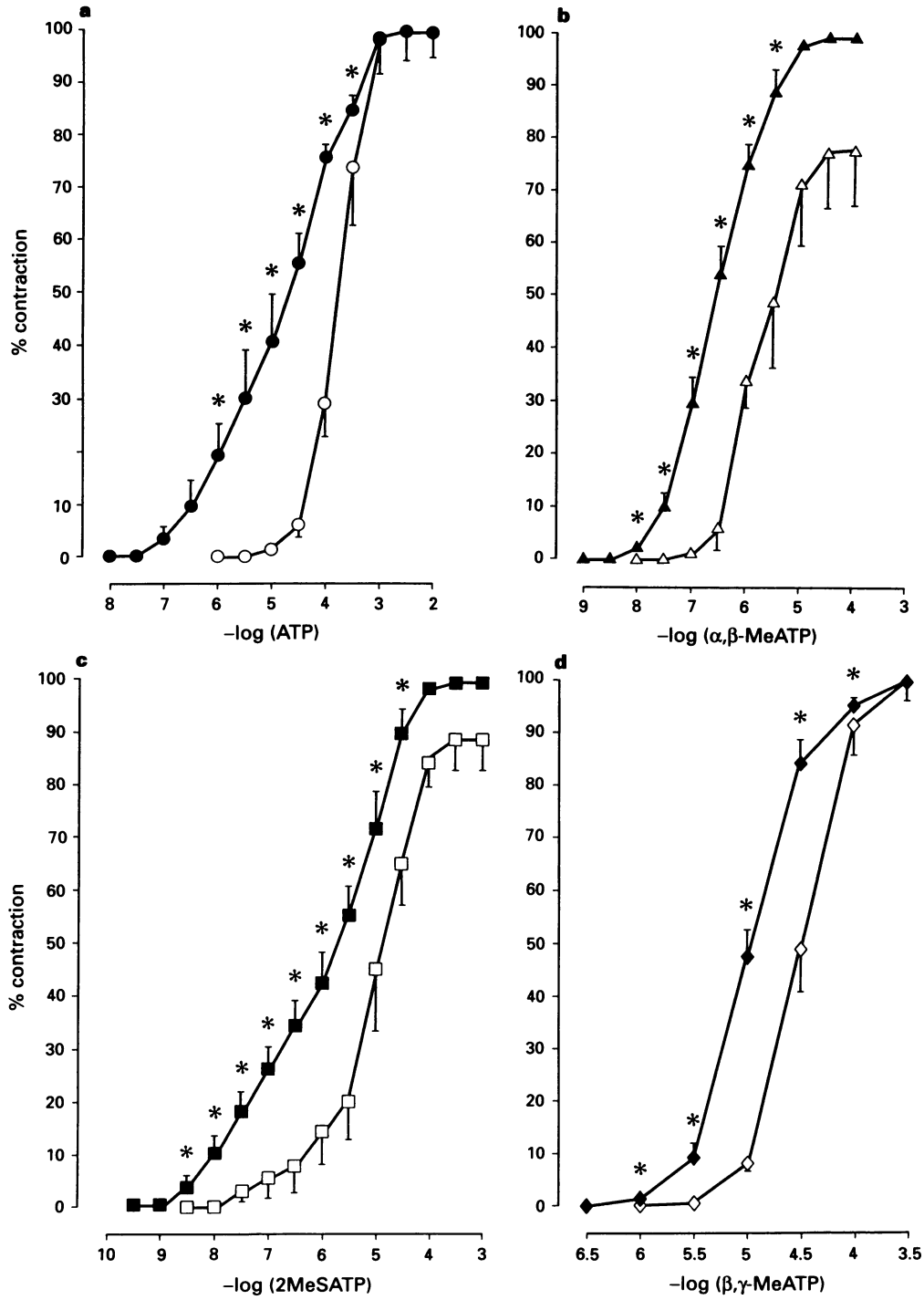


**Figure 3** Effect of suramin (0.1 mM) on the concentration-response relationships for ATP, α,β-MeATP, 2MeSATP and β,γ-MeATP, on the dorsal aorta of the garter snake. (a) Concentration-response relationship for ATP in the absence (●, $n = 5$) and presence (○, $n = 5$) of suramin (0.1 mM). (b) Concentration-response relationship for α,β-MeATP in the absence (▲, $n = 6$) and presence (△, $n = 6$) of suramin (0.1 mM). (c) Concentration-response relationship for 2MeSATP in the absence (■, $n = 6$) and pressence (□, $n = 6$) of suramin (0.1 mM). (d) Concentration-response relationship for β,γ-MeATP in the absence (◆, $n = 5$) and presence (◇, $n = 5$) of suramin (0.1 mM). All symbols show mean % contraction ± s.e. (unless masked by the symbol). *Indicates significance, $P < 0.05$. For abbreviations, see text.

from this study is of the $A_3$ type, proposed for those receptors that cannot be classified according to classical criteria.

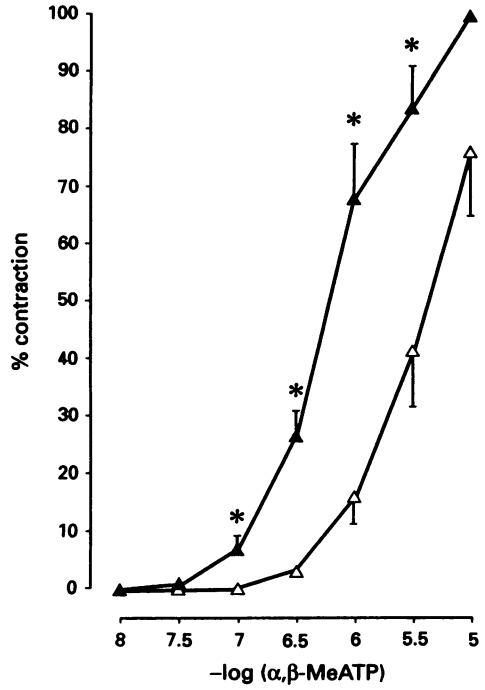


**Figure 4** Concentration-response relationship for α,β-MeATP in the absence (▲, $n = 6$) and presence (△, $n = 6$) of PPADS (30 μM), on the dorsal aorta of the garter snake. All symbols show mean % contraction ± s.e. (unless masked by the symbol). *Indicates significance, $P<0.05$. For abbreviations, see text.

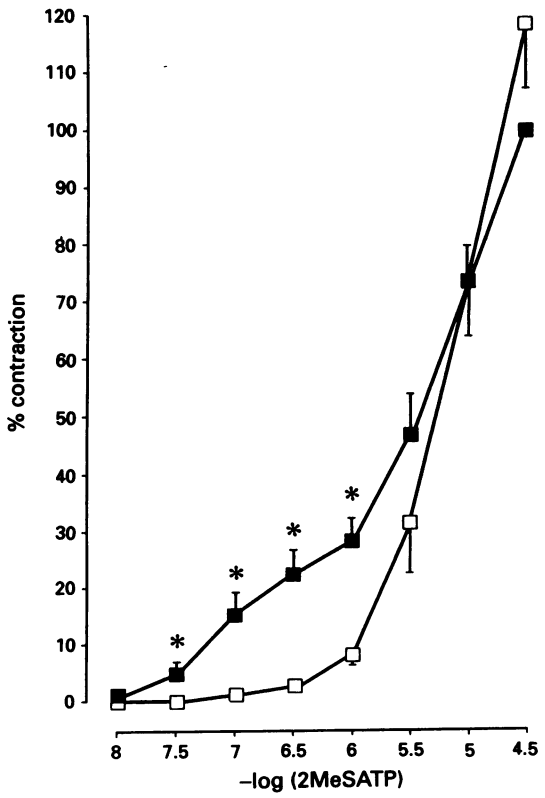


**Figure 5** Concentration-response relationship for 2MeSATP in the absence (■, $n = 6$) and presence (□, $n = 6$) of RB2 (30 μM), on the dorsal aorta of the garter snake. All symbols show mean % contraction ± s.e. (unless masked by the symbol). *Indicates significance, $P<0.05$. For abbreviations, see text.

The adenosine antagonists examined in this study inhibited the constrictor activity of NA by an unknown mechanism, classification of the receptor by the use of selective antagonists was therefore denied and as such the receptor can only be classified by the use of the somewhat misleading agonist potency order. Lack of activity of selective agonists and antagonists is not uncommon when studying more primitive organisms. For instance the adenosine antagonist

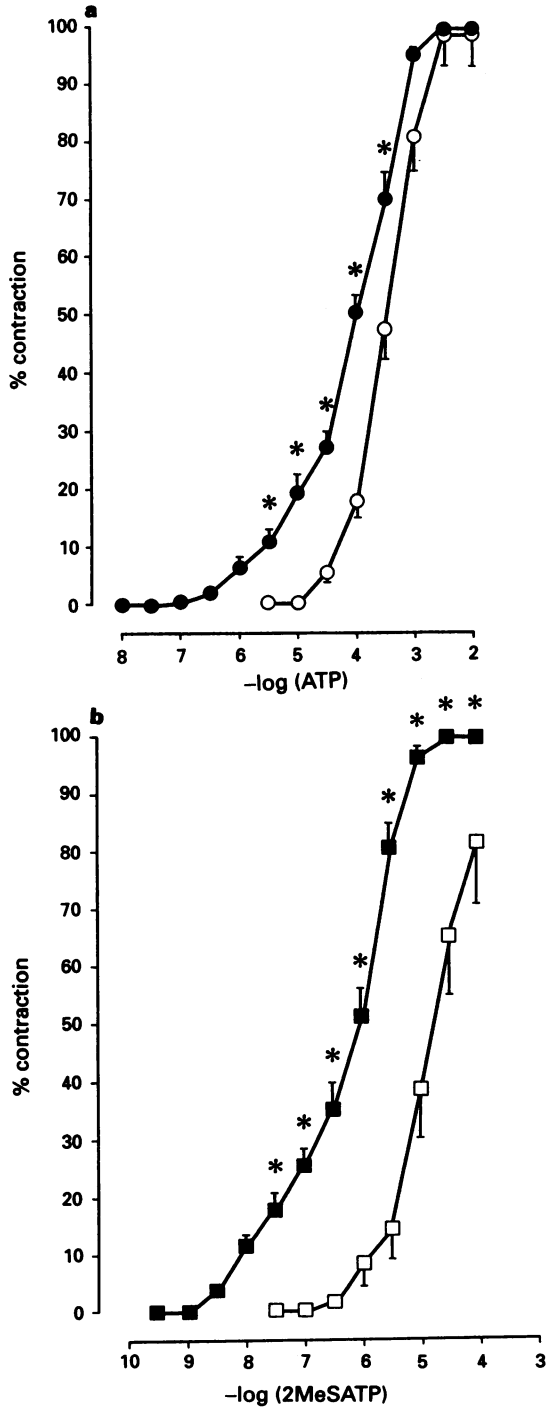


**Figure 6** Effect of indomethacin (30 μM) on the concentration-response relationships for ATP and 2MeSATP, on the dorsal aorta of the garter snake. (a) Concentration-response relationship for ATP in the absence (●, $n = 5$) and presence (○, $n = 5$) of indomethacin (30 μM). (b) Concentration-response relationship for 2MeSATP in the absence (■, $n = 5$) and presence (□, $n = 5$) of indomethacin (30 μM). All symbols show mean % contraction ± s.e. (unless masked by the symbol). *Indicates significance, $P<0.05$. For abbreviations, see text.

1,3-dipropyl-8-cyclopentylxanthine (DPCPX) was found to be ineffective at blocking adenosine in the stomach and intestine of the stickleback (Knight & Burnstock, 1993).

There are many instances in the literature where subtypes of adenosine receptors have been identified although the potency order does not agree with the classical definition. For instance, the receptor inhibiting nerve evoked contractions of the rat vas deferens has an agonist profile of NECA > **R**-PIA > 2-CA, the **R/S** ratio being 100 and the receptor is classified by the authors as an $A_1$ subtype on this basis, even though NECA is the more potent (Paton & Webster, 1984). Similarly, in bovine coronary arteries, the inhibitory adenosine receptor is classified as an $A_2$-receptor, the potency order being NECA > **R**-PIA > 2-CA although the **R/S** ratio is much greater than 10, being in fact 32 (Mustafa & Askar, 1985). Clearly, identification of adenosine receptor subtypes on agonist potency alone, particularly when the order does not agree exactly with those described for classical $A_1$, $A_2$ or $A_3$ subtypes is inappropriate. considering the ambiguity of the interpretation of the potency order of the adenosine analogues, it may be appropriate to conclude that the receptor identified in this study is unique in nature possessing characteristics of all 3 adenosine receptors when defined by the order of potency alone.

This study has revealed the presence of $P_2$-purinoceptors on the aorta that are also independent of an intact endothelium. The order of potency: $\alpha,\beta$-MeATP = 2MeSATP > ATP > $\beta,\gamma$-MeATP > UTP, would suggest an undifferentiated $P_2$-purinoceptor. The possibility of the receptor being specific for UTP, a $P_{2U}$ subtype (O'Connor, 1992) is discounted since UTP was significantly less potent than all the ATP analogues.

Selective blockade of the ATP analogues indicates the presence of both $P_{2X}$- and $P_{2Y}$-purinoceptors. All of the ATP analogues were inhibited by suramin (0.1 mM) which blocks both $P_{2X}$- and $P_{2Y}$-purinoceptors (Dunn & Blakeley, 1988; Hoyle *et al.*, 1990). Thus, all the ATP analogues mediated vasoconstriction via $P_2$-purinoceptors.

The recently developed compound PPADS has shown specificity for the $P_{2X}$-purinoceptor on the vas deferens of the rabbit and guinea-pig (Lambrecht *et al.*, 1992; McLaren *et al.*, 1994), rabbit bladder (Ziganshin *et al.*, 1993) and ear and saphenous artery (Ziganshin *et al.*, 1994). PPADS (30 $\mu$M) was found to inhibit selectively contractions in response to $\alpha,\beta$-MeATP while having no effect on 2MeSATP. Interestingly, PPADS was ineffective in blocking the response to ATP itself. In addition, the presence of a $P_{2X}$-purinoceptor is confirmed since the repeated administration of $\alpha,\beta$-MeATP resulted in desensitization of the response; ATP may or may not be acting at this receptor, since its continued activity after desensitization with $\alpha,\beta$-MeATP or blockade with PPADS could indicate a lack of activity at this receptor, or an action on a separate receptor.

The activity of RB2 indicates that there is also a $P_{2Y}$-purinoceptor present on the aorta of the snake. RB2 (30 $\mu$M) selectively attenuated responses to 2MeSATP only, without an effect on $\alpha,\beta$-MeATP or ATP. Contractile responses to 2MeSATP are also inhibited by indomethacin, indicating that 2MeSATP stimulated the production of a prostanoid; similarly ATP contractions were also sensitive to indomethacin, indicating that it too was activating the production of a prostanoid, although whether via an action on a separate, unidentified purinoceptor subtype is still open to conjecture.

The presence of a dual population of receptor subtypes producing the same response is somewhat unusual; however, examples can be found: $A_1$- and $A_2$-purinoceptors have been identified in the rat duodenum, both causing relaxation (Nicholls *et al.*, 1992). Commonly, vascular responses to $\alpha,\beta$-MeATP and 2MeSATP are antagonistic in nature; $\alpha,\beta$-MeATP mediating vasoconstriction and 2MeSATP mediating vasodilatation; however there was no evidence of vasodilatation to purine compounds.

While this study has not revealed the mechanism of action for the response to $\alpha,\beta$-MeATP, it has revealed that stimulation of the $P_{2Y}$-purinoceptor results in the formation of a prostanoid, since ATP and 2MeSATP were inhibited by indomethacin. This is consistent with a number of mammalian vascular systems (Needleman *et al.*, 1974; Boeynaems & Galand, 1983) where prostaglandin synthesis is initiated by ATP at the endothelial level, mediating vasodilatation (see Moncada, 1982). However, in this preparation, the prostaglandin is not endothelial in origin, since vasoconstriction to 2MeSATP occurred even when the endothelium was not intact (as revealed by the lack of vasodilatation to ACh). In a number of vascular systems. some members of the prostaglandin family can initiate vasoconstriction, for example, the rabbit aorta constricts to prostaglandin $E_1$ ($PGE_1$) and $PGE_{F1\alpha}$ (Khairallah *et al.*, 1967; Strong & Bohr, 1967). The nature of the agonist also appears to be important, since prostaglandin formation occurred only in response to an agonist possessing an unaltered phosphate chain of the purine molecule (Brown & Burnstock, 1981); $\alpha,\beta$-MeATP and $\beta,\gamma$-MeATP did not stimulate prostaglandin formation.

There is evidence that both adenosine and ATP play a part in vascular control of lower vertebrate animals. Adenosine has been shown to dilate the perfused gills of the hagfish (Axelsson *et al.*, 1990) and have effects upon the heart of the trout, frog and toad (Meghji & Burnstock, 1983b; 1984b; Hoyle & Burnstock, 1986; Lazou & Beis, 1987). Both an $A_1$- and an $A_2$-receptor have been identified in the aorta of the dogfish (Evans, 1992). ATP has been shown to have activity in a variety of different vessels from lower vertebrates, such as the systemic vasculature and coronary artery of the rainbow trout (Wood, 1977; Small *et al.*, 1990) and steelhead trout (Small & Farrell, 1990), mako shark coronary artery (Farrell & Davie, 1991b) and gill vascular bed (Colin & Leray, 1979; Colin *et al.*, 1979). However, a full characterization of these responses was not performed and as such the subtype, if any, of the purinoceptor is unknown. The dual activity of ATP, vasodilatation and vasoconstriction, on the coeliamesenteric artery and anterior cardinal vein of the trout, suggested to Olson & Villa (1991) the possibility of a dual population of ATP receptors that were antagonistic in nature.

In summary, the results from the present study have shown the presence of both $P_1$- and $P_2$-purinoceptors on the smooth muscle of the garter snake aorta. The $P_1$-purinoceptor, mediating vasodilatation, exhibiting the characteristics of an $A_1$-, an $A_2$- and the less accepted $A_3$-receptor such that its classification is limited to that of a unique or unclassified adenosine receptor. The presence of $P_{2X}$- and $P_{2Y}$-purinoceptors mediating vasoconstriction was revealed by selective antagonism by PPADS and RB2, and desensitization by $\alpha,\beta$-MeATP. The $P_{2Y}$-purinoceptor is somewhat unusual in that it mediates vasoconstriction via the production of a prostaglandin. The possibility of a receptor specific to ATP is postulated, since the response to ATP was not affected by PPADS or RB2, but, by the action of suramin, was acting via a $P_2$-purinoceptor. This study has revealed that antagonistic control of vascular smooth muscle by separate receptors for both adenosine and ATP, is a primitive feature which has now been demonstrated in a variety of lower vertebrates.

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# Characterization of the interaction between muscarinic $M_2$ receptors and β-adrenoceptor subtypes in guinea-pig isolated ileum

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**1** Contraction of guinea-pig ileum to muscarinic agonists is mediated by $M_3$ receptors, even though they account for only 30% of the total muscarinic receptor population. The aim of this study was to characterize the biochemical and functional effects of stimulation of the predominant $M_2$ muscarinic receptor (70%) and to investigate the hypothesis that $M_2$ receptors specifically oppose β-adrenoceptor-mediated effects in the ileum.

**2** In guinea-pig ileal longitudinal smooth muscle slices, isoprenaline, a non-selective β-adrenoceptor agonist, and BRL 37344 (sodium-4-[2-[2-hydroxy-2-(3-chlorophenyl)ethylamino]propyl]-phenoxyacetate sesquihydrate), a $\beta_3$-adrenoceptor selective agonist, increased cyclic AMP accumulation with $-\log EC_{50}$ values of $6.6 \pm 0.1$ and $5.8 \pm 0.1$ respectively. Maximal stimulation by BRL 37344 (10 μM) was $26.4 \pm 5.2\%$ of that observed with isoprenaline (10 μM). Isoprenaline (10 μM)-stimulated cyclic AMP accumulation was significantly, but not completely, inhibited by propranolol (5 μM), with a propranolol-resistant component of $28.2 \pm 6.8\%$ of the maximal stimulation to isoprenaline. In contrast, basal and BRL 37344 responses were resistant to this antagonist. These data provide evidence that both $\beta_1$- and $\beta_3$-adrenoceptors activate adenylyl cyclase in guinea-pig ileum.

**3** Isoprenaline (10 μM)-stimulated cyclic AMP accumulation was inhibited ($67.4 \pm 0.9\%$) by the muscarinic agonist (+)-*cis*-dioxolane ($-\log EC_{50} = 7.3 \pm 0.1$). The rank order of antagonist affinities against the (+)-*cis*-dioxolane response was ($-\log K_B$ values in parentheses): atropine ($9.0 \pm 0.2$) > methoctramine ($7.1 \pm 0.1$) > *p*-fluoro-hexa-hydrosilaphenidol (*p*-F-HHSiD; $6.5 \pm 0.2$) ≥ pirenzepine ($6.3 \pm 0.2$). (+)-*cis*-dioxolane also significantly inhibited BRL 37344 (10 μM; $56.5 \pm 2.4\%$) stimulated cyclic AMP accumulation. These data suggest that $M_2$ receptors mediate inhibition of cyclic AMP accumulation in response to both $\beta_1$- and $\beta_3$-adrenoceptor stimulation in guinea-pig ileum.

**4** 5-Hydroxytryptamine (5-HT), vasoactive intestinal peptide, prostaglandins $E_2$ and $E_1$, all at 10 μM, significantly increased cyclic AMP accumulation. (+)-*cis*-Dioxolane (10 μM) inhibited both basal and agonist-induced cyclic AMP accumulation. Thus the inhibitory effect of $M_2$ receptor agonism does not appear to be restricted to β-adrenoceptor-stimulated cyclic AMP accumulation.

**5** The potential for involvement of activation of $M_2$ receptors on responses to β-adrenoceptor agonists was also studied functionally. Selective $M_3$ receptor alkylation was achieved by pretreatment of tissues with 4-DAMP mustard (40 nM), in the presence of methoctramine (1 μM; to protect $M_2$ receptors). After washing, tissues were pre-contracted with histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM). Under these conditions, oxotremorine M caused concentration-dependent contractions ($-\log EC_{50}$ of $7.8 \pm 0.1$), that were surmountably antagonized by methoctramine (1 μM) with a $-\log K_B$ estimate of $7.4 \pm 0.1$. Similar observations were seen versus relaxation produced by BRL 37344 (1 μM), where the $-\log K_B$ value for methoctramine was $7.8 \pm 0.2$. These data suggest that $M_2$ receptors mediate a functional inhibition of relaxant responses to isoprenaline and BRL 37344.

**6** These findings are consistent with $\beta_1$- and $\beta_3$-adrenoceptors coupling to stimulation of adenylyl cyclase in guinea-pig ileum; a response that is inhibited by $M_2$ receptor stimulation. Concordantly, $M_2$ receptor stimulation also inhibits relaxation to both $\beta_1$- and $\beta_3$-adrenoceptor stimulation. These results implicate $M_2$ receptors in the modulation of sympathetic control of ileal motility.

**Keywords:** $M_2$ muscarinic receptors; β-adrenoceptors; $\beta_3$-adrenoceptors; guinea-pig ileum; cyclic AMP; receptor alkylation

## Introduction

Several smooth muscles, including guinea-pig ileum, contain heterogeneous populations of muscarinic receptors (see Eglen *et al.*, 1994 for a review). Radioligand binding studies have demonstrated that the majority of guinea-pig ileal muscarinic receptors are of the $M_2$ subtype (~70%), while a minority are of the $M_3$ subtype (~30%), with no measurable quantities of $M_1$ or $M_4$ receptors (Giraldo *et al.*, 1987; Ford *et al.*, 1991). Activation by muscarinic agonists of the minority $M_3$ receptor population is associated with longitudinal and circular smooth muscle contraction and electrolyte transport (Kachur *et al.*, 1990; Ford *et al.*, 1991). Inactivation of $M_2$ receptors by either selective alkylation (Eglen & Harris, 1993) or by treatment with pertussis toxin, which inactivates the guanine nucleotide regulatory protein $G_i$ (Eglen *et al.*, 1988), has minimal effects on guinea-pig ileal contractile responses to muscarinic agonists. These observations suggest that $M_2$ receptors are not directly involved in contraction; thus, a role for the majority $M_2$ receptor population in ileal tissue is unclear.

In some smooth muscle tissues, such as guinea-pig uterus, $M_2$ receptors directly mediate contractile responses to muscarinic agonists (Eglen *et al.*, 1989; Doods *et al.*, 1993). However, in general, smooth muscle contractile tone can be relaxed by increased levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Berridge, 1975). Consequently, as

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$M_2$ receptors have been shown to inhibit adenylyl cyclase activity in canine colon, rat ileum and bovine trachea (Zhang & Buxton, 1991; Griffin & Ehlert, 1992; Challiss *et al.*, 1993), it has been proposed that $M_2$ receptors may modulate ileal relaxation. In guinea-pig and canine tracheal smooth muscle the relaxant potency of isoprenaline in tissues pre-contracted with muscarinic agonists, is less than in tissues precontracted with histamine (Russell, 1984; Watson & Eglen, 1994). Furthermore, the relaxant potency of isoprenaline in tracheal tissues is enhanced by either selective $M_2$ receptor antagonism or pertussis toxin pretreatment (Fernandes *et al.*, 1992; Mitchell *et al.*, 1993; Watson & Eglen, 1994). Together, these findings indicate an inhibitory effect of $M_2$ receptors on relaxant responses to β-adrenoceptor stimulation.

Although no direct contractile response to $M_2$ receptor activation can be demonstrated in guinea-pig ileum (Eglen & Harris, 1993), an indirect influence on contraction via inhibition of β-adrenoceptor-mediated relaxation remains possible. In support of this proposal, Griffin & Ehlert (1992) demonstrated, in rat ileum, that $M_2$ receptors specifically inhibit β-adrenoceptor-mediated cyclic AMP accumulation. In contractile studies, Thomas *et al.* (1993) have shown that, in guinea-pig ileum, after selective $M_3$ receptor alkylation, $M_2$ receptor stimulation offsets β-adrenoceptor-mediated relaxation; an effect which manifests as re-contraction. Non-selective β-adrenoceptor agonists mediate relaxation of guinea-pig ileum via activation of both $\beta_1$- and $\beta_3$-adrenoceptor subtypes (Grassby & Broadley, 1984; Mian *et al.*, 1984; Bond & Clarke, 1988). The contribution of distinct β-adrenoceptor subtypes involved in the interaction between $M_2$ receptors and β-adrenoceptors in this tissue has not been addressed.

The aim of this study was to investigate the effect of stimulation of $\beta_1$- and $\beta_3$-adrenoceptors on guinea-pig ileal adenylyl cyclase activity and to characterize the effect thereupon of muscarinic receptor agonism. A further aim was to investigate the functional interactions occurring between muscarinic $M_2$ receptors and $\beta_1$- and $\beta_3$-adrenoceptors at the level of contraction in guinea-pig ileum. A preliminary account of this work has been presented to the British Pharmacological Society (Reddy *et al.*, 1994).

## Methods

### *Measurement of [$^3$H]-cyclic AMP accumulation*

Male guinea-pigs (300–400 g) were killed by carbon dioxide asphyxiation and ileal tissue removed into modified Krebs-Henseleit solution (containing mM: KCl 4.6, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, NaCl 118.2, glucose 10.0, $NaHCO_3$ 24.8 and $CaCl_2$ 2.5). Longitudinal smooth muscle from the ileum was isolated by rubbing the ileum with a cotton swab (Rang, 1964). Slices were then prepared by cross-chopping the tissue on a McIlwain tissue chopper and placed into gassed (95% $O_2$/5% $CO_2$) Krebs solution. The slices were then labelled with 1 μM [$^3$H]-adenine (2 μCi ml$^{-1}$) and subsequently washed, as described by Griffin & Ehlert (1992).

[$^3$H]-adenine labelled slices, in gassed Krebs solution containing 3-isobutyl-1-methylxanthine (1 mM, final assay concentration), were pre-incubated in either the presence or absence of antagonists for 10 min at 37°C. This was followed by a further pre-incubation for 3 min at 37°C in the presence or absence of muscarinic agonists as indicated. In most studies the non-selective muscarinic agonist (Grana *et al.*, 1986) (+)-*cis*-dioxolane was used. Forskolin (0.1 μM) was then added to each sample unless otherwise indicated (this significantly increased the stimulation of cyclic AMP accumulation by isoprenaline (10 μM), above basal levels from 2.5 to 3.1 fold). The assay was started by the addition of β-adrenoceptor agonist (or other agonist as indicated) giving a total assay volume of 300 μl. The accumulation of cyclic AMP was terminated after 10 min, shaking at 37°C, by the addition of 30 μl of 2.2 N HCl containing [$^{14}$C]-cyclic AMP (~1000 d.p.m./tube). In initial studies, idazoxan (3 μM) was included to minimize the potential for effects of isoprenaline at $\alpha_2$-adrenoceptors (Drew, 1978). However, as no significant effect of idazoxan (3 μM) on basal or isoprenaline-stimulated cyclic AMP accumulation was observed (results not shown), it was omitted from further studies.

Chromatographic separation of [$^3$H]-cyclic AMP from substrate and other adenine metabolites was achieved by the method of Alvarez & Daniels (1992). An aliquot was removed from each tube as an estimate of the total radioactivity present and the remaining contents applied to columns containing acidic alumina (1.3 g). The columns were washed with 12 ml HCl (0.005 N) followed by 1.5 ml ammonium acetate (0.1 mM; unbuffered) and the eluant discarded. The columns were placed over scintillation vials and [$^3$H]-cyclic AMP was eluted by the addition of 4.5 ml ammonium acetate (0.1 mM; unbuffered). After addition of scintillant, quantitation of radioactivity was determined by liquid scintillation counting (Packard Tri-Carb). Assays were carried out in triplicate and results were corrected for [$^{14}$C]-cyclic AMP recovery from the alumina columns and expressed as a percentage of total [$^3$H]-adenine counts converted to [$^3$H]-cyclic AMP.

### *Contractile studies*

Whole ileum was obtained from guinea-pigs as described above, and placed into gassed (95% $O_2$/5% $CO_2$), modified Krebs solution (the composition of the Krebs solution was as above except that 1.8 mM $CaCl_2$ was used). Ileal segments (~2 cm in length) were suspended, under 0.5 g tension, in 10 ml organ baths containing gassed Krebs solution (pH 7.4, 37°C) and changes in isometric tension recorded. All preparations were equilibrated for 60 min prior to construction of a concentration-response curve to oxotremorine M, a non-selective muscarinic agonist (Grana *et al.*, 1986), and then treated as described below before performing a second concentration-response curve to oxotremorine M. Antagonists when used were equilibrated with tissues for 60 min before construction of the second curve to oxotremorine M.

Certain conditions have been described as optimal for revealing indirect contractile responses to $M_2$ receptor-activation: selective alkylation of $M_3$ receptors, followed by induction of tone by histamine and subsequent relaxation with isoprenaline, prior to addition of a muscarinic agonist (Thomas *et al.*, 1993). Under these conditions a reversal of the isoprenaline-induced relaxation can be seen. In the present studies a similar protocol was used and preliminary experiments, to optimize these conditions, were undertaken. For the sake of clarity, these are discussed separately. In addition, in order to adhere to the protocol developed by Thomas *et al.* (1993), oxotremorine M rather than *cis*-dioxolane was employed. Both agonists exhibit a high intrinsic efficacy and do not discriminate between muscarinic receptor subtypes.

### *Effects of histamine precontraction and β-adrenoceptor relaxation on the contractile response to muscarinic receptor stimulation*

Tissues were washed at 15 min intervals for 60 min and then precontracted with histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM). When a steady level of isometric tension was attained under these conditions, a second concentration-response curve to oxotremorine M was constructed.

In some studies, the above protocol was repeated but in the presence of histamine (1 μM) and the $\beta_3$-adrenoceptor agonist BRL 37344 (Arch *et al.*, 1964) at 1 μM. All contractile studies with BRL 37344 were carried out in the presence of propranolol (5 μM) to antagonize $\beta_1$-adrenoceptors. In order to achieve the same precontractile response, a histamine con-

centration of 1 μM was used in this study since an inhibitory effect of propranolol on the histamine contraction was observed (data not shown; see also Bond & Clarke, 1988).

### *Effect of selective $M_3$ receptor alkylation on the contractile response to muscarinic receptor stimulation*

Tissues were washed and exposed to the slightly $M_3$-selective irreversible receptor antagonist 4-DAMP mustard (40 nM), for 60 min, in the presence of methoctramine (1 μM). Tissues were then exposed to sodium sulphite (0.5 mM) for an additional 10 min to inactivate the mustard. Methoctramine was present to protect $M_2$ receptors from alkylation, thereby enhancing the selective $M_3$ inactivation by 4-DAMP mustard. Tissues were then washed at 10 min intervals for 90 min to remove both 4-DAMP mustard and methoctramine and a second concentration-response curve to oxotremorine M established.

### *Effect of selective $M_3$ receptor alkylation, histamine precontraction and β-adrenoceptor relaxation on the contractile response to muscarinic receptor stimulation*

A schematic outline of the protocol used is shown in Figure 1. Concentration-response curves to oxotremorine M were established, tissues washed and then exposed to 4-DAMP mustard (40 nM) under the conditions described above. Tissues were then exposed to histamine (0.3 μM), followed by isoprenaline (0.6 μM), and a second concentration-response curve to oxotremorine M constructed. To investigate the potential interaction between $M_2$ receptors and $\beta_3$-adrenoceptors, the above protocol was repeated in separate tissues with histamine (1 μM) and BRL 37344 (1 μM).

### *Chemicals*

The following compounds were used: vasoactive intestinal peptide was from Bachem Inc. (Torrance, CA, U.S.A.). Prostaglandins $E_1$ and $E_2$ were from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Alumina (Acidic, Activity grade I) was from ICN Biomedicals GmbH (Eschwege, Germany). [2-$^3$H]-Adenine (specific activity 15–25 Ci mmol$^{-1}$) and [8-$^{14}$C]-cyclic AMP (specific activity 50–60 mCi mmol$^{-1}$) were obtained from Moravek Biochemicals Inc. (Brea, CA, U.S.A.). Atropine sulphate, (+)-*cis*-dioxolane (L-(+)-*cis*-2-methyl-4-trimethylammonium methyl-1,3-dioxolane iodide, a 60:40 mixture of *cis*:*trans*), 4-DAMP mustard (4-diphenylacetoxy-N-(2-chloroethyl) piperidine), *p*-F-HHSiD (*para*-fluoro-hexa-hydro-siladiphenidol), histamine, idazoxan hydrochloride, methoctramine tetrachloride, oxotremorine methiodide (oxotremorine M) and pirenzepine dihydrochloride were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Adenine, ammonium acetate, 5-HT, 3-isobutyl-1-methylxanthine, (±)-isoprenaline sulphate, forskolin, (±)-propranolol hydrochloride and sodium sulphite were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BRL 37344 (sodium-4-[2-[2-hydroxy-2-(3-chlorophenyl)ethylamino] propyl]-phenoxyacetate sesquihydrate (**RR.SS** diastereoisomer) was synthesized by the Institute of Organic Chemistry (Syntex Discovery Research, Palo Alto, CA, U.S.A.).

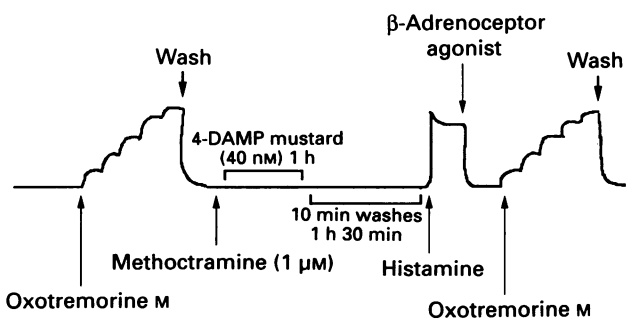


**Figure 1** Schematic representation of the protocol used. Concentration-response curves to oxotremorine M were constructed. Tissues were then alkylated with 4-DAMP mustard in the presence of $M_2$ receptor protection and the response to oxotremorine M assessed in the presence of histamine and β-adrenoceptor agonism.

Solutions were prepared in distilled water with the following exceptions: stock solutions of BRL 37344, *p*-F-HHSiD, prostaglandins $E_1$ and $E_2$ were prepared in ethanol. Isoprenaline was dissolved in ascorbic acid (5 mM). Vasoactive intestinal peptide and 4-DAMP mustard were prepared in distilled water and subsequently acidified with dilute acetic acid.

### *Data analysis*

Agonist potencies ($-\log EC_{50}$) were estimated using the relationship of Parker & Waud (1971) by a non-linear iterative curve fitting procedure (Kaleidagraph, Synergy software, Reading, PA, U.S.A.). Antagonist affinities ($-\log K_B$ values) were estimated as described by the relationship of Furchgott (1972). All values quoted are mean ± s.e.mean. Statistical significance was determined by Student's unpaired *t* test, a probability level of $P<0.05$ being considered significant.

## Results

### *β-Adrenoceptor stimulation of [$^3$H]-cyclic AMP accumulation*

Isoprenaline caused concentration-dependent increases in cyclic AMP accumulation above basal levels in longitudinal smooth muscle slices (Figure 2a). The potencies ($-\log EC_{50}$) of isoprenaline and BRL 37344 were 6.6 ± 0.1 and 5.8 ± 0.3, respectively ($n = 5$). The maximal stimulation of cyclic AMP accumulation by BRL 37344 was 26.4 ± 5.2% of the maximal response to isoprenaline. Propanolol (1 μM) caused a dextral shift in portions of the concentration-response curve to isoprenaline and the resultant curve was best described by iterative curve fitting to a two-site model (Figure 2a). This analysis revealed a propanolol-resistant component, with a maximum comprising 19 ± 9% of the total response to isoprenaline. The magnitude of the propranolol-resistant component was similar to the response to BRL 37344 alone (26.4 ± 5.2% of the maximum response to isoprenaline). The apparent affinity ($-\log K_B$) of propanolol on the sensitive component of the isoprenaline-response curve was approximately 8.3. Propanolol at a concentration of 5 μM did not significantly alter either basal or BRL 37344 (10 μM)-stimulated cyclic AMP levels (Figure 2b). By contrast, the response to isoprenaline (10 μM) was significantly reduced by 5 μM propranolol, but with a resistant component of 28.2 ± 6.8% of the maximal isoprenaline stimulation (5 μM; Figure 2b).

### *Effect of muscarinic agonists on β-adrenoceptor stimulated [$^3$H]-cyclic AMP accumulation*

(+)-*cis*-Dioxolane (10 μM) significantly inhibited basal, BRL 37344 (10 μM) and isoprenaline (10 μM)-stimulated cyclic AMP accumulation (Figure 3a). Similar results were obtained with oxotremorine M (10 μM) which is also a non-selective muscarinic agonist (results not shown). The inhibition by (+)-*cis*-dioxolane of isoprenaline (67%) and BRL 37344 (56%) stimulated cyclic AMP accumulation was significantly greater than the inhibitory effect on basal (45%). In the absence of forskolin (0.1 μM), (+)-*cis*-dioxolane (10 μM) significantly reduced the cyclic AMP response to basal, BRL 37344 and isoprenaline-stimulated cyclic AMP accumulation (data not shown).

(+)-*cis*-Dioxolane produced a concentration-dependent

inhibition (−log $EC_{50}$ of 7.3 ± 0.1) of isoprenaline (10 μM)-stimulated cyclic AMP accumulation (Figure 3b). Several antagonists were used to characterize the muscarinic receptor subtype responsible for this inhibitory effect: atropine (10 nM), pirenzepine (1 μM), methoctramine (1 μM) and *p*-F-HHSiD (3 μM) as shown in Figure 3b and Table 1. All antagonists caused parallel dextral shifts in concentration-response curves to (+)-*cis*-dioxolane, with no significant effect on either basal cyclic AMP accumulation or the maximal response to (+)-*cis*-dioxolane. The rank order of apparent antagonist affinities (−log $K_B$) at receptors mediating the (+)-*cis*-dioxolane response was atropine > methoctramine > *p*-F-HHSiD ≥ pirenzepine (Table 1). Due to the small amount of cyclic AMP accumulation induced by both basal and BRL 37344-stimulation, the muscarinic receptor subtype involved in inhibition of these responses was not investigated.

### *Specificity of the inhibitory effect of (+)-cis-dioxolane on [$^3$H]-cyclic AMP accumulation*

To investigate the specificity of the inhibitory effect of (+)-*cis*-dioxolane on cyclic AMP accumulation, other agents known to stimulate adenylyl cyclase were studied. Thus, 5-HT, vasoactive intestinal peptide, prostaglandin $E_1$ and $E_2$, all at 10 μM, caused significant increases in cyclic AMP

**Table 1** Muscarinic receptor antagonist affinities against (+)-*cis*-dioxolane inhibition of isoprenaline (10 μM)-stimulated cyclic AMP accumulation

| *Antagonist* | *Concentration* | *Affinity* (estimated −log $K_B$) | n |
|---|---|---|---|
| Atropine | 10 nM | 9.0 ± 0.2 | 4 |
| Methoctramine | 1 μM | 7.1 ± 0.1 | 5 |
| *p*-F-HHSiD | 3 μM | 6.5 ± 0.2 | 5 |
| Pirenzepine | 1 μM | 6.3 ± 0.2 | 5 |

Antagonists were studied at single concentrations and −log $K_B$ values estimated by the method of Furchgott (1972). Values are means ± s.e.mean.

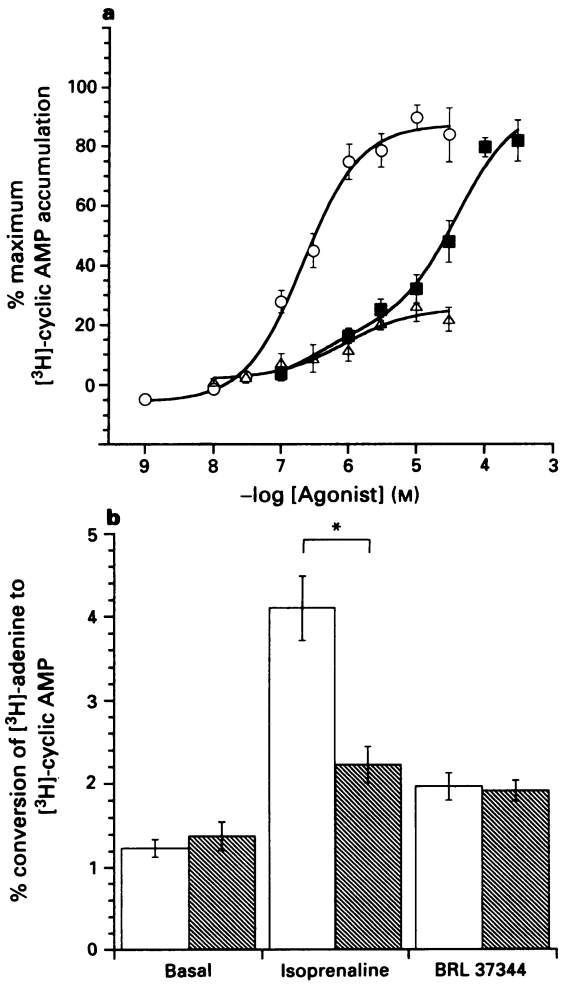


**Figure 2** (a) Accumulation of [$^3$H]-cyclic AMP in longitudinal smooth muscle slices in response to β-adrenoceptor agonism: stimulation by isoprenaline in the absence (○) and presence (■) of propranolol (1 μM), or stimulation by BRL 37344 (△). Results are expressed as a percentage of maximal stimulation of isoprenaline (basal levels subtracted). Values are the mean ± s.e.mean, $n = 5$. (b) The effect of propranolol on basal and β-adrenoceptor stimulated [$^3$H]-cyclic AMP accumulation in longitudinal smooth muscle slices. Histograms showing cyclic AMP accumulation in the presence (hatched columns) and absence (open columns) of propranolol (5 μM) on basal, isoprenaline (10 μM) and BRL 37344 (10 μM)-stimulated levels. Values are the mean ± s.e.mean, $n = 5$–6, $^*P < 0.05$.

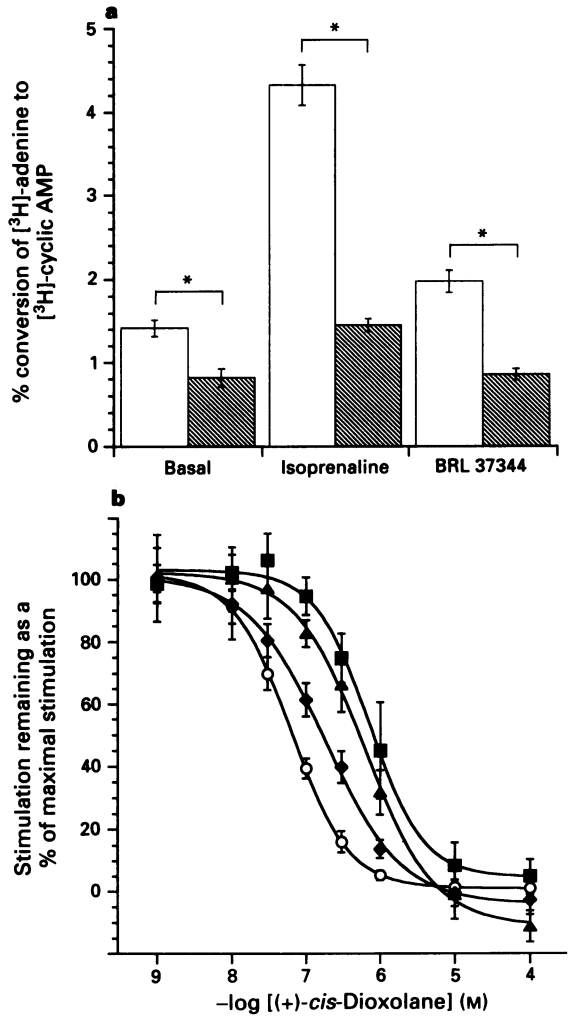


**Figure 3** (a) The effect of the presence (hatched columns) and absence (open columns) of (+)-*cis*-dioxolane (10 μM) on basal, isoprenaline (10 μM) and BRL 37344 (10 μM)-stimulated [$^3$H]-cyclic AMP accumulation in longitudinal smooth muscle slices. Values are the mean ± s.e.mean, $n = 5$, $^*P < 0.05$. (b) The effect of muscarinic antagonism on (+)-*cis*-dioxolane inhibition of isoprenaline (10 μM) stimulated [$^3$H]-cyclic AMP accumulation in longitudinal smooth muscle slices. Slices were incubated with isoprenaline (10 μM) and increasing concentrations of (+)-*cis*-dioxolane in the absence (○) and presence of pirenzepine (◆: 1 μM), *p*-F-HHSiD (△: 3 μM) and methoctramine (■: 1 μM). Results are expressed as a percentage of maximal stimulation with isoprenaline (10 μM) with basal levels subtracted. −log $K_B$ estimates calculated for these antagonists are shown in Table 1.

**Table 2** Effect of muscarinic agonism with (+)-*cis*-dioxolane on basal and stimulated cyclic AMP accumulation

| Condition | % conversion to cyclic AMP Control | (+)-*cis*-Dioxolane | % inhibition (+)-*cis*-Dioxolane | n |
|---|---|---|---|---|
| Basal | 1.42 ± 0.12 | 0.82 ± 0.10 | 45.0 ± 4.0 | 8 |
| BRL 37344 (10 μM) | 1.98 ± 0.13 | 0.86 ± 0.07 | 56.4 ± 2.4 | 6 |
| Isoprenaline (10 μM) | 4.33 ± 0.24 | 1.46 ± 0.08 | 67.4 ± 0.9 | 8 |
| 5-HT (10 μM) | 2.41 ± 0.07 | 1.30 ± 0.09 | 46.0 ± 2.3 | 3 |
| $PGE_1$ (10 μM) | 7.48 ± 0.95 | 2.96 ± 0.63 | 61.2 ± 3.7 | 3 |
| $PGE_2$ (10 μM) | 4.87 ± 0.61 | 1.83 ± 0.19 | 62.2 ± 1.7 | 3 |
| VIP (10 μM) | 3.20 ± 0.55 | 1.95 ± 0.37 | 38.8 ± 5.1 | 3 |

The percentage of total [$^3$H]-adenine counts converted to [$^3$H]-cyclic AMP in response to several agents, in the presence and absence of (+)-*cis*-dioxolane (10 μM); $PGE_1$, prostaglandin $E_1$; $PGE_2$, prostaglandin $E_2$; VIP, vasoactive intestinal peptide. Values are means ± s.e.mean and $n$ = number of experiments.

**Table 3** Affinity of methoctramine (1 μM) at receptors mediating ileal contractions to oxotremorine M under several conditions

| Condition | Affinity (estimated $-\log K_B$) | n |
|---|---|---|
| Control | 6.2 ± 0.2 | 6 |
| Histamine/isoprenaline | 6.5 ± 0.1 | 6 |
| Histamine/BRL 37344 | 6.5 ± 0.1 | 6 |
| 4-DAMP mustard | 6.4 ± 0.1 | 5 |
| 4-DAMP must/histamine/isoprenaline | 7.4 ± 0.1* | 6 |
| 4-DAMP must/histamine/BRL 37344 | 7.8 ± 0.2* | 5 |

Histamine/isoprenaline; tissues contracted with histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM) prior to contraction with oxotremorine M. Histamine/BRL 37344; tissues contracted with histamine (1 μM) and relaxed with BRL 37344 (1 μM) prior to contraction with oxotremorine M. 4-DAMP mustard; tissues treated with 4-DAMP mustard (described in Methods) before contraction with oxotremorine M. Values are means ± s.e.mean. *$-\log K_B$ values are significantly greater than control value.

accumulation in longitudinal smooth muscle slices, all of which were inhibited by (+)-*cis*-dioxolane (10 μM; Table 2).

### *Effects of histamine precontraction and β-adrenoceptor relaxation on the contractile response to oxotremorine M*

In contractile studies the potency ($-\log EC_{50}$) of oxotremorine M was 7.7 ± 0.1 and the maximal response was 3.1 ± 0.2 g ($n$ = 6). Methoctramine (1 μM) reduced the potency of oxotremorine M to 7.3 ± 0.1, with no significant effect on maximum contraction. The apparent affinity of methoctramine estimated against contraction to oxotremorine M ($-\log K_B$ value) was 6.2 ± 0.2 ($n$ = 6; Table 3). In tissues precontracted to histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM), there was no significant effect on either potency (7.7 ± 0.1) or the maximal response to oxotremorine M (2.6 ± 0.3 g, $n$ = 6; Figure 4a). Methoctramine antagonized the response to oxotremorine M in the presence of histamine and isoprenaline resulting in a reduced potency (7.1 ± 0.1) with no significant effect on the maximal response (2.8 g ± 0.2 g) (Figure 4a; Table 3). Likewise, the potency and maximal contractile response to oxotremorine M were unaltered by pretreatment with histamine (1 μM) and BRL 37344 (1 μM). (These studies, using BRL 37344 were conducted in the presence of 5 μM propranolol, to antagonize $\beta_1$-adrenoceptors.) Methoctramine (1 μM) had no significant effect on the magnitude of the responses to histamine, isoprenaline or BRL 37344 under the conditions discussed above. There was no significant difference in the apparent affinity of methoctramine (1 μM) between control contractions to oxotremorine M and on the contractile responses in the presence of histamine and isoprenaline or BRL 37344 (Table 3).

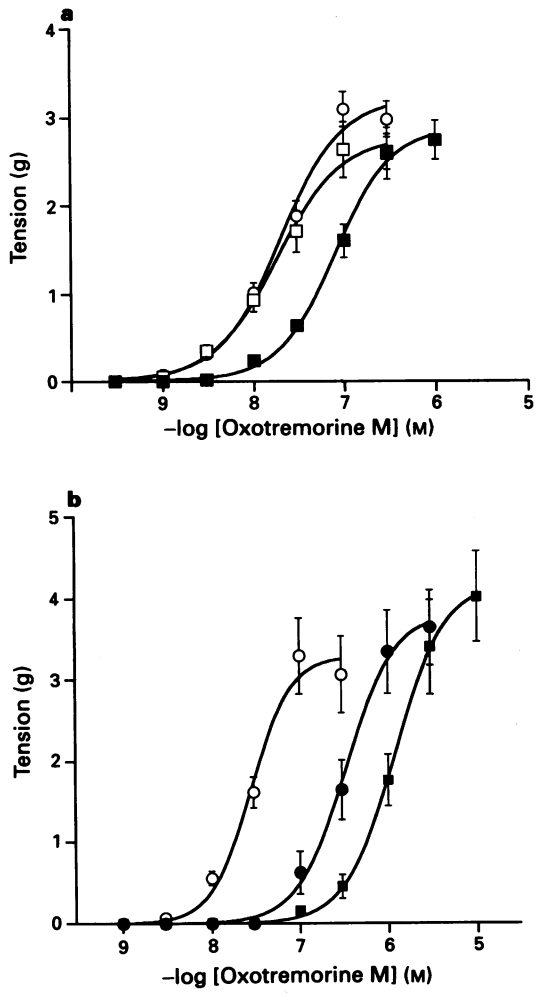


**Figure 4** (a) Contractile responses of guinea-pig ileum to oxotremorine M in control tissues (○) and responses to oxotremorine M in tissues pre-exposed to histamine (0.3 μM) and isoprenaline (0.6 μM) in the absence (□) and presence (■) of methoctramine (1 μM). Values are means ± s.e.mean ($n$ = 6). (b) Contractile responses of guinea-pig ileum to oxotremorine M in control tissues (○) and after pretreatment with 4-DAMP mustard (in the presence of $M_2$ receptor protection) (closed symbols). Effect of the presence (■) and absence (●) of methoctramine (1 μM) on tissues pretreated with 4-DAMP mustard (in the presence $M_2$ receptor protection). Values are means ± s.e.mean ($n$ = 5).

### *Effects of $M_3$ receptor alkylation on the contractile response to muscarinic receptor stimulation*

Pretreatment with 4-DAMP mustard (40 nM for 60 min, in the presence $M_2$ receptor protection; see Methods) significantly reduced the potency of oxotremorine M to a value

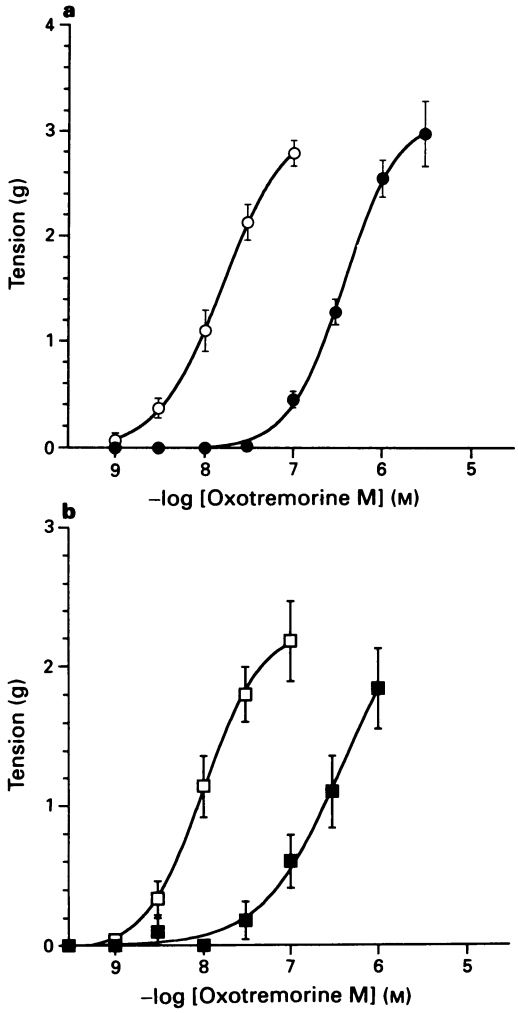


**Figure 5** (a) Contractions to oxotremorine M in the absence (○) and presence of methoctramine (1 μM) (●) in guinea-pig ileum pre-exposed to 4-DAMP mustard (in the presence of $M_2$ receptor protection). Contractions to oxotremorine M were recorded in the presence of histamine (0.3 μM) and isoprenaline (0.6 μM). Values are means ± s.e.mean ($n = 6$). (b) Contractions to oxotremorine M in the absence (□) and presence of 1 μM methoctramine (■) in guinea-pig ileum pre-exposed to 4-DAMP mustard (in the presence of $M_2$ receptor protection). Contractions to oxotremorine M were recorded in the presence of histamine (1.0 μM), BRL 37344 (1.0 μM) and propranolol (5 μM). Values are means ± s.e.mean ($n = 5$).

of 6.5 ± 0.1, with no significant effect on the maximal contraction (3.6 ± 0.5 g; Figure 4b). Methoctramine (1 μM) surmountably antagonized this response, resulting in a $-\log K_B$ value of 6.4 ± 0.1 ($n = 5$, Table 3). Consequently, there was no significant difference in the affinity ($-\log K_B$) of methoctramine at muscarinic receptors mediating ileal contraction before or after alkylation by 4-DAMP mustard.

### *Effects of selective $M_3$ alkylation, histamine precontraction and β-adrenoceptor relaxation on the contractile response to muscarinic receptor stimulation*

In separate tissues, the control contractile potency estimate for oxotremorine M was 7.7 ± 0.1, with maximal contraction of 3.1 ± 0.1 g ($n = 6$). In tissues alkylated with 4-DAMP mustard (in the presence of $M_2$ receptor protection), precontracted with histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM), oxotremorine M gave a contractile potency of 7.8 ± 0.1 ($n = 6$) with a maximum response of 2.7 ± 0.1 g ($n = 6$). Methoctramine (1 μM) surmountably antagonized this concentration-response curve more potently, resulting in a potency estimate for oxotremorine M of 6.4 ± 0.1, yielding a $-\log K_B$ value of 7.4 ± 0.1 (Figure 5a; Table 3).

In separate tissues, the above experimental protocol was repeated except that, after alkylation, tissues were precontracted to histamine (1 μM) and relaxed with BRL 37344 (1 μM). Under these conditions, the potency of oxotremorine M was 8.0 ± 0.1 with a maximal response of 2.4 ± 0.3 g ($n = 5$). Methoctramine (1 μM) also surmountably antagonized this response, reducing the potency of oxotremorine M to 6.2 ± 0.1, yielding a $-\log K_B$ value of 7.8 ± 0.2 (Figure 5b; Table 3). These affinity values for methoctramine are significantly greater than the value obtained on the contractile response to oxotremorine M alone (Table 3).

## Discussion

This study has investigated the effect of β-adrenoceptor stimulation on cyclic AMP accumulation in ileal longitudinal smooth muscle of guinea-pig and the inhibitory effect, thereupon, of muscarinic receptor agonism. Furthermore, the functional sequelae of this interaction were characterized by investigation of the effect of selective muscarinic $M_2$ receptor stimulation on β-adrenoceptor-mediated relaxation.

Isoprenaline and BRL 37344 both stimulated cyclic AMP accumulation in ileal smooth muscle slices, presumably through activation of $G_s$ and stimulation of adenylyl cyclase (Emorine *et al.*, 1991). The potency of isoprenaline ($-\log EC_{50} = 6.6$) at receptors mediating isoprenaline-stimulated cyclic AMP accumulation is lower than the potency of isoprenaline causing relaxation of guinea-pig ileum ($-\log EC_{50} = 7.3$; data not shown). This suggests that there is a receptor reserve for isoprenaline-mediated relaxation; however, it is likely that responses to isoprenaline were mediated through both $\beta_1$- and $\beta_3$-adrenoceptors (Arch & Kauman, 1993). Further evidence to support the latter was seen in the biphasic nature of concentration-response curve to isoprenaline in the presence of propranolol (1 μM). The affinity of propranolol at the propranolol-sensitive component of the isoprenaline response (8.3) is similar to the affinity of propranolol at either $\beta_1$- or $\beta_2$-adrenoceptors (~8.7; Farmer & Levy, 1970). Although BRL 37344 was less potent (5.8) than isoprenaline on cyclic AMP accumulation, BRL 37344 is equipotent compared to isoprenaline in relaxing guinea-pig ileum and, furthermore maximally relaxes this tissue (data not shown) despite a sub-maximal stimulation of cyclic AMP accumulation (26% of that observed with isoprenaline). Similar observations regarding BRL 37344 effects on relaxation and cyclic AMP accumulation have been made in rat oesophageal smooth muscle (Ford *et al.*, 1992). In the present study the cyclic AMP response to BRL 37344 was resistant to propranolol (5 μM); this has also been observed with respect to the relaxant effect of BRL 37344 in guinea-pig ileum (Bond & Clarke, 1988). Taken together, these results support the presence of both $\beta_1$- and $\beta_3$-adrenoceptors in guinea-pig ileum as suggested by Bond & Clarke (1988), and demonstrate a positive coupling of each of these subtypes to adenylyl cyclase.

(+)-*cis*-Dioxolane inhibited basal, isoprenaline and BRL 37344-stimulated cyclic AMP accumulation. This inhibitory effect was partial and was also observed in the absence of forskolin, where the isoprenaline response was inhibited by 33% (data not shown). In rat ileum, an inhibitory effect of a similar magnitude has been reported (Griffin & Ehlert, 1992). Muscarinic receptor agonism inhibited both $\beta_1$- and $\beta_3$-stimulated cyclic AMP levels but was not specific as an inhibitory effect was also seen against activation of other receptors mediating increases in cyclic AMP. Thus, basal and cyclic AMP levels stimulated by 5-HT, vasoactive intestinal peptide and prostanoid receptors were also inhibited. This suggests that $M_2$ receptor-mediated inhibition of adenylyl cyclase, via $G_i$, modulates the responses to other agents which increase cyclic AMP levels. However, in contrast,

Griffin & Ehlert (1992) have shown that rat ileal $M_2$ receptors interact specifically with β-adrenoceptors. Further experiments are required to define the role of muscarinic receptors in this respect.

The potency of (+)-*cis*-dioxolane at receptors inhibiting cyclic AMP accumulation (7.3, this study) was greater than the potency previously reported at receptors stimulating phosphoinositide hydrolysis in ileum (5.6; Ford *et al.*, 1991). In order to identify the muscarinic subtype responsible for the inhibition of adenylyl cyclase a range of antagonists were investigated. It is unlikely that $M_4$ receptors are involved, given the lack of expression of mRNA in guinea-pig ileum (Ford *et al.*, 1991) and the low affinity for pirenzepine (this study; Caulfield, 1993). In general $M_3$ and $M_1$ muscarinic receptors do not couple preferentially to adenylyl cyclase and therefore $M_2$ receptors are the most likely subtype responsible for inhibition of adenylyl cyclase in this tissue. The apparent affinity of methoctramine (7.1) obtained in this study is somewhat lower than reported values for methoctramine at other $M_2$ receptors (7.8; Melchiorre *et al.*, 1993; Eglen *et al.*, 1988). However, the affinity was estimated from only a single concentration and may not be conclusive. Nevertheless, the rank order of antagonist affinities at receptors inhibiting cyclic AMP accumulation was atropine > methoctramine > *p*-F-HHSiD ≥ pirenzepine. From the literature this rank order of antagonist affinities is entirely consistent with the involvement of $M_2$ receptors (Caulfield, 1993). This conclusion is in agreement with Griffin & Ehlert (1992) who have suggested that $M_2$ receptors inhibit adenylyl cyclase in rat ileum.

The apparent affinity of methoctramine at receptors mediating contraction to oxotremorine M in guinea-pig ileum was 6.2, a value that is consistent with contraction mediated through $M_3$ receptors and which is in agreement with previous studies (Ford *et al.*, 1991). $M_3$ receptor inactivation by 4-DAMP mustard, in conjunction with AFDX-116 to protect $M_2$ receptors, has been used to study the functional role of $M_2$ receptors in ileum (Thomas *et al.*, 1993). In the present study the selectivity of 4-DAMP mustard was enhanced by the more selective $M_2$ antagonist, methoctramine (Melchiorre *et al.*, 1993), which has an $M_2/M_3$ fold selectivity of approximately 100, compared with the selectivity ratio for AFDX 116 (Micheletti *et al.*, 1987) which is approximately 10 fold. Selective alkylation of $M_3$ receptors by 4-DAMP mustard dextrally shifted concentration-response curves to oxotremorine M (~16 fold), with no decrease in maxima. Therefore, under the alkylation conditions used, there was incomplete alkylation of the $M_3$ receptor population. However, use of higher concentrations of 4-DAMP mustard, in an attempt to increase the proportion of $M_3$ receptors alkylated, would progressively alkylate more $M_2$ receptors (Thomas *et al.*, 1992). Nonetheless, under the conditions used in the present study, the population of $M_3$ receptors was reduced sufficiently to reveal a role for $M_2$ receptors, as described below. After partial $M_3$ receptor alkylation, contractile responses were antagonized by methoctramine with an affinity of 6.4, suggesting that, after alkylation, the contractile response was still mediated by $M_3$, and not $M_2$ receptors. Clearly, even after selective, albeit incomplete, $M_3$ receptor alkylation and *in the absence of β-adrenoceptor stimulation*, a contractile role for $M_2$ receptors was absent.

Consequently, studies were also performed using $M_3$ receptor inactivation followed by relaxation of histamine-induced tone with β-adrenoceptor agonists. Preliminary studies showed that, in the absence of $M_3$ receptor alkylation, there was no significant effect of pretreatment with histamine and isoprenaline or BRL 37344 on the subsequent responses to oxotremorine M. This showed that functional antagonism of the contractile response to oxotremorine M did not occur under any of the conditions investigated. Furthermore, the affinity of methoctramine under these conditions was not significantly different from tissues treated with oxotremorine M alone. However, in the presence of β-adrenoceptor stimulation with isoprenaline, following partial $M_3$ receptor inactivation, a role for $M_2$ receptors was disclosed, since the observed affinity ($-\log K_B$) for methoctramine increased from 6.2 to 7.4, in agreement with the findings of Thomas *et al.* (1993). Similar effects were seen when $\beta_3$-adrenoceptors were selectively stimulated by BRL 37344 to relax the histamine-induced tone. The demonstration of a response antagonized with high affinity by methoctramine ($-\log K_B$ values of 7.4 and 7.8), under conditions of partial $M_3$ alkylation along with β-adrenoceptor stimulation, reflects a role for the $M_2$, but not the residual $M_3$ population, in modulation of the response to β-adrenoceptor agonists.

The potency of oxotremorine M at $M_2$ receptors mediating this 're-contraction' under these conditions was significantly more potent ($-\log EC_{50} = 7.8$) than the potency of oxotremorine M under the alkylation conditions alone ($-\log EC_{50} = 6.5$). Indeed, the potency was similar to that mediating control oxotremorine M contractions ($-\log EC_{50} = 7.7$). Consequently, it is possible that inhibition of β-adrenoceptor relaxation, by $M_2$ receptors, may occur at similar levels of acetylcholine release from parasympathetic nerves which mediate contraction, assuming, that $M_2$ and $M_3$ receptors are equally accessed by neuronally released acetylcholine. Hypothetically then, the role of $M_2$ and $M_3$ receptors may vary, depending upon the prevailing level of sympathetic tone, with $M_2$ receptor agonism inhibiting the action of catecholamines, and thereby aiding contraction to $M_3$ receptor stimulation. The ability of $M_2$ receptor activation to inhibit cyclic AMP levels stimulated by a variety of neurotransmitters further suggests that $M_2$ receptors may provide an important modulatory control over ileal motility under many different circumstances.

In conclusion, evidence has been found that $M_2$ receptors inhibit β-adrenoceptor stimulation of cyclic AMP accumulation and that, after inactivation of $M_3$ muscarinic receptors, $M_2$ receptors inhibit $\beta_1$- and $\beta_3$-adrenoceptor relaxation of guinea-pig isolated ileum. Thus, both $M_2$ and $M_3$ muscarinic receptors play a role in controlling motility in this tissue.

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# Production of antinociception by peripheral administration of [Lys$^7$]dermorphin, a naturally occurring peptide with high affinity for $\mu$-opioid receptors

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**1** The opioid activity of the amphibian peptide, [Lys$^7$]dermorphin, was studied in rats and mice. When administered intracerebroventricularly (i.c.v.), intravenously (i.v.) or subcutaneously (s.c.) it produced a long lasting analgesia. Its antinociceptive potency exceeded that of morphine 290 times by i.c.v. injection, and 25-30 times by peripheral administration.

**2** The dose-response curves of [Lys$^7$]dermorphin antinociception were shifted to the right by the pretreatment with naloxone (0.1 mg kg$^{-1}$, s.c.) or with the $\mu_1$-selective antagonist, naloxonazine (10 mg kg$^{-1}$, i.v. 24 h before peptide injection).

**3** The peptide also displayed potent antinociceptive effects in a chronic inflammatory pain model (rat Freund's adjuvant arthritis). In this pain model, systemic administration of the peptide raised the nociceptive threshold more in inflamed than in healthy paw.

**4** High central and peripheral doses of [Lys$^7$]dermorphin in rats produced catalepsy. The cataleptic response was antagonized by naloxone but left unchanged by naloxonazine pretreatment.

**5** In rats and mice, central or peripheral administration of [Lys$^7$]dermorphin induced a significantly slower development of tolerance to the antinociceptive effect than did morphine.

**6** Upon naloxone precipitation of the withdrawal syndrome, [Lys$^7$]dermorphin-dependent mice made fewer jumps and lost less weight than the morphine-dependent animals. Withdrawal hyperalgesia did not develop in [Lys$^7$]dermorphin-dependent mice.

**7** In conclusion, [Lys$^7$]dermorphin seems to be a unique opioid peptide having a high penetration into the blood-brain barrier despite its low lipid solubility. This peptide causes fewer side-effects than other opioids and appears less likely than morphine to cause physical dependence in rats and mice.

**Keywords:** [Lys$^7$]dermorphin; peripheral administration; opioid activity; tolerance; physical dependence

## Introduction

Dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-$NH_2$) was the first opioid peptide to be discovered in the skin of South American frogs belonging to the subfamily Phyllomedusinae (Montecucchi *et al.*, 1981). Dermorphin binds with high affinity and selectivity to $\mu$-opioid receptors (de Castiglione & Rossi, 1985) and produces antinociceptive effects in laboratory animals and man (Broccardo *et al.*, 1981; Basso *et al.*, 1985; Sandrini *et al.*, 1986). More recently, three other dermorphin-like peptides have been identified (Richter *et al.*, 1990) and isolated from the skin of *Phyllomedusa bicolor* and their amino acid sequences determined (Mignogna *et al.*, 1992; Negri, 1993). In a previous paper (Negri *et al.*, 1992) we reported the receptor-binding profile of these peptides and briefly described their biological activities. Among the amphibian opioids isolated, [Lys$^7$]dermorphin shows an affinity and selectivity for $\mu$-opioid receptors one order of magnitude higher than that of DAMGO and dermorphin and two orders of magnitude higher than that of morphine. The analysis of the displacement of [$^3$H]-DAMGO binding to rat brain membranes by graded concentrations of [Lys$^7$]dermorphin revealed two $\mu$-binding sites, one of high and the other of low affinity (Negri *et al.*, 1992). In this paper, we describe the antinociception, tolerance and physical dependence produced by [Lys$^7$]dermorphin and compare these effects with those of dermorphin and morphine in rats and mice.

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## Methods

### *Animals*

Male CD-1 mice (25-30 g) and male Wistar rats (250-300 g), were used for the experiments. They were housed at 22 ± 2°C, with food and water *ad libitum*. A standard light/dark cycle was maintained with a time-regulated light period from 06 h 00 min to 18 h 00 min. The IASP guidelines on ethical standards for investigations of experimental pain in animals were followed.

### *Synthesis of [Lys$^7$]dermorphin and dermorphin*

Peptides were synthesized by fluorenylmethoxy-carbonyl-polyamide active-ester chemistry on a Biolynx automated peptide synthesizer (Pharmacia LKB Biochrom, Cambridge, U.K.). The product was purified by preparative high performance liquid chromatography (h.p.l.c.) on a Vydac reverse phase column (22 mm × 250 mm; 10 µm particle size; 300 Å pore diameter; C18 packing; The Separations Group, Hesperia, CA, USA) with a linear gradient from 10% to 90% of solvent B (5 mM trifluoroacetic acid in acetonitrile) in solvent A (5 mM trifluoroacetic acid in water) over 60 min. After 6 M HCl hydrolysis (24 h, 110°C, *in vacuo*), amino acid analyses gave ratios consistent with the desired structure. The expected amino acid sequence was confirmed by automated Edman degradation performed with an Applied Biosystems 470A protein sequencer (Negri *et al.*, 1992).

### *Intracerebroventricular (i.c.v.) injections*

For i.c.v. injection, compounds were dissolved in sterile Ringer solution. Under ethyl ether anaesthesia, rats were

placed in a Kopf stereotaxic instrument according to Paxinos & Watson (1982) and a 2 mm diameter hole was drilled into the skull over the left brain ventricle, 1 mm caudal to and 1.8 mm lateral to the bregma. When the two sides of the coronal suture met the sagittal suture at different points, the bregma was assumed to lie midway between the two junctions. A plastic guide cannula 7 mm in length (Linca, Tel-Aviv, Israel) was inserted into the skull hole perpendicular to the horizontal plane passing through bregma and lambda, to a depth of 0.6 mm below the surface of the skull. The tip of the cannula never entered the brain, but grazed the meningeal layer. The cannula was secured to the skull with dental cement. After surgery, the rats were allowed to recover for 7 days in individual plastic cages. A 10 µl Hamilton syringe fitted with a 26 gauge needle was used for i.c.v. injections. The needle was inserted through the guide cannula to a depth of 3.6 mm below the external surface of the skull in conscious rats. Drugs and control solutions were injected slowly (60 s) in a constant volume of 5 µl. After drug testing, the proper position of the cannula was ascertained by injecting 5 µl of methylene blue solution through the cannula and verifying the colour filling of the brain ventricles. Rats were killed by inspiration of $CO_2$ at a concentration of 75% in air.

### *Tests of antinociception*

Nociceptive responses were evoked in rats by exposing the tail to radiant heat (D'Amour & Smith, 1941) or by applying a mechanical stimulus to the dorsal surface of the hindpaw (Randall & Selitto, 1957). In mice the nociceptive threshold was measured by immersion of the tail in hot (52°C) water (Janssen *et al.*, 1963). The latency to tail withdrawal was taken as a measure of the nociceptive response to heat exposure. Before peptide administration, each animal was tested and the latency to tail-flick recorded (control latency; CL). Animals not flicking their tails within 5 s were not used. The test was repeated at 15 min intervals during the first hour after drug administration and every 30 min thereafter for a total period of 4 h. The latency to tail-flick of each drug-injected animal was defined as the test latency (TL). To avoid tissue damage, animals with a test latency of more than 12 s (cut-off time), were removed from the nociceptive stimulus and assigned a TL value of 12. For drawing the dose- and time-response curves, the antinociceptive response was expressed as MPE, calculated by the following equation:

$$\text{MPE} = 100 \times (\text{TL-CL})/(12\text{-CL})$$

Each dose was tested in five to eight animals. $A_{50}$ was defined as the opioid dose that produced a peak effect equal to 50 MPE. When drugs were administered the i.c.v. $A_{50}$ value was expressed as nmol per rat, otherwise it was expressed as nmol kg$^{-1}$ of body weight. A computer programme (InPlot, GraphPad, CA, U.S.A.) was used to calculate the area under the time-response curve (AUC) for each animal and for all opioid doses. The time-response relationship was analysed further by plotting the mean peak effect after a given dose against the mean ratio peak/AUC for that dose. A regression line was then fitted to data by a linear regression computer programme (InPlot, GraphPad, CA, U.S.A.). In the resulting plot the Y intercept is a function of the duration of the response. A curve slope significantly different from zero indicates a non-linear relationship between the duration of the response and the peak effect.

When nociceptive responses were induced with a mechanical painful stimulus, the rat were gently restrained under paper wadding and incremental pressure was applied to a 1.75 mm$^2$ area of the dorsal surface of the hindpaw via a wedge-shaped blunt piston connected to an automated gauge, designed to generate a linearly increasing mechanical force (Ugo Basile, Comerio, VA, Italy). The pressure required to elicit paw withdrawal, the paw pressure threshold, was determined and expressed in g. In the absence of a nociceptive response, cutoff pressure was 250 g. Pressure was applied to normal (left) and to inflamed (right) hindpaws of rats. Inflammation was produced by injecting 0.15 ml of modified Freund's complete adjuvant (Stein *et al.*, 1988) into the plantar region of the right hindfoot under brief ether anaesthesia. In all rats studied, the inflammation remained confined to the inoculated paw and all testing was conducted between 4 and 6 days after inoculation. Three consecutive trials, separated by 10 s intervals, were conducted and the average pressure was determined. The same procedure was applied to the untreated and inflamed hindfoot of each rat. To preclude order effects, the sequence of feet tested was alternated between subjects. Separate groups (8 rats per group) were used for each treatment. A further group of 8 rats with inflamed hindpaw received vehicle alone and acted as controls.

### *Test of catalepsy*

Catalepsy was evaluated by placing both front limbs of the rat over a 10 cm high horizontal bar and measuring the time the animal maintained this posture. Rats remaining more than 30 s on the bar were defined cataleptic (positive bar test). The cataleptic $ED_{50}$ ($CD_{50}$) of each opioid was defined as the dose that produced a positive bar test in 50% of the animals tested. Four scalar doses of each opioid were used and each dose was tested in eight rats.

### *Opioid antagonists*

To confirm the opioid origin of antinociception and catalepsy, separate rat groups (5 rats per group) were pretreated with opioid antagonists. Naloxone (0.1 or 1 mg kg$^{-1}$) was injected s.c. 15 min before i.c.v. opioid injection or 15 min after s.c. opioid administration. Naloxone antagonism was tested against one dose of each opioid in a 5 animals group. Naloxonazine (10 mg kg$^{-1}$) was injected i.v. in rats or i.p. in mice 24 h before opioid administration. The $A_{50}$ and $CD_{50}$ values of the opioids were determined in naloxonazine-pretreated animals using four scalar doses of each opioid and five animals for each dose.

### *Single-dose tolerance*

When tolerance was induced with a single opioid dose, morphine 133 µmol kg$^{-1}$, dermorphin 50 µmol kg$^{-1}$ or [Lys$^7$]-dermorphin 13 µmol kg$^{-1}$ were administered subcutaneously to separate groups of 15 mice. Five hours later, the $A_{50}$ value of the tolerance-producing opioid was determined with three graded s.c. doses of the opioid (5 mice per dose).

### *Single-dose physical dependence*

To induce physical dependence, the same doses of the three opioids used in the single-dose tolerance protocol were administered subcutaneously to three groups of mice (15 mice per group). Five hours later physical dependence was assessed by precipitating a withdrawal syndrome with s.c. naloxone.

### *Tolerance in chronically treated animals*

In rats, tolerance was induced by once daily (10 h 00 min) i.c.v. injection of the opioid. Two groups of rats (20 rats per group) were used. One group received i.c.v. 0.24 nmol of [Lys$^7$]dermorphin for seven days and the other the vehicle alone for the same period. On day 7, rats were challenged by subcutaneous injection of four graded doses of [Lys$^7$]dermorphin. Five rats were used for each dose level and the antinociceptive response was measured over a period of 4 h. From the dose-response curves the s.c. $A_{50}$ value of [Lys$^7$]dermorphin was calculated in saline- and in opioid-treated animals.

In mice, tolerance was induced by infusing [Lys$^7$]der-

morphin and morphine s.c. for eight days. Mini-osmotic pumps (Alzet Model 2001, Alza Co., CA. U.S.A.) were filled with the opioid solution and implanted s.c. into two mouse-groups (60 animals per group, one pump per animal). One mouse-group received [Lys7]dermorphin-filled pumps, the other group morphine-filled pumps.

Infusion rates were: [Lys7]dermorphin, 27 nmol $\mu l^{-1} h^{-1}$; morphine, 620 nmol $\mu l^{-1} h^{-1}$. Opioids were dissolved in dimethyl sulphoxide (DMSO) and the solution was diluted with distilled water to give a 25% DMSO concentration. A third group of 60 mice was implanted with mini-osmotic pumps prefilled with the vehicle alone (25% DMSO), which was infused at a rate of 1 $\mu l\, h^{-1}$. Pumps remained active throughout the eight day-experimental period. On the morning of each day after pump implantation, the tail-flick test latency and the body weight of the vehicle- and opioid-infused mice were measured. For assessment of tolerance on the morning of day 4 after pump implant, two groups of 20 mice randomly selected from the opioid-infused animals received four graded s.c. doses of their own opioid (5 mice per dose). From the dose-response relationship the $A_{50}$ values of both opioids were calculated. On day 7 after implant, this paradigm was repeated using two different animal groups (20 animals per group) randomly selected from the opioid infused mice. On day 7 after pump implant, 40 mice infused with the vehicle alone were injected with four graded doses of [Lys7]dermorphin or morphine (5 mice per dose) and the $A_{50}$ values of the two opioids was determined.

### *Cross-tolerance between [Lys7]dermorphin and morphine*

On day 7 after implant, the untested animals from the two opioid-infused groups received four graded s.c. doses of the other opioid. Five mice were used for each dose level. The $A_{50}$ value of morphine was calculated in [Lys7]dermorphin-tolerant mice, and the $A_{50}$ of [Lys7]dermorphin in morphine-tolerant mice.

### *Assessment of tolerance*

Tolerance to antinociceptive effects were expressed by a ratio defined as the $A_{50}$ value of the agonist in opioid-treated animals divided by the $A_{50}$ value of the same agonist in vehicle-treated animals.

### *Assessment of dependence*

In single-dose tolerance experiments, the degree of physical dependence on morphine on [Lys7]dermorphin was measured by estimating the amount or naloxone needed to induce withdrawal jumping (Way *et al.*, 1969) in tolerant mice. Five hours after receiving the opioid agonist, mice were injected with naloxone and immediately placed singly in 30 × 40 cm plexiglass cylinders. The number of vertical jumps occurring during the succeeding 15 min was recorded. Because a small percentage of animals displayed spontaneous jumping before naloxone injection, the criterion for a positive jumping response was that a mouse had to jump more than four times during the 15 min observation period. The $ED_{50}$ of naloxone for inducing withdrawal jumping was estimated by the method of Dixon (1965), using 5 animals for each determination.

The $ED_{50}$ of naloxone was determined three times in separate groups of tolerant mice and the mean values ± s.e.mean were reported. The degree of physical dependence in chronically opioid-infused mice was measured by precipitating the withdrawal syndrome with a full dose of naloxone. Except for animal groups used for cross-tolerance studies, in which dependence was not assayed, mice made tolerant with pump-infused opioids were injected with naloxone (1 mg $kg^{-1}$, s.c.) on day 8 after pump-implantation. On the same day, the remaining 20 vehicle-infused mice, which never received opioids, were also injected with naloxone and acted as opioid-nondependent controls. In all animal groups, jumps were counted for a period of 30 min after the naloxone injection. The pumps were then removed under light ether anaesthesia. Twelve hours after pump removal the body weight and the tail-flick latency of each mouse were measured. Animals were killed by inhalation of 75% $CO_2$ in air.

### *Drugs*

Morphine HCl and naloxone HCl were bought from SALARS (Como, Italy). Naloxonazine dihydrate (NXZ) was obtained from RBI (Natick, MA, U.S.A.). Freund's complete adjuvant was bought from Calbiochem (La Jolla, CA, U.S.A.).

### *Statistics*

A linear regression computer programme (InPlot, GraphPad, CA, U.S.A.) was used for analysing the dose response curves and calculating the curve slopes with 95% confidence limits and the *P* values for slope difference from zero (two-tailed test). The $A_{50}$ values with their associated 95% confidence intervals and the relative potencies of the opioids were calculated with a pharmacological software programme written from published procedures (Tallarida & Murray, 1986). Values within square brackets are 95% confidence limits [lower, higher]. For statistical analysis the CSS: STATISTICA software package (StatSoft, Tulsa, OK, U.S.A.) was used. Bartlett's test was used for preliminary analysis of the homogeneity of variance. The data were then compared with a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

## Results

### *Single i.c.v. injection*

In the tail-flick test in rats, i.c.v. administration of [Lys7]dermorphin, dermorphin and morphine elicited a dose-dependent antinociception. Doses that produced peak effects between 20 and 80 MPE were plotted into a log dose-response curve and the curve was analysed with least-squares linear regression yielding a positive correlation between dose and effect (Figure 1a). The $A_{50}$ value of [Lys7]dermorphin did not significantly differ from that of dermorphin, but was 290 [256–386] times lower than that of morphine (Table 1). At a dose of 0.059 nmol, [Lys7]dermorphin produced an antinociceptive response that peaked (MPE = 75) at 60 min and lasted about 180 min. A dose of 18.6 nmol of morphine or 0.062 nmol of dermorphin elicited an antinociceptive response that had the same intensity (MPE = 75) but peaked earlier (from 30 to 45 min) and lasted from 90 to 120 min (Figure 2a). The Y intercepts for the regression lines of the three opioids in a peak vs peak/AUC plot (Figure 3a) did not differ significantly (dermorphin, 2.64 ± 0.15; [Lys7]dermorphin, 2.26 ± 0.15; morphine 2.07 ± 0.09) indicating that the antinociceptive responses to morphine and peptides equal to or lower than 20 MPE had a similar duration of action. But because the peptide line slopes were significantly lower than zero and lower than the morphine line slope, the higher antinociceptive responses to the two peptides had a longer duration of action than those to morphine. Naloxone (0.1 mg $kg^{-1}$ s.c.) completely reversed the antinociception produced by 0.12 nmol of [Lys7]dermorphin (data not shown). Pretreatment with naloxonazine (10 mg $kg^{-1}$, i.v.) produced a significant rightward displacement in the dose-response curve of [Lys7]dermorphin and increased the $A_{50}$ value of the peptide by 10 times.

In the rat, single i.c.v. doses of the opioids produced catalepsy. The $CD_{50}$ value of [Lys7]dermorphin was about 15

times higher than the [Lys⁷]dermorphin $A_{50}$ value. In comparison the $CD_{50}$ of morphine was only three times higher than the morphine $A_{50}$, and the dermorphin $A_{50}$ was four times greater than the dermorphin $CD_{50}$ (Table 1). Naloxone (1 mg kg$^{-1}$, s.c.) completely antagonized the cataleptic response to the [Lys⁷]dermorphin and morphine $CD_{50}$. Naloxonazine pretreatment (10 mg kg$^{-1}$, i.v.) increased slightly but not significantly the [Lys⁷]dermorphin $CD_{50}$ value (0.62 [0.39, 0.81] nmol), whereas it significantly attenuated morphine catalepsy ($CD_{50}$ = 45.2 [19.8, 61.5], $P < 0.01$ vs control $CD_{50}$).

### *Single intravenous or subcutaneous injection*

[Lys⁷]dermorphin produced dose-related antinociceptive effects in the tail-flick test after i.v. administration in rats. When i.v. doses of the peptide were plotted on a log dose-response curve and the curve was analysed with a least-square linear regression computer programme, a positive correlation (slope = 67.45 [31.25, 121.24]; $r = 0.943$; $P = 0.0032$, two-tailed) was found between dose and effect. The i.v. $A_{50}$ value of [Lys⁷]dermorphin was 0.06 [0.03, 0.11] μmol kg$^{-1}$.

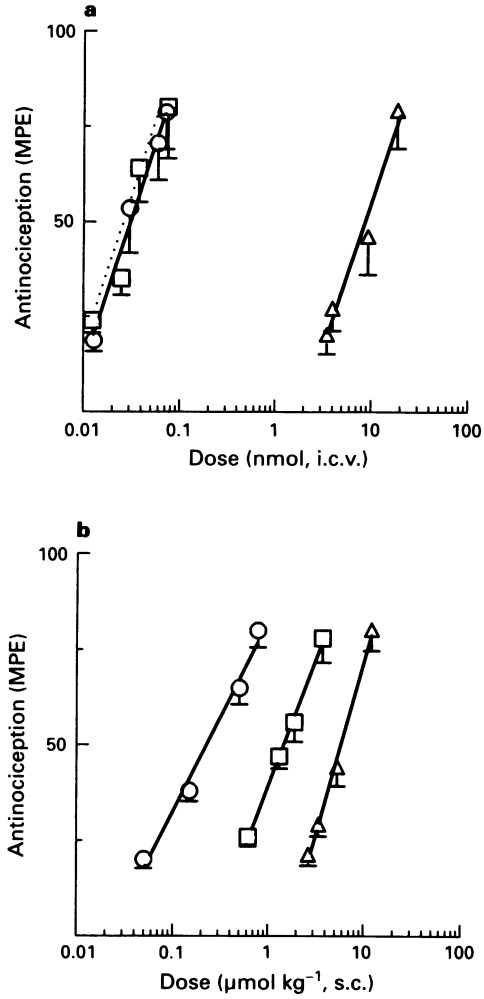


**Figure 1** Dose-response relationship of the antinociception produced by [Lys⁷]dermorphin (○), dermorphin (□) and morphine (△) in rats: (a) i.c.v. administration; (b) s.c. administration. Curves were fitted to data points and analysed by a linear regression computer programme. Each point represents the mean antinociceptive peak effect of 5–8 rats ± s.e.mean. (a) [Lys⁷]dermorphin, slope = 78.98 [51.55, 106.40], $r = 0.994$, $P = 0.0064$; dermorphin, slope = 76.48 [38.38, 121.69], $r = 0.966$, $P = 0.0332$; morphine, slope = 76.69 [36.36, 117.02] $r = 0.971$, $P = 0.0146$. (b) [Lys⁷]dermorphin, slope = 47.55 [6.65, 88.461], $r = 0.962$, $P = 0.038$; dermorphin, slope = 66.24 [55.51, 76,98], $r = 0.986$, $P = 0.0014$; and morphine, slope = 90.54 [69.91, 111.17], $r = 0.987$, $P = 0.0028$.

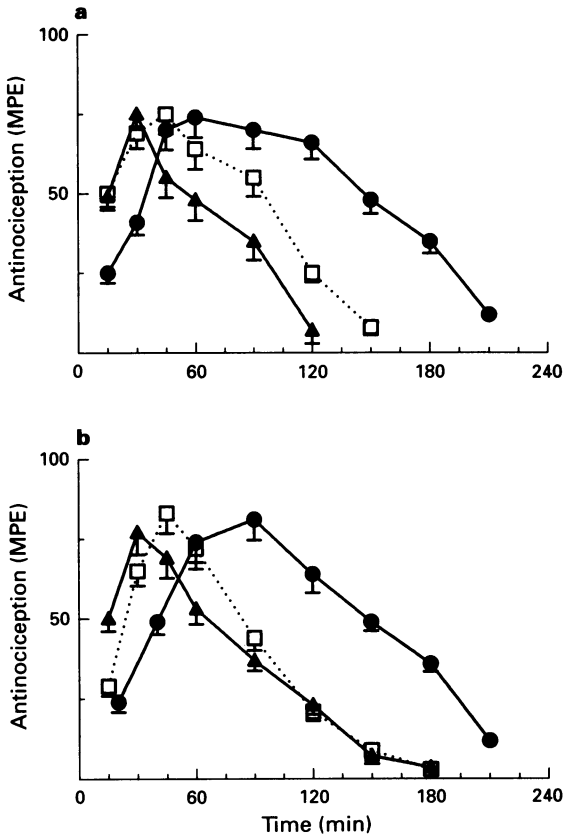


**Figure 2** Time-course of the antinociception produced by [Lys⁷]dermorphin (●), dermorphin (□) and morphine (▲), in the rat. I.c.v. doses (a): [Lys⁷]dermorphin, 0.059 nmol; dermorphin, 0.062 nmol and morphine 18.6 nmol. S.c. doses (b): [Lys⁷]dermorphin, 0.5 μmol kg$^{-1}$; dermorphin, 3.7 μmol kg$^{-1}$ and morphine, 13.3 μmol kg$^{-1}$. Each point represents the mean antinociceptive effect of 5 rats ± s.e.mean. Two-way ANOVA with repeated measures showed significant interactions between time and dose ($P < 0.01$) for the following time intervals: (a) [Lys⁷]dermorphin, from 15 to 180 min; dermorphin, from 15 to 120 min and morphine, from 15 to 90 min; (b) [Lys⁷]dermorphin, from 20 to 180 min; dermorphin, from 15 to 120 min and morphine, from 15 to 20 min.

**Table 1** Antinociception and catalepsy produced by i.c.v. and s.c. injection of morphine, dermorphin and [Lys7]dermorphin in rats

| | *Antinociception* $A_{50}$ *[95% confidence limits]* | | *Catalepsy* $CD_{50}$ *[95% confidence limits]* | |
|---|---|---|---|---|
| *Treatment* | *i.c.v. (nmol/rat)* | *s.c. (μmol kg$^{-1}$)* | *i.c.v. (nmol/rat)* | *s.c. (μmol kg$^{-1}$)* |
| Morphine | 8.66 [5.96, 12.56] | 5.8 [5.0, 6.6] | 26.3 [10.5, 38.4] | 13.4 [7.7, 24.1] |
| Dermorphin | 0.030 [0.018, 0.051] | 1.46 [1.31, 1.62] | 0.13 [0.06, 0.21] | ND |
| [Lys⁷]dermorphin | 0.030 [0.024, 0.038] | 0.22 [0.15, 0.33] | 0.44 [0.28, 0.68] | 8.3 [5.3, 17.9] |

ND: not determined.

the i.v. $A_{50}$ value of morphine was 1.6 [0.7, 2.2] and that of dermorphin 0.46 [0.15, 0.84] μmol kg$^{-1}$.

In rats (Figure 1b) single s.c. doses of [Lys$^7$]dermorphin resulted in an antinociceptive response to radiant heat (tail-flick test) that correlated with the log dose. The s.c. $A_{50}$ value of [Lys$^7$]dermorphin was 6.4 [5.3, 7.8] times lower than that of dermorphin and 26.9 [22.0, 33.16] times lower than that of morphine (Table 1). Naloxonazine pretreatment shifted the dose-response curve to the right by 8.9 [3.9, 19.5] times and increased the s.c. $A_{50}$ value of [Lys$^7$]dermorphin to 1.96 [0.59, 6.43]. Even when given in doses that produced a similar peak effect (about 75 MPE), the three opioids elicited antinociceptive responses that had dissimilar time courses. The [Lys$^7$]dermorphin antinociception lasted 180 min, whereas dermorphin and morphine antinociception lasted 120 min (Figure 2b). When the mean peak after a given dose was plotted against the mean ratio peak/AUC for that dose, the following rank ordering of Y intercepts was produced: [Lys$^7$]dermorphin (0.56 [0.31, 0.82]) <dermorphin (1.55 [1.19, 1.90]) = morphine (1.64 [1.08, 2.20]). Thus, also by this route of administration, [Lys$^7$]dermorphin showed the longest duration of action. Line slopes were not significantly different from zero (Figure 3b).

In the paw-withdrawal test, changes in the nociceptive thresholds of untreated and chronically inflamed rat hindpaw were measured after s.c. administration of 3.6 or 1.8 μmol kg$^{-1}$ of [Lys$^7$]dermorphin to two groups of 8 rats. Saline-injected rats (8 animals) showed a lower pain threshold in the right inflamed than in left normal hindpaw (Figure 4a). As expected, the peptide produced an intense antinociceptive response to the noxious pressure stimulus in healthy as well as in chronically inflamed paw. The peptide raised the nociceptive threshold more in the inflamed than in the untreated paw, however, so that it reversed the basal threshold difference between the two paws (Figure 4b).

High subcutaneous doses of [Lys$^7$]dermorphin in rats produced catalepsy (Table 1). Naloxone (1 mg kg$^{-1}$) completely antagonized catalepsy produced by 10 μmol kg$^{-1}$ of the peptide (data not shown). Naloxonazine pretreatment did not significantly modify the [Lys$^7$]dermorphin $CD_{50}$ value (12.3 [7.5, 20.8] μmol kg$^{-1}$), but it increased four times the morphine $CD_{50}$ value (45.2 [31.6, 91.2]).

In CD1 mice, [Lys$^7$]dermorphin given by s.c. injection, increased the latency to hot water tail-flick in a dose-dependent manner (Figure 5). Its relative potency versus dermorphin and morphine equalled that calculated in rats, though CD1 mice appeared less sensitive to morphine antinociception than Wistar rats (Table 2). In mice [Lys$^7$]dermorphin, dermorphin and morphine had a similar duration of action. In a peak vs peak/AUC plot, the Y intercepts of the three opioid curves were similar ([Lys$^7$]dermorphin 0.75 [0.20, 1.31]); dermorphin 1.18 [0.40,

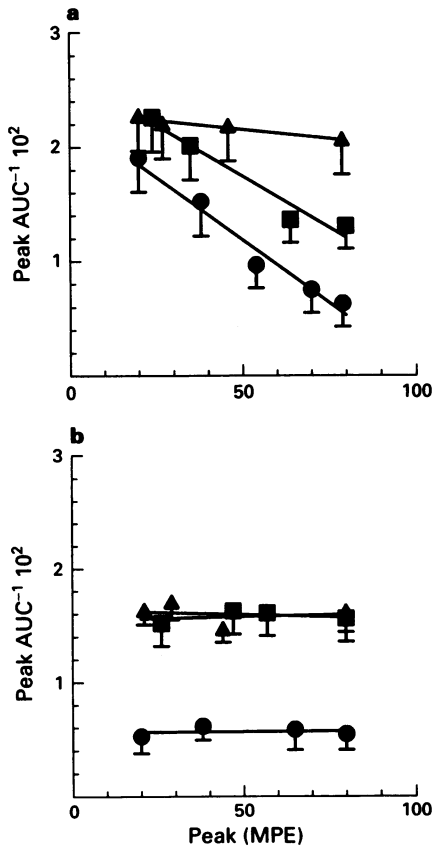


**Figure 3** The relationship between the antinociceptive peak effect and the ratio between the peak effect and the area under the time-response curve: (a) I.c.v. administration; (b) s.c. administration. Each point represents the mean (peak AUC$^{-1}$ 10$^2$) of 5 rats ± s.e.mean, plotted against the mean peak effect calculated in the same rat group. Linear regression analysis revealed that the slopes of [Lys$^7$]dermorphin (●) (− 0.0224 [− 0.0292, − 0.0156]) and dermorphin (■) (− 0.0179 [− 0.0297, − 0.0062]) were significantly different ($P<0.01$, two-tailed) from zero and from the morphine (▲) slope (− 0.0002 [− 0.0005, 0.0120]), only in i.c.v. injected rats (a); (b) yielded the following Y intercepts: [Lys$^7$]dermorphin (0.56 [0.31, 0.82], $P<0.01$ vs morphine); dermorphin (1.55 [1.19, 1.90]) and morphine (1.64 [1.08, 2.20]).

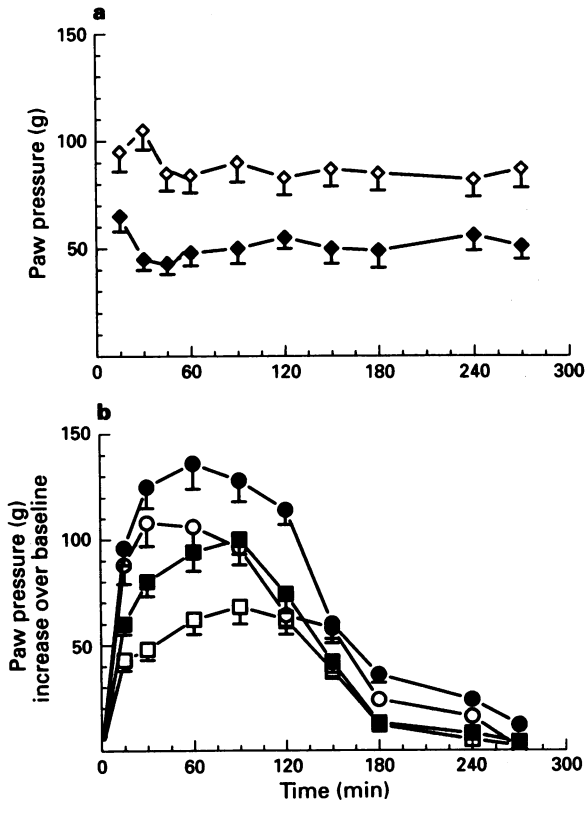


**Figure 4** (a) Time course of the pressure thresholds of the left healthy (◇) and the right inflamed (◆) paw of saline-injected rats. (b) Increase in the pressure threshold of the healthy (open symbols) and inflamed (solid symbols) paw in rats injected s.c. with 1.8 (■) and 3.6 (●) μmol kg$^{-1}$ of [Lys$^7$]dermorphin. Each point represents the mean paw pressure threshold of 8 rats ± s.e.mean. A two-way ANOVA with repeated measures showed a significant interaction between time and dose ($P<0.001$) for both peptide doses, and for time interval from 30 to 150 min. Tukey's test revealed a significant difference ($P<0.05$) between the two mean pressure thresholds of the left and right paw of saline-injected rats at all time points. A reverse significant difference between the two paw thresholds ($P<0.05$) was found at 60 and 90 min after s.c. injection of 1.8 μmol kg$^{-1}$ and at 90 and 120 min after s.c. injection of 3.6 μmol kg$^{-1}$ of [Lys$^7$]dermorphin.

1.95] and morphine 0.69 ([0.14, 1.34]). The curve slopes did not differ from zero. In mice, naloxonazine pretreatment shifted the dose-response curve to the right by 3.5 times only and increased the $A_{50}$ value of [Lys⁷]dermorphin to 1.26 [0.69, 2.29].

In mice, tolerance to the antinociceptive response was tested 5 h after single s.c. injection of 13 μmol kg$^{-1}$ of [Lys⁷]dermorphin, 50 μmol kg$^{-1}$ of dermorphin or 133 μmol kg$^{-1}$ of morphine (Table 2). The peptide $A_{50}$ values did not differ significantly between saline and peptide pretreated animals; however, the morphine $A_{50}$ values in morphine pretreated mice and in naive mice had nonoverlapping confidence intervals. Single s.c. injections of 13 μmol kg$^{-1}$ of [Lys⁷]dermorphin, 50 μmol kg$^{-1}$ of dermorphin or 133 μmol kg$^{-1}$ of morphine also produced physical dependence. The naloxone $ED_{50}$ values that precipitated a withdrawal syndrome 5 h after opioid injection were as follows: 0.45 ± 0.18 μmol kg$^{-1}$ in morphine-injected mice; 3.5 ± 1.2 μmol kg$^{-1}$ in dermorphin-injected mice and 7.6 ± 2.1 in [Lys⁷]dermorphin-injected mice. The naloxone $ED_{50}$ in [Lys⁷]dermorphin-injected mice was significantly lower than that in morphine-injected mice ($P < 0.05$).

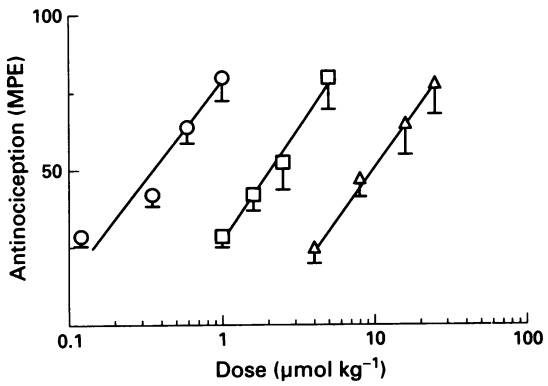


**Figure 5** Dose-response relationship of the antinociception produced by [Lys⁷]dermorphin (○), dermorphin (□) and morphine (△) after s.c. administration in mice. Each point represents the mean antinociceptive peak effect of 5 mice ± s.e.mean. Curves were fitted to data points and analysed by a linear regression computer programme. [Lys⁷]dermorphin: slope = 56.21 [10.45, 101.97], $r = 0.966$, $P = 0.034$; dermorphin: slope = 72.76 [46.98, 98.54], $r = 0.993$, $P = 0.0067$ and morphine: slope = 66.35 [57.02, 75.68], $r = 0.998$, $P = 0.0011$.

### *Repeated i.c.v. injections*

Preliminary experiments in rats demonstrated that the antinociceptive responses elicited by i.c.v. 0.24 nmol of [Lys⁷]dermorphin lasted 7 h. We therefore chose to induce

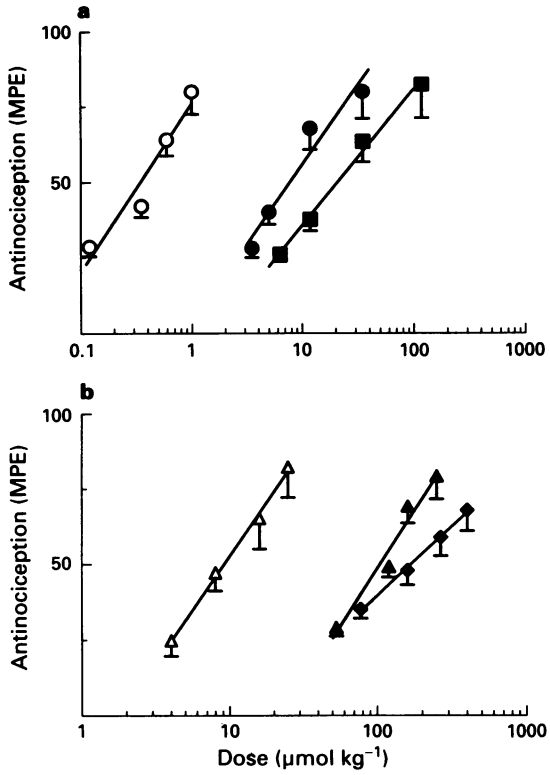


**Figure 6** Dose-response curves of antinociception produced by s.c. doses of [Lys⁷]dermorphin (a) and morphine (b) in vehicle- and opioid-infused mice. Infusion rate (s.c. implanted osmotic pumps): vehicle (25% DMSO), 1 μl h$^{-1}$ for 7 days (○, △); [Lys⁷]dermorphin, 27 nmol h$^{-1}$ for 4 days (●) and 7 days (■) and morphine, 620 nmol h$^{-1}$ for 4 days (▲) and 7 days (◆).

**Table 2** Antinociception produced by s.c. injections of [Lys⁷]dermorphin, dermorphin and morphine in naive and tolerant mice

| *Tolerance-inducing procedure* | | *Assessment of tolerance* | |
|---|---|---|---|
| *Treatment* (dose) | *Agent* (test time) | $A_{50}$ [95% confidence limits] (μmol kg$^{-1}$) | *Tolerance* ($A_{50}$ ratio) |
| *Single injection* | | | |
| Saline (4 ml kg$^{-1}$) | [Lys⁷]dermorphin | 0.36 [0.15, 0.83] | |
| [Lys⁷]dermorphin (13 μmol kg$^{-1}$) | [Lys⁷]dermorphin (5th h) | 0.85 [0.42, 2.1] | NS |
| Saline (4 ml kg$^{-1}$) | Dermorphin | 2.06 [1.67, 2.55] | |
| Dermorphin (50 μmol kg$^{-1}$) | Dermorphin (5th h) | 5.27 [1.93, 7.34] | NS |
| Saline (4 ml kg$^{-1}$) | Morphine | 9.33 [8.52, 10.02] | |
| Morphine (133 μmol kg$^{-1}$) | Morphine (5th h) | 37.21 [13.8, 48.7]* | 4.27 [3.17, 5.63] |
| *Chronic infusion (7 days)* | | | |
| Saline (1 μl h$^{-1}$) | [Lys⁷]dermorphin (7th day) | 0.31 [0.18, 0.95] | |
| [Lys⁷]dermorphin (27 nmol h$^{-1}$) | [Lys⁷]dermorphin (4th day) | 7.75 [3.90, 13.39]* | 21.9 [10.5, 34.9] |
| | [Lys⁷]dermorphin (7th day) | 21.5 [14.4, 31.9]* | 62.1 [39.3, 134.2] |
| | Morphine (7th day) | 372 [340, 408]* | 46.8 [41.3, 53.05] |
| Saline | Morphine (7th day) | 9.50 [7.45, 15.12] | |
| Morphine (620 nmol h$^{-1}$) | Morphine (4th day) | 103.7 [72.6, 148.2]* | 10.8 [8.5, 22.7] |
| | Morphine (7th day) | 169.3 [154.6, 185.4]* | 19.1 [17.6, 20.7] |
| | [Lys⁷]dermorphin (7th day) | 9.58 [5.66, 16.22]* | 27.9 [12.4, 64.3] |

*$P < 0.05$ v.s. saline; NS: not significant.

**Table 3** Chronic treatment of rats with daily i.c.v. injection of 0.24 nmol of [Lys⁷]dermorphin for seven days

| | Day of treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | *1st* | *2nd* | *3rd* | *4th* | *5th* | *6th* | *7th* |
| Antinociception (MPE) | 100 | 89 ± 3 | 83 ± 5 | 71 ± 9 | 75 ± 10 | 65 ± 8 | 69 ± 5 |
| Catalepsy (% animals) | 55 | 45 | 20 | 5 | 0 | 0 | 0 |

$n = 20$. Antinociception = mean peak effect ± s.e.mean.

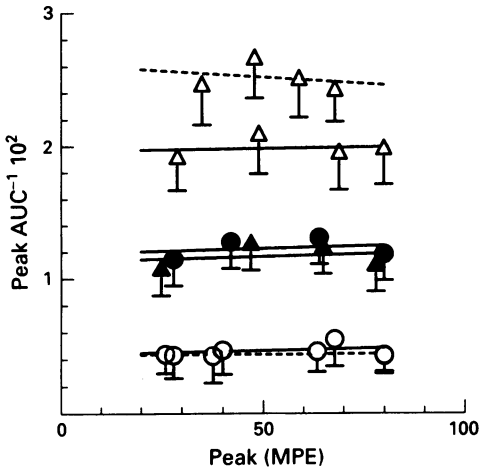


**Figure 7** The relationship between the antinociceptive peak effect and the ratio between the peak effect and the area under the time-response curve in naive and opioid tolerant mice. The opioids were infused s.c. in mice for 7 days. The time-course of the antinociceptive responses to four s.c. graded doses of the toleragen was recorded on day 4 and day 7. Each point represents the mean (peak $AUC^{-1}$ $10^2$) of 5 mice ± s.e.mean, plotted against the mean peak effect calculated in the same mouse group. Linear regression analysis yielded the following Y intercept values: naive mice, [Lys⁷]dermorphin (●) (1.19 [0.73, 2.12]) morphine (▲) (1.17 [0.83, 1.94]); 4-day tolerance mice, [Lys⁷]dermorphin (○, continuous line) (0.40 [0.29, 0.68], morphine (△, continuous line) 1.96 [1.35, 2.58]; 7-day tolerant mice, [Lys⁷]dermorphin (○, dashed line) 0.43 [0.33, 0.53], and morphine (△, dashed line) 2.62 [1.45, 3.78].

tolerance to [Lys⁷]dermorphin in rats with one daily i.c.v. injection of 0.24 nmol of the peptide. According to this schedule, seven-day administration of the peptide caused a progressive decrease in the antinociceptive and cataleptic responses (Table 3). The antinociceptive response reached a minimum after 4 days, thereafter it remained unchanged up to the 7th day. In contrast, catalepsy faded out within the first three days of treatment. After seven daily i.c.v. injections of [Lys⁷]dermorphin, rats were tested for antinociception with four graded s.c. doses of [Lys⁷]dermorphin and the $A_{50}$ value was calculated from the dose-response curves. The s.c. $A_{50}$ value of [Lys⁷]dermorphin was 2.95 [2.05, 3.17] $\mu$mol $kg^{-1}$ and was 13.9 (9.3, 21.4) times higher than the $A_{50}$ value calculated in saline-injected rats (0.22 [0.15, 0.33] $\mu$mol $kg^{-1}$).

### *Continuous subcutaneous infusion*

In mice, 4-day s.c. infusion of [Lys⁷]dermorphin (27 nmol $\mu l^{-1}$ $h^{-1}$) or morphine (620 nmol $\mu l^{-1}$ $h^{-1}$) produced a significant rightward shift of the dose-antinociception curves (Figure 6a,b). Only the slope of the morphine dose-response curve was significantly lower in 7-day tolerant mice (slope = 46.14 [38.07, 54.21], $r = 0.998$, $P = 0.0016$) than in 4-day tolerant (slope = 79.99 [56.42, 123.55] and in naive mice (slope = 66.35 [57.02, 75.68]). [Lys⁷]dermorphin tolerance, defined as the $A_{50}$ of opioid-infused mice divided by the $A_{50}$ of saline-infused mice, was 21.9 [10.5, 34.9]. Morphine tolerance, calculated as above, was 10.8 [8.5, 13.7]. Further 3-day infusion, at the same rate, raised the $A_{50}$ values of both the opioids, but [Lys⁷]dermorphin tolerance alone increased significantly ($P < 0.05$), reaching 62.1 [39.3, 134.2] (Table 2). In peak vs peak/AUC plots the regression line slopes of morphine and [Lys⁷]dermorphin were not different from zero, but the Y intercepts of the [Lys⁷]dermorphin lines were significantly lower in tolerant than in naive mice. By contrast, the Y intercepts of the morphine lines were higher in tolerant than in naive mice (Figure 7). In conclusion, in tolerant mice antinociception produced by the peptide lasted longer than it did in naive mice whereas morphine antinociception had a shorter duration.

Cross-tolerance was tested on day 7 in separate opioid-infused animal groups. In morphine-infused mice the $A_{50}$ value of [Lys⁷]dermorphin was 9.58 [5.66, 16.22] and tolerance to peptide was 27.9 [12.4, 64.3], whereas in [Lys⁷]dermorphin-infused animals the $A_{50}$ value of morphine was 372 [340, 408] and tolerance to the alkaloid was 46.8 [41.3, 53.05] (Table 2). The two levels of cross-tolerance did not differ significantly. In addition to tolerance, chronic infusion of morphine but not of [Lys⁷]dermorphin produced a shortening of the basal tail-flick latency from the 4th day of infusion onward (Figure 8b). It also produced a significantly higher daily body-weight loss than the infusion of [Lys⁷]dermorphin (Figure 8a).

Upon precipitation of the withdrawal syndrome by naloxone, morphine-dependent mice displayed more severe abstinence signs that [Lys⁷]dermorphin-dependent animals. The morphine-dependent mice made a greater number of jumps and lost more weight than the [Lys⁷]dermorphin-dependent animals (Figure 9a,b). In morphine but not in [Lys⁷]dermorphin-dependent mice a 39% decrease in the tail-flick latency was observed 12 h after naloxone injection (Figure 9c).

## Discussion

When injected i.c.v., i.v. and s.c., the $\mu$-selective opioid agonist [Lys⁷]dermorphin produced dose-dependent antinociception in rats and mice. After i.c.v. injection, the $A_{50}$ value of the peptide was 290 times lower than that of morphine. Whereas the ratio between the peak effect and the time-response area (AUC) decreased linearly with the increasing peak effect of the peptide, it remained constant over the entire range of morphine effects. This finding means that the increasing peak effect prolongs the duration of action more after peptide administration than after morphine. Although we did not attempt to obtain pharmacokinetic measurements of blood and brain concentrations of [Lys⁷]dermorphin, our tentative explanation for this dose-dependent higher increase in duration of action than in peak effect is that opioid peptides are transported in the direction of brain to blood by a saturable efflux system (PTS – 1) as claimed by Banks & Kastin (1993). Because PTS – 1 does not transport morphine, this alkaloid diffuses out of the brain at a low rate and in a concentration-gradient dependent manner. If [Lys⁷]dermorphin is transported out of the CNS by a saturable system, high brain concentrations of the peptide

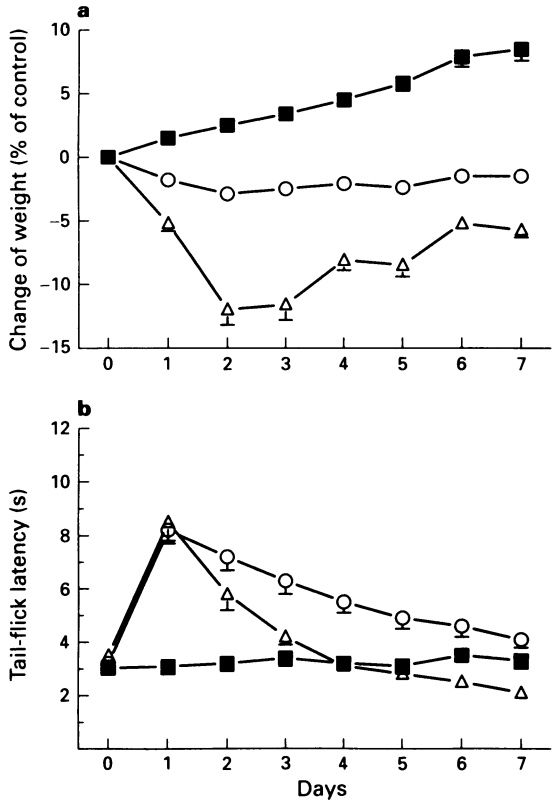


**Figure 8** Time course of percentage body weight variation (a) and tail-flick latency (b) in mice infused with vehicle (■), [Lys⁷]dermorphin (○) and morphine (△). Vehicle and opioids were s.c. infused for 7 days. (a) Percentage body-weight variation was calculated as the percentage difference between the body weight on each treatment day and the body weight before the treatment. Each point represents the mean percentage body-weight variation of 20 mice ± s.e.mean. From day 2 to day 7 of treatment, the body weights of opioid-infused mice were significantly lower than those of naive mice (ANOVA, $P<0.01$), but the body weight losses produced by morphine were significantly higher than those produced by [Lys⁷]dermorphin (ANOVA, $P<0.05$). (b) Each point represents tail-flick latency of 20 mice ± s.e.mean, ANOVA with repeated measures showed a significant difference between the mean antinociceptive responses of [Lys⁷]dermorphin- and vehicle-infused mice at all time points ($P<0.01$). Significant differences between morphine- and vehicle-infused animals ($P<0.05$) occurred on days 1, 2, 3, 6 and 7.

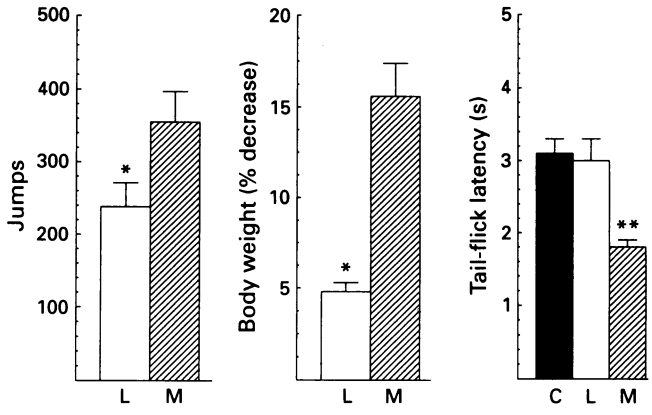


**Figure 9** Signs of naloxone-precipitated withdrawal syndrome in [Lys⁷]dermorphin- and morphine-dependent mice. Opioids were infused s.c. for eight days as follows: [Lys⁷]dermorphin (L) 27 nmol $h^{-1}$ for 8 days, morphine (M) 620 nmol $h^{-1}$ for 8 days. C = vehicle-infused mice. ANOVA: $*P<0.05$ vs morphine-infused mice; $**P<0.05$ vs vehicle-infused mice.

could inhibit peptide passage from brain to blood and could prolong the duration of antinociception.

As a peripheral antinociceptive agent, [Lys⁷]dermorphin is still from 20 to 30 times more potent than morphine. Its i.v. or s.c. potency also greatly exceeds that of dermorphin and TAPS, a synthetic tetrapeptide dermorphin analogue (Sato *et al.*, 1987; Paakkari *et al.*, 1993). This probably indicates that peripheral [Lys⁷]dermorphin has a relatively high blood to brain rate of influx, which cannot be attributed to the poor lipid solubility of the peptide. This high rate of brain entry may be due, at least in part, to a saturable blood-brain transport system. Because [Lys⁷]dermorphin is not a glycosilated peptide this transport system must be different from the glucose transporter GLUT-1 which is reported to act as the blood-brain transporter of the 2β-D-glucoside analogue of [Ser²,Met⁵]enkephalin (Polt *et al.*, 1994). More probably, the [Lys⁷]dermorphin transporter is similar to that described for other peptides (Barrera *et al.*, 1989; Banks & Kastin, 1990). If two opposite saturable transport systems regulate the brain influx and efflux of [Lys⁷]dermorphin, their concurrent saturation by high peripheral doses of the peptide would explain why, unlike intracerebral doses, subcutaneous doses of the opioid did not alter the antinociceptive peak/AUC ratio (Figure 3b).

In rats, and to a lesser extent in mice, [Lys⁷]dermorphin antinociception is highly sensitive to naloxonazine antagonism. This suggests that the antinociceptive response is mediated, at least in part, by $\mu_1$ opioid receptor subtypes because naloxonazine pretreatment 24 h before the opioid injection produces a long-lasting irreversible occupation of $\mu_1$ sites (Hahn *et al.*, 1982; Ling *et al.*, 1986). Yet despite being highly sensitive to naloxone, [Lys⁷]dermorphin catalepsy was not prevented by naloxonazine pretreatment. This finding is rather surprising in view of the fact that morphine catalepsy is significantly attenuated by naloxonazine, as is TAPS catalepsy (Paakkari *et al.*, 1993). On the other hand, in a previous paper (Negri *et al.*, 1992) we showed that another dermorphin analogue, [Trp⁴, Asn⁷] dermorphin, is 40 times more potent in producing naloxone-sensitive catalepsy than it is in producing antinociception. Thus we suggest that opioid antinociception and opioid catalepsy are modulated by different μ-opioid subtypes and that naloxonazine pretreatment is not sufficiently selective to distinguish between them.

Subcutaneous administration of [Lys⁷]dermorphin produced a stronger antinociceptive response to mechanical pressure in the rat inflamed hindpaw than in normal paw. Stein *et al.* (1989) described an antinociceptive response which occurred within 5 min from intraplantar injection of opioids in the rat inflamed paw and lasted 15 min only. Preliminary experiments with local injections (1–2 nmol) of [Lys⁷]dermorphin in the inflamed paw produced no antinociceptive response at 30, 60, 90 and 120 min intervals (data not shown). Thus in this model of inflammatory pain the 30–120 min antinociceptive response recorded after the systemic administration of the peptide appears to be mainly of central origin.

As expected, [Lys⁷]dermorphin produced tolerance, physical dependence, as well as a full cross tolerance with morphine. However [Lys⁷]dermorphin and morphine appear to differ in the degree and time-course of tolerance development. Tolerance to morphine developed at higher speed than peptide tolerance. It reached a maximum after 4-day continuous infusion of morphine, whereas [Lys⁷]dermorphin tolerance progressively increased over the 7-day infusion of the peptide. An evaluation of the antinociceptive effects of morphine in 7-day morphine tolerant mice indicates a decrease in the maximum achievable antinociceptive response, as shown by the reduced slope of the dose-response curve (Figure 6). A number of reports have noted a similar nonparallel shift after morphine treatment. Both in

laboratory animals (Theiss *et al.*, 1975; Mucha *et al.*, 1978; 1979; Blasing *et al.*, 1979; Mucha & Kalant, 1980) and in patients (Jaffe & Martin, 1985; Stimmel, 1989) highly tolerant to opiates a decreased maximal relief of pain has been reported after morphine treatment. The decrease in maximal achievable antinociceptive effect may suggest that in highly tolerant subjects, morphine does not have the efficacy necessary to provide complete antinociception. In contrast to the results seen with morphine, an even higher degree of tolerance did not alter the ability of [Lys$^7$]dermorphin to produce a maximal antinociceptive effect. These results would indicate that [Lys$^7$]dermorphin is a compound with greater efficacy than morphine or that in tolerant animals the peptide is acting in a fashion similar to a full agonist whereas morphine behaves as a partial agonist. It is known that the classification of a compound as either full agonist or partial agonist or even antagonist depends upon the effector system under study. Thus, in the rat vas deferens preparation *in vitro*, morphine acts as antagonist in a medium containing 2.5 mM $Ca^{2+}$ and as partial agonist in a medium containing 0.625 mM $Ca^{2+}$ (Miller *et al.*, 1986; Sheehan *et al.*, 1988). In the same preparation, [Lys$^7$]dermorphin acts as full agonist in both high and low $Ca^{2+}$ concentrations but it displays higher potency in the low calcium medium (data not shown). There is an apparent increase in the effective receptor reserve of the tissue in low calcium medium, so that an antagonist reveals an agonist effect, a partial agonist becomes able to inhibit the muscle twitch completely and a full agonist is about 10 times more potent. Presumably there is no increase in receptor number in low calcium, more probably, there is a change in receptor-effector coupling or in the ability of the agonist-receptor complex to transduce the measured effect. Changes in the opioid receptor system of tolerant animals have been controversial although a decrease in the fraction of spare opioid receptors (Chavkin & Goldstein, 1982), in the opioid receptor density (Rogers & El-Fakahany, 1986; Tao *et al.*, 1987) or in receptor-effector coupling (Werling *et al.*, 1989) have been reported. If a functional down-regulation of receptors occurs during opioid tolerance development, agents with low efficacy (i.e. morphine), which need a high receptor occupancy to produce maximal antinociception, may become partial agonists if the population of receptors falls below that necessary for the agent to evoke the maximal response. The differential slope reported for the morphine versus the [Lys$^7$]dermorphin curve in 7-day tolerant mice could correspond to the differences in the fractional receptor occupancy associated with the effects of the two opioids to modulate equally nociceptive processing in the CNS. It should be stressed however that these results are completely parallel with what one anticipates from the case of any non-competitive antagonist. Because morphine is a far less selective agonist for $\mu$ opioid receptors than [Lys$^7$]dermorphin, the high dose of morphine used in our infusion experiments could produce the recruitment of other receptor types. For example, rats infused chronically with morphine displayed supersensitivity to the activation of $\delta$ opioid receptors (Melchiorri *et al.*, 1992). Acting at $\delta$ sites, [Met$^5$]enkephalin can negatively modulate morphine efficacy in the mouse tail-flick assay (Vaught *et al.*, 1979; Lee *et al.*, 1980). Naltrindole, a $\delta$-selective opioid antagonist (Portoghese *et al.*, 1988), prevents the development of morphine tolerance in mice (Abdelhamid *et al.*, 1991). Mao *et al.* (1994) showed that coactivation of central NMDA and non-NMDA receptors is crucial for the development and expression of tolerance in morphine-exposed rats and that the block of NMDA receptors with MK 801 can delay tolerance development. Preliminary results in our laboratory (Negri, unpublished data) show that neither naltrindole nor MK 801 are able to prevent tolerance to [Lys$^7$]dermorphin. This indicates that [Lys$^7$]dermorphin tolerance, unlike morphine tolerance, involves a distinct mechanism of development or distinct opioid receptor subtypes. The antinociceptive response produced by [Lys$^7$]dermorphin lasted longer in tolerant mice than in naive mice. Presumably the high doses necessary to produce antinociception in tolerant animals resulted in high brain concentrations of the peptide which saturated the brain-blood transport system, slowed the efflux rate and prolonged antinociception.

As reported by others (Mao *et al.*, 1994), thermal hyperalgesia developed in association with the development of morphine tolerance, but it did not develop in [Lys$^7$]dermorphin-tolerant animals. Central NMDA and non-NMDA receptors appear to modulate the development and expression of thermal hyperalgesia in morphine-tolerant animals (Mao *et al.*, 1994). In a previous paper (Melchiorri *et al.*, 1992) we showed that $\delta$-selective opioid agonists also potentiated the hyperalgesic response in morphine-tolerant rats.

Owing to the long-lasting opioid action, the cessation of [Lys$^7$]dermorphin treatment was not *per se* sufficiently abrupt to precipitate a frank abstinence syndrome. We decided therefore to precipitate an acute withdrawal syndrome by injecting naloxone. In the acute tolerance model, naloxone precipitating dose was significantly higher when the tolerance-producing opioid was [Lys$^7$]dermorphin than when it was morphine. In the chronic tolerance model, the number of jumps and the weight loss were greater in morphine- than in [Lys$^7$]dermorphin-abstinent mice. Thermal hyperalgesic responses were recorded only in morphine-abstinent mice 12 h after opioid withdrawal. They were absent in [Lys$^7$]dermorphin-abstinent animals.

Preliminary experiments in rats also indicate that low and medium doses of [Lys$^7$]dermorphin produce respiratory stimulation, whereas high doses produce respiratory depression. Naloxonazine antagonizes the respiratory stimulation but has no effect on the respiratory depression (Negri *et al.*, unpublished data). These findings seem to confirm $\mu_1$-opioid receptors as the preferred sites of action of [Lys$^7$]dermorphin for the respiratory stimulation. They also suggest that respiratory depression induced by higher doses of the peptide may be mediated by a $\mu_2$-opioid receptor subtype.

In conclusion, the present data show that [Lys$^7$]dermorphin produces a strong long-lasting antinociception in rats and mice. It seems to be a unique opioid peptide having a high penetration into the blood-brain barrier despite its low lopid solubility.

[Lys$^7$]dermorphin also produces fewer side-effects than other opioids and appears less likely than morphine to cause physical dependence in rats and mice.

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# Pharmacological characterization of bradykinin receptors in canine cultured tracheal smooth muscle cells

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1 [$^3$H]-bradykinin was used to characterize the bradykinin receptors associated with canine cultured tracheal smooth muscle cells (TSMCs). Receptor binding assay showed that TSMCs had specific, saturable, high-affinity binding sites for [$^3$H]-bradykinin.

2 The specific [$^3$H]-bradykinin binding increased linearly with increasing cell concentrations. The equilibrium for association of [$^3$H]-bradykinin with the bradykinin receptors was attained within 2 h at 4°C and 1 h at room temperature, respectively.

3 Analysis of binding isotherms yielded an apparent equilibrium dissociation constant ($K_D$) of $2.5 \pm 0.3$ nM and a maximum receptor density ($B_{max}$) of $25.1 \pm 0.3$ fmol mg$^{-1}$ protein. The Hill coefficient for [$^3$H]-bradykinin binding was $1.00 \pm 0.02$. The association ($K_1$) and dissociation ($K_{-1}$) rate constants were $(8.67 \pm 2.60) \times 10^6$ M$^{-1}$ min$^{-1}$ and $0.024 \pm 0.005$ min$^{-1}$, respectively. $K_D$, calculated from the ratio of $K_{-1}$ and $K_1$ was $2.8 \pm 0.5$ nM, a value close to that of $K_D$ calculated from Scatchard plots of binding isotherms.

4 The $B_1$ receptor selective agonist, (des-Arg$^9$-bradykinin, 0.1 nM–10 μM) and antagonist ([Leu$^8$, des-Arg$^9$]-bradykinin, 0.1 nM–10 μM) did not inhibit the [$^3$H]-bradykinin binding to TSMCs, which excludes the presence of $B_1$ receptors in canine TSMCs.

5 The specific binding of [$^3$H]-bradykinin to canine TSMCs was inhibited by $B_2$ receptor selective antagonists ([D-Arg$^0$, Hyp$^3$, Thi$^5$, D-Tic$^7$, Oic$^8$]-bradykinin, Hoe 140, 0.1 nM–10 μM and [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-bradykinin, 0.1 nM–10 μM) and agonists (bradykinin and kallidin, 0.1 nM–10 μM) with a best fit by a one-binding site model. The order of potency for the inhibition of [$^3$H]-bradykinin binding was kallidin = bradykinin = Hoe 140 > [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-bradykinin.

6 Preincubation of TSMCs with forskolin for 24 h led to an up-regulation of $B_2$ receptors, increasing in $B_{max}$ from $25.1 \pm 0.3$ to $218 \pm 24$ fmol mg$^{-1}$ protein without changing the $K_D$ values. [$^3$H]-bradykinin binding to TSMCs was inhibited by the $B_2$ receptor selective antagonists and agonists, but not by the $B_1$ receptor selective reagents. The up-regulation of the $B_2$ receptor by forskolin was mediated through protein synthesis, since cycloheximide blocked this response.

7 It is concluded that the pharmacological characteristics of the bradykinin receptors in canine cultured TSMCs are primarily of the $B_2$ receptor subtype.

**Keywords:** Bradykinin receptor; canine tracheal smooth muscle cells; cyclic AMP elevating agents; kinins

## Introduction

Bradykinin is a classic mediator of inflammatory diseases of the airways and is implicated in allergic asthma (Christiansen *et al.*, 1987; Farmer *et al.*, 1991). In the airways, bradykinin causes bronchoconstriction, pulmonary and bronchial vasodilatation, mucus secretion and microvascular leakage (Barnes, 1992). It is well established that the kinins, bradykinin, kallidin, and des-Arg$^9$-bradykinin, interact with two bradykinin receptor subtypes, which have been classified as $B_1$ and $B_2$ (Regoli *et al.*, 1990). On the basis of the relative potencies of these three agonists, preparations with higher sensitivity to bradykinin than to des-Arg$^9$-bradykinin are considered to express $B_2$ receptor activity, while preparations having opposite sensitivity are considered to express $B_1$ receptor activity (Proud & Kaplan, 1988; Regoli *et al.*, 1990). The use of selective antagonists has further strengthened the existence of such receptor subtypes (Regoli *et al.*, 1990). In addition, studies with $B_2$ receptor selective antagonists have revealed that further heterogeneity exists among bradykinin receptors (Farmer *et al.*, 1989; Regoli *et al.*, 1990; Stewart & Vavrek, 1990). The existence of a $B_3$ receptor subtype has been proposed in guinea-pig trachea and lung, principally due to a lack of activity of $B_2$ receptor-selective antagonists (Farmer *et al.*, 1989).

Binding and functional studies have provided evidence for a $B_2$ receptor subtype in guinea-pig ileum and lung and in rat myometrium membranes and vas deferens (Manning *et al.*, 1986; Plevin & Owen, 1988; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991). In previous studies, we have reported that bradykinin can induce an increase in phosphoinositide hydrolysis and a rise in intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) in canine cultured TSMCs which appear to be mediated via the activation of $B_2$ receptors (Yang *et al.*, 1994a,b). However, studies on guinea-pig (Farmer *et al.*, 1991) and bovine (Marsh & Hill, 1993) TSMCs suggested that activation of $B_3$ and $B_2$ receptors were responsible for the bradykinin-induced increase in $[Ca^{2+}]_i$, respectively. However, it is possible that differences in affinities of $B_2$ receptor antagonists are due to species homologues of $B_2$ receptors (Hall *et al.*, 1993). Consequently, the pharmacological characteristics of the bradykinin receptor subtypes in canine cultured TSMCs need to be defined.

It has been shown that long-term cholera toxin treatment potentiates the bradykinin-stimulated phosphoinositide turnover in canine TSMCs, human foreskin fibroblasts, osteoblast-like cell line MC3T3-E1 and BALB/c/3T3 cells (Banno *et al.*, 1993; Etscheid *et al.*, 1991; Olashaw & Pledger, 1988; Yang *et al.*, 1994c). This potentiating action was inhibited by cycloheximide, suggesting that it involves an increase in protein synthesis following elevation of the con-

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centration of adenosine 3':5'-cyclic monophosphate (cyclic AMP), probably through an increase in the density of bradykinin receptors (Etscheid *et al.*, 1991; Banno *et al.*, 1993; Yang *et al.*, 1994c). Although only $B_2$ receptor density has been shown to increase in osteoblast-like MC3T3-E1 cells treated with cholera toxin (Banno *et al.*, 1993), the changes in pharmacological properties of bradykinin receptor subtypes are not clearly delineated in canine TSMCs treated with cyclic AMP elevating agents.

The purpose of this study was to characterize the bradykinin receptors in canine cultured TSMCs by radioligand [$^3$H]-bradykinin binding assay. To determine whether bradykinin receptor subtypes are present in canine TSMCs, competitive inhibition of [$^3$H]-bradykinin binding was performed using $B_1$ and $B_2$ receptor selective agonists and antagonists as competing ligands.

## Methods

### *Animals*

Mongrel dogs, 10–20 kg, both male and female were purchased from a local supplier. Dogs were housed indoors in the animal facilities under automatically controlled temperatures and light cycle conditions and fed standard laboratory chow and tap water *ad libitum*. Dogs were anaesthetized with ketamine (20 mg kg$^{-1}$, i.m.) and pentobarbitone (30 mg kg$^{-1}$, i.v.). The tracheae were surgically removed.

### *Isolation of tracheal smooth muscle cells*

The TSMCs were isolated according to the methods previously described (Yang *et al.*, 1991). The muscle was dissected, minced and transferred to the dissociation medium containing 0.1% collagenase IV, 0.025% deoxyribonuclease I, 0.025% elastase IV, and antibiotics (100 u ml$^{-1}$ penicillin G, 100 μg ml$^{-1}$ streptomycin, 250 ng ml$^{-1}$ fungizone) in physiological solution. The physiological solution contained (mM): NaCl 137, KCl 5, $CaCl_2$ 1.1, $NaHCO_3$ 20, $NaH_2PO_4$ 1, glucose 11 and HEPES 25 (pH 7.4). The tissue pieces were gently agitated at 37°C in a rotary shaker for 1 h. The released cells were collected and the residuum was again digested with fresh enzyme solution for an additional 1 h at 37°C. The released cells were washed twice with Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F-12 (F-12) medium (1:1, vol/vol). The cells, suspended in DMEM/F-12 containing 10% foetal bovine serum (FBS), were plated onto a 60 mm culture dish and incubated at 37°C for 1 h to remove fibroblasts. The cells were diluted with DMEM/F-12 to a final concentration of $2 \times 10^5$ cells ml$^{-1}$. The cells (0.5 ml/well) were plated onto 24-well culture plates for receptor binding assay. The medium was changed after 24 h and then every 3 days. After 5 days, the cells were cultured in DMEM/F-12 containing 1% FBS for 24 h at 37°C. Then, the cells were grown in DMEM/F-12 containing 1% FBS supplemented with insulin-like growth factor I (IGF-I, 10 ng ml$^{-1}$) and insulin (1 μg ml$^{-1}$) for 12–14 days.

In order to characterize the isolated and cultured TSMCs and to exclude contamination by epithelial cells and fibroblasts, the cells were identified by an indirect immunofluorescence method using a monoclonal antibody of light chain myosin (Gown *et al.*, 1985). Under the above culture conditions, over 95% of the cells were smooth muscle cells.

### *[$^3$H]-bradykinin binding assay*

Binding assays were performed with confluent TSMCs in 24-well culture plates, with or without forskolin in DMEM/F-12 containing 1% FBS for 24 h prior to the binding experiments, as described by Yang *et al.* (1994c). Culture medium was removed and 1 ml of binding buffer (composition, mM: HEPES 20, pH 7.4, NaCl 17, KCl 5.4, $KH_2PO_4$ 0.44, $CaCl_2$ 0.63, $MgSO_4$ 0.21, $Na_2HPO_4$ 0.34, N-methylglucamine 110, 0.1% (w/v) BSA and bacitracin 2) was added to each well. Cells were equilibrated on ice for 10 min, after which the binding buffer was replaced with 0.25 ml of binding buffer containing the appropriate concentration of [$^3$H]-bradykinin in the absence or presence of unlabelled bradykinin (10 μM). After 4 h incubation at 4°C, the binding buffer was removed, cells were washed three times with 2 ml of binding buffer at 4°C, suspended in 0.25 ml of 0.1 N NaOH and counted in a radiospectrometer. The amount of specific binding was calculated as the total binding minus the binding in the presence of 10 μM unlabelled bradykinin. Total receptor density ($B_{max}$) and dissociation constant ($K_D$) were calculated by Ligand programme, as described previously (Yang *et al.*, 1991). Protein concentration was measured by the method of Bradford (1976).

For cell concentration binding experiments, the cells were grown onto 100 mm culture dishes and released by 0.25% trypsin and 0.5 mM EDTA. In the assay, 50 μl cell suspension in binding buffer ($10^3$ to $10^6$ cells) was added to culture test tubes in triplicate each containing 0.2 ml binding buffer with 3 nM [$^3$H]-bradykinin. Nonspecific binding was determined in the presence of 10 μM unlabelled bradykinin. The mixture was incubated at 4°C for 4 h. The reaction was terminated by rapid filtration under vacuum through Whatman GF/C fibre filters, using a cell harvester manifold, followed by four washes with 5 ml of chilled binding buffer. After the filtration step, each filter was transferred to a vial containing 5 ml scintillation solution, and the radioactivity was determined in a liquid scintillation counter (Beckman LS5000TA).

### *Kinetic assays*

The kinetic studies were performed at room temperature and at 4°C. For the association rate constant and time course, 250 μl of [$^3$H]-bradykinin (3 nM) was added to the cells at different time intervals. The dissociation rate constant was determined by first equilibrating the cells with 3 nM [$^3$H]-bradykinin at room temperature and 4°C for 1 h and 2 h, respectively. At this time (time 0), 10 μM unlabelled bradykinin was added and determinations were made at various time interals over a 2 h period.

### *Analysis of binding data*

Equilibrium dissociation constant ($K_D$) and maximal receptor density ($B_{max}$) were calculated by Graph Pad Programme (Graph Pad, San Diego, U.S.A.) in a linear regression analysis of the transformed data. Half-maximal inhibitory concentration ($IC_{50}$) values were calculated from competition experiments by Graph Pad Programme. $IC_{50}$ values were transformed to apparent inhibitory constant ($K_i$) values. Subtype analysis was performed by fitting the competitive inhibition curves with either a one- or a two-binding site model using an iterative least-squares fit by Graph Pad Programme that corrected for occupancy of [$^3$H]-bradykinin with statistical significance established by Fisher's $F$ test (Zar, 1974).

### *Chemicals*

DMEM/F-12 medium and FBS were purchased from J.R. Scientific (Woodland, CA, U.S.A.). Insulin and IGF-I were from Boehringer Mannheim (GmbH, Germany). [$^3$H]-bradykinin (67 Ci mmol$^{-1}$) was from Dupont NEN (Boston, MA, U.S.A.). Bradykinin, des-Arg$^9$-bradykinin, [Leu$^8$, des-Arg$^9$]-bradykinin, [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-bradykinin and Hoe 140 ([D-Arg$^0$, Hyp$^3$, Thi$^5$, D-Tic$^7$, Oic$^8$]-bradykinin) were from Peninsula Laboratories (Belmont, CA, U.S.A.). Enzymes and other chemicals were from Sigma Co (St. Louis, MO, U.S.A.). All of the reagents were prepared as 1 mM stock solutions and diluted with deionized water to the appropriate concentration.

## Results

### *Kinetic constants of [$^3$H]-bradykinin binding to TSMCs*

Specific [$^3$H]-bradykinin binding increased linearly with increasing cell concentrations (from $10^3$ to $10^6$ cells), when the cells were incubated with 3 nM [$^3$H]-bradykinin at 4°C for 4 h (Figure 1). Specific [$^3$H]-bradykinin binding to TSMCs was time- and temperature-dependent. Binding of [$^3$H]-bradykinin to TSMCs reached an apparent equilibrium within 2 h at 4°C and 1 h at room temperature (Figure 2a). Half maximal bindings occurred within 21 and 8 min at 4°C and room temperature, respectively. At 4°C, no [$^3$H]-bradykinin dissociation from TSMCs was observed (Figure 2b). After 60 min association at room temperature, dissociation was initiated by addition of 10 μM unlabelled bradykinin which resulted in a reduction of specifically bound [$^3$H]-bradykinin. Half dissociation occurred within 20 min. The observed association rate constant ($K_{obs}$) was $2.625 \pm 0.126$ min$^{-1}$ and the dissociation rate constant ($K_{-1}$) was $0.024 \pm 0.005$ min$^{-1}$. The value of the association rate constant ($K_1$) calculated from the equation $K_1 = (K_{obs} - K_{-1})/[\text{radioligand}]$ was $(8.67 \pm 2.60) \times 10^6$ M$^{-1}$ min$^{-1}$. A $K_D$ value of $2.8 \pm 0.5$ nM calculated from the ratio of the rate constants, $K_{-1}/K_1$, ageed reasonably well with the $K_D$ ($2.5 \pm 0.3$ nM) determined by Scatchard analysis of saturation isotherms, as shown in Figure 3b.

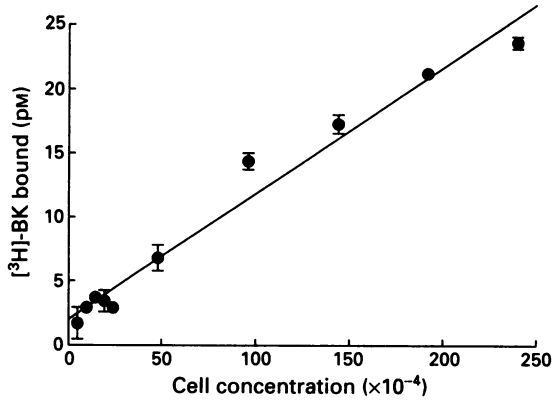


**Figure 1** Specific binding of [$^3$H]-bradykinin as a function of cell concentration. Various concentrations of TSMCs were incubated with 3 nM [$^3$H] bradykinin at 4°C for 4 h in 0.25 ml binding buffer. The relationship between cell concentration and specific binding of [$^3$H]-bradykinin is linear in the range from $10^3$ to $10^6$ cells.

### *Saturability of [$^3$H]-bradykinin binding*

The saturability of [$^3$H]-bradykinin binding was measured by incubating the cultured TSMCs with various concentrations of [$^3$H]-bradykinin from 0.1 to 11 nM (Figure 3a). The saturation isotherm is a rectangular hyperbola, suggesting that a single population of saturable high affinity bradykinin binding sites exists. Nonspecific binding, on the other hand, increased linearly with increasing [$^3$H]-bradykinin concentra-

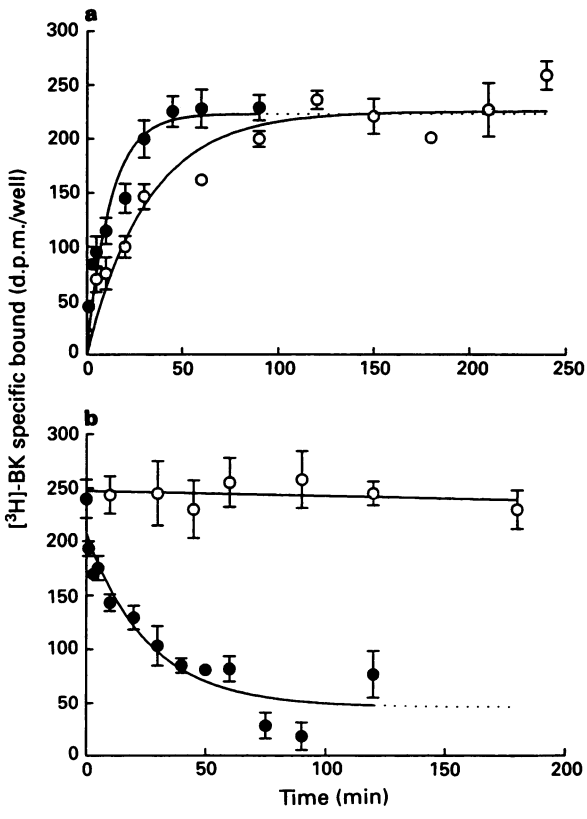


**Figure 2** Time course of association and dissociation of specific [$^3$H]-bradykinin binding to cultured TSMCs. For association (a), the cells were incubated with 3 nM [$^3$H]-bradykinin for the various periods of time indicated at room temperature (●) and 4°C (○). For determination of dissociation rate constant (b), unlabelled bradykinin at a final concentration 10 μM was added after incubation with [$^3$H]-bradykinin at room temperature (●) and 4°C (○) for 1 and 2 h, respectively. The data shown are the average of triplicate determinations from one experiment, representative of three separate experiments.

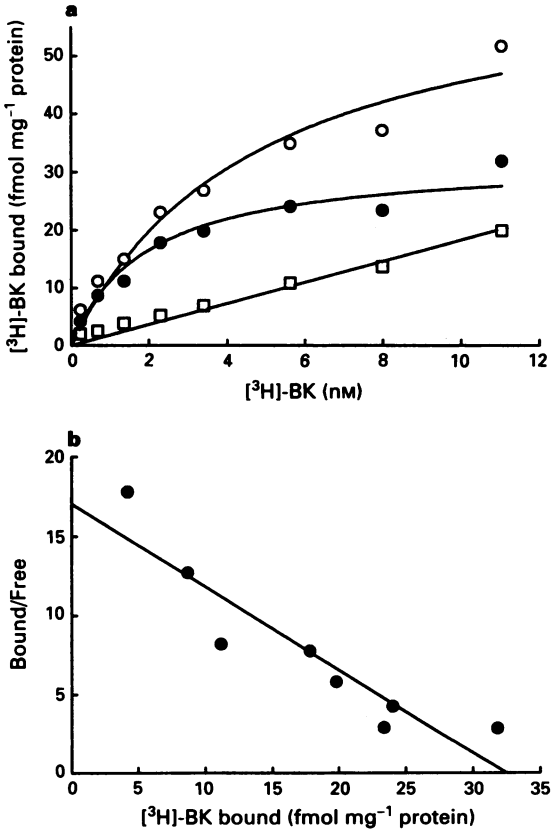


**Figure 3** (a) Saturation isotherms of [$^3$H]-bradykinin binding to canine cultured TSMCs incubated in triplicates with 0.1 to 11 nM of [$^3$H]-bradykinin at 4°C for 4 h as described under Methods. Specific binding (●) was determined as the difference between total (○) and nonspecific binding (□) in the absence and presence of 10 μM unlabelled bradykinin. (b) Scatchard plots of specific [$^3$H]-bradykinin binding data in (a). X-intercept of least-squares fit to Scatchard plot is a measure of maximal receptor density ($B_{max}$); negative reciprocal of the slope is the dissociation constant ($K_D$). Data are expressed as one of six separate experiments.

tion up to 11 nM. Scatchard plot analysis of specific bound [$^3$H]-bradykinin in the cultured TSMCs gave an apparent dissociation constant ($K_D$) of 2.5 ± 0.3 nM and a maximal receptor density ($B_{max}$) of 25.1 ± 0.3 fmol mg$^{-1}$ protein, $n = 6$ (Figure 3b). A plot of binding data for [$^3$H]-bradykinin according to the Hill equation gave a straight line with a Hill coefficient of 1.00 ± 0.02, suggesting the existence of one population of binding sites.

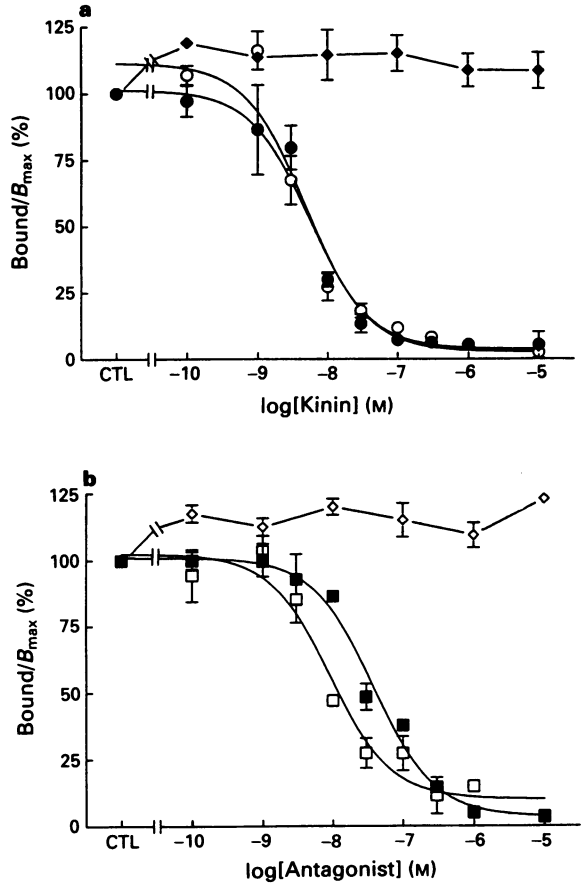


**Figure 4** Competition for [$^3$H]-bradykinin binding to canine cultured TSMCs by bradykinin receptor (a) agonists; (◆) des-Arg$^9$-bradykinin; (○) kallidin; (●) bradykinin and (b) antagonists; (◇) [Leu$^8$, des-Arg$^9$]-bradykinin; (■) [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe]-bradykinin; (□) Hoe 140. Cells were incubated with various concentrations of the competing ligand in the presence of 3 nM [$^3$H]-bradykinin. Nonspecific binding, determined in the presence of 10 μM unlablled bradykinin, was subtracted from total binding. The points shown are averages of triplicate determinations from one representative of three experiments. The curves are best non-linear least squares, computer generated fits to the data, as described under Methods.

### *Pharmacological specificity of [$^3$H]-bradykinin binding*

The pharmacological specificity of binding was defined by studying the inhibition of [$^3$H]-bradykinin binding by bradykinin receptor agonists and antagonists. Figure 4 shows that all of the drugs tested displaced [$^3$H]-bradykinin binding at bradykinin receptors in a concentration-related manner except the $B_1$ receptor-selective agonist des-Arg$^9$-bradykinin and antagonist [Leu$^8$, des-Arg$^9$]-bradykinin, even at the highest concentration tested (10 μM). The concentration of these drugs required to inhibit 50% of the specific binding ($IC_{50}$) and inhibition constant ($K_i$) in competition with [$^3$H]-bradykinin are listed in Table 1. The order of potency for the inhibition of [$^3$H]-bradykinin binding was kallidin = bradykinin = Hoe 140 > [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-bradykinin >> des-Arg$^9$-bradykinin, [Leu$^8$, des-Arg$^9$]-bradykinin. The competition curves were steep and could be adequately described by a one binding site model for these drugs (Figure 4). The pharmacological properties of the bradykinin receptors are consistent with those of $B_2$ receptors (Farmer *et al.*, 1989; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991; 1994).

### *Effect of forskolin on [$^3$H]-bradykinin binding*

Cyclic AMP elevating agents have been shown to exert their effect by receptor up-regulation (Etscheid *et al.*, 1991; Banno *et al.*, 1993). We previously reported that pretreatment of TSMCs with cholera toxin, forskolin, and dibutyryl cyclic AMP enhanced bradykinin-induced inositol phosphates accumulation and a rise in $[Ca^{2+}]_i$ through an increase in density of the bradykinin receptor (Yang *et al.*, 1994c). To examine further which bradykinin receptor subtype was increased in the cells treated with forskolin for 24 h, we chose des-Arg$^9$-bradykinin, [Leu$^8$, des-Arg$^9$]-bradykinin, bradykinin, Hoe 140 and [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-bradykinin to differentiate between the bradykinin binding sites. All tested drugs inhibited [$^3$H]-bradykinin binding with the same affinities as those of the control (Table 1). [$^3$H]-bradykinin binding to TSMCs treated with forskolin was inhibited by the $B_2$ receptor-selective antagonists and agonist, but was not affected by the $B_1$ receptor-selective agonist and antagonist. Moreover, preincubation with forskolin led to an increase in $B_{max}$ from 25.1 ± 0.3 to 218 ± 24 fmol mg$^{-1}$ protein without changing the $K_D$ value. These results suggest that the increase in receptor density is primarily due to the $B_2$ receptor.

## Discussion

It is well established that bradykinin-induced tracheal smooth muscle contraction contributes to an increase in phos-

**Table 1** Relative potencies of drugs for [$^3$H]-bradykinin binding to canine cultured TSMCs preincubated with forskolin

| | *Control* | | *Forskolin* | |
|---|---|---|---|---|
| *Drug* | $IC_{50}$ (nM) | $K_i$ (nM) | $IC_{50}$ (nM) | $K_i$ (nM) |
| *Agonists* | | | | |
| des-Arg$^9$-bradykinin | > 10000 | > 10000 | > 10000 | > 10000 |
| Kallidin | 5.6 ± 1.4 | 2.4 ± 0.8 | – | – |
| Bradykinin | 4.6 ± 0.6 | 1.9 ± 0.2 | 5.2 ± 0.3 | 2.2 ± 0.4 |
| *Antagonists* | | | | |
| [Leu$^8$, des-Arg$^9$]-bradykinin | > 10000 | > 10000 | > 10000 | > 10000 |
| [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$,D-Phe$^7$]-bradykinin | 40 ± 9 | 21 ± 5 | 52 ± 9 | 25 ± 7 |
| Hoe 140 | 5.3 ± 0.9 | 2.7 ± 0.4 | 6.1 ± 1.6 | 3.0 ± 0.4 |

Cultured TSMCs were preincubated in the absence or presence of forskolin (10 μM) for 24 h. [$^3$H]-bradykinin (3 nM) binding was determined as described in Methods in the presence of increasing concentrations of drugs. Values are the mean ± s.e.mean of three separate experiments.

phoinositide hydrolysis and a rise in $[Ca^{2+}]_i$ in cultured canine and bovine TSMCs which appears to be mediated via the activation of the $B_2$ receptors (Marsh & Hill, 1992; 1993; Yang *et al.*, 1994a,b). In this study, we have characterized the pharmacological properties of bradykinin receptors in canine cultured TSMCs by receptor binding assay with [$^3$H]-bradykinin. [$^3$H]-bradykinin has been shown to label bradykinin receptors in guinea-pig lung and trachea, and rat myometrium (Farmer *et al.*, 1989; Liebmann *et al.*, 1991; Trifilieff *et al.*, 1991; 1994). Since [$^3$H]-bradykinin is a peptide which does not penetrate the cell membrane, it allows the determination of cell surface bradykinin receptors on intact TSMCs. It is important to characterize the bradykinin receptors on intact TSMCs, because the initiation of target cell response is dependent on the number and affinity of the cell surface receptors for its agonists. Therefore, this study helps to clarify the role of these receptors in tracheal functions.

The interaction of [$^3$H]-bradykinin with bradykinin receptors occurs rapidly at room temperature. The half-time for association of [$^3$H]-bradykinin with TSMCs was 8 min. Dissociation occurred slowly with a half-life of approximately 20 min at room temperature. The equilibrium dissociation constant ($K_D$) of $2.8 \pm 0.5$ nM as determined by kinetic experiments was in reasonable agreement with the $K_D$ value of $2.5 \pm 0.3$ nM derived from saturation isotherms, which indicates that the assumption of a single biomolecular reaction is valid.

Canine TSMCs were found to be rich in [$^3$H]-bradykinin-specific, saturable, high-affinity bradykinin receptor-like binding sites. Scatchard analysis of binding data indicated a $K_D$ value of $2.5 \pm 0.3$ nM and a $B_{max}$ value of $25.1 \pm 0.3$ fmol mg$^{-1}$ protein. The binding sites most likely represent a single population without significant cooperative interactions, since Scatchard plots were linear and the Hill coefficients were near unity. Furthermore, the bradykinin receptor acting drugs competed with [$^3$H]-bradykinin binding in a concentration-related manner. The order of potency was bradykinin = kallidin >> des-Arg$^9$-bradykinin (Table 1). It has been suggested that bradykinin induces its effects through at least two receptor subtypes which have been characterized as $B_1$ and $B_2$ reeptors (Regoli *et al.*, 1990). Bradykinin and kallidin have high affinity for $B_2$ and low affinity for $B_1$ receptors (Regoli *et al.*, 1990). In contrast, des-Arg$^9$-bradykinin has a high affinity for $B_1$, but low affinity for $B_2$ receptors (Regoli *et al.*, 1990). In this study, the $B_1$ receptor-selective antagonist, [Leu$^8$, des-Arg$^9$]-bradykinin and agonist des-Arg$^9$-bradykinin, triggered no displacement at concentrations up to 10 $\mu$M, thus excluding the presence of $B_1$ receptors in canine cultured TSMCs. The binding characteristics are similar to those of the bradykinin receptors described for guinea-pig lung and trachea, and rat myometrium (Farmer *et al.*, 1989; Liebmann *et al.*, 1991; Trifillieff *et al.*, 1991; 1994).

Moreover, the competitive inhibition of specific [$^3$H]-bradykinin binding by the $B_2$ receptor-selective antagonists, Hoe 140 and [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-bradykinin showed a single population of bradykinin binding sites in canine cultured TSMCs (Table 1). The order of potency of antagonists in this binding assay was Hoe 140 > [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-bradykinin >> [Leu$^8$, des-Arg$^9$]-bradykinin (Table 1). The highest affinity was obtained with Hoe 140, a potent $B_2$ receptor-selective antagonist (Hock *et al.*, 1991). The binding affinities for Hoe 140 and [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-bradykinin were $2.7 \pm 0.4$ and $21 \pm 5$ nM, respectively, confirming their high affinity for $B_2$ receptors described for other tissues (Eggerickx *et al.*, 1992; Hess *et al.*, 1994).

We found that forskolin increased the total number of [$^3$H]-bradykinin binding sites without changing their affinity in canine TSMCs. The increase in [$^3$H]-bradykinin binding was inhibited by $B_2$ receptor-selective antagonists (Hoe 140 and [D-Arg$^0$, Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-bradykinin) and bradykinin, but not by the $B_1$ receptor-selective agonist, des-Arg$^9$-bradykinin and antagonist [Leu$^8$, des-Arg$^9$]-bradykinin (Table 1). We speculate that these cyclic AMP elevating agents affect the $B_2$ receptor number at the gene level, and are associated with the sustained elevation of intracellular cyclic AMP, since TSMCs have to be exposed to cholera toxin, forskolin, or dibutyryl cyclic AMP for several hours before any enhancing effect occurs (Yang *et al.*, 1994c).

In conclusion, our experiments demonstrate that radioligand binding assays are effective means of directly measuring parameters of receptor occupancy in canine cultured TSMCs. Canine TSMCs possess high-affinity, specific [$^3$H]-bradykinin binding sites. The bradykinin receptors in canine TSMCs are primarily $B_2$ receptors which may regulate the activity of tracheal smooth muscle through activation of this receptor subtype (Yang *et al.*, 1994a,b,c).

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# An enhancing effect of 5-hydroxytryptamine on electrically evoked atropine-resistant contraction of guinea-pig proximal colon

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**1** In the presence of atropine (0.2 μM) and indomethacin (2 μM), the effects of 5-hydroxytryptamine (5-HT) have been studied on electrically-evoked, neurogenic contractions of the guinea-pig proximal colon *in vitro*.

**2** 5-HT, at higher concentrations than 1 nM, caused an increase in electrically (1 Hz, 0.3 ms, 160 mA)-evoked, atropine-resistant contractions in a concentration-dependent manner and at 30 nM produced a maximal effect ($pEC_{50}$ value of 8.20 ± 0.11, $n = 6$). The enhancing effects of 5-HT on the electrically evoked contractions were mimicked by α-methyl-5-HT ($pEC_{50}$ value of 6.59 ± 0.05, $n = 6$).

**3** Both hexamethonium (100 μM) and spantide (10 μM), selective antagonists for nicotinic and tachykinin receptors respectively, significantly reduced the enhancement of the electrically evoked contractions by 5-HT (30 nM).

**4** DAU 6285 (3 μM), a $5\text{-HT}_4$ receptor antagonist, abolished the enhancing action of 5-HT (30 nM), but metitepine (0.03 μM), a $5\text{-HT}_1/5\text{-HT}_2$ receptor antagonist, ketanserin (0.01 μM), a $5\text{-HT}_2$ receptor antagonist, and ondansetron (1 μM), a $5\text{-HT}_3$ receptor antagonist, had no effect on the enhancement. The enhancing effects of α-methyl-5-HT (1 μM) were also abolished by DAU 6285 (3 μM).

**5** Both 5-HT (30 nM) and α-methyl-5-HT (1 μM) had no effect on contractions to exogenous substance P (0.15–0.3 nM).

**6** These results indicate that in the guinea-pig proximal colon, 5-HT produced an enhancement of atropine-resistant neurogenic contraction induced by electrical field stimulation through pre-junctional mechanisms and that the enhancement is mediated by the stimulation of $5\text{-HT}_4$ receptors located on intramural preganglionic cholinergic neurones and tachykininergic neurones.

**Keywords:** 5-Hydroxytryptamine (5-HT); $5\text{-HT}_4$ receptors; atropine-resistant contraction; proximal colon (guinea-pig)

## Introduction

5-Hydroxytryptamine (5-HT) can exert multiple actions on the mammalian digestive tract, including contraction or relaxation of the smooth muscle and stimulation of the intramural nerve plexus (Furness & Costa, 1982). Nevertheless, the precise role of 5-HT in the control of gastrointestinal motility is not clear.

In isolated preparations of the guinea-pig proximal colon, 5-HT produces both relaxations and contractions (Costa & Furness, 1979). Our previous study demonstrated that the 5-HT-evoked relaxations are mediated by the stimulation of $5\text{-HT}_1$-like receptors located on non-adrenergic, non-cholinergic inhibitory neurones (Kojima, 1991). The 5-HT-evoked contractions appear to be mediated by stimulation of cholinergic and non-cholinergic excitatory neurones (Costa & Furness, 1979). Atropine-sensitive contractions induced by 5-HT are mediated by the stimulation of $5\text{-HT}_4$ receptors located on cholinergic neurones (Elswood *et al.*, 1991), while the receptor subtypes responsible for atropine-resistant neurogenic contractions remain to be determined. The aim of the present study was to provide evidence for understanding the mechanism of the excitatory action of 5-HT on non-cholinergic neurones by elucidating the effects of 5-HT on electrically-evoked, atropine-resistant contractions of guinea-pig proximal colon.

## Methods

### *Experimental set-up*

Male Dunkin Hartley guinea-pigs, weighing 250–650 g, were stunned and bled. A segment of the proximal colon, 2–9.5 cm away from the caecum was removed, and the luminal contents were washed out with a modified Tyrode solution (composition mM: NaCl 136.8, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaH_2PO_4$ 0.42, $NaHCO_3$ 11.9, glucose 5.56, ascorbic acid 0.12). The colon was divided into three segments of circa 2.5 cm and opened longitudinally. After careful dissection of the underlying mucosa, muscle strips were suspended in longitudinal direction under a 0.5 g load in 15 ml tissue baths filled with modified Tyrode solution at 37°C and bubbled with 5% $CO_2$:95% $O_2$. The modified Tyrode solution always contained atropine (0.2 μM) and indomethacin (2 μM), to minimize cholinergic activity and endogenous prostanoid biosynthesis in response to electrical field stimulation (EFS). Tissues were allowed to equilibrate for at least 60 min before the start of experiments. During the equilibration period, the bathing medium was replaced every 15 min. Changes in mechanical activity of the tissue were recorded isotonically (isotonic transducer, Nihon Kohden, TD-112S; Nihon Kohden recticoder, RJG-3006). To obtain nerve-mediated contractions of guinea-pig colon, the tissue was placed between bipolar platinum ring electrodes (5 mm internal diameter, 6 mm apart) and connected to a Nihon Kohden stimulator (SEN-3201). Optimal stimulus parameters (rectangular pulse with 1 Hz, 0.3 ms duration, 160 mA, 37 V, for 10 s every 10 min) to obtain tetrodotoxin-sensitive, submaximal contractions (approximately 30% of histamine (10 μM)-induced maximal contraction) with stable amplitude were determined in a preliminary study. When control responses to EFS were stable for at least 30 min, the colonic strips were incubated with a single concentration (1–3000 nM) of 5-HT or α-methyl-5-HT for 5 min and further 3–5 stimulations performed. In each experiment, histamine 10 μM was added at the first stage of the experimental protocol. When the effects of 5-HT receptor antagonists, hexamethonium or

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spantide were evaluated, the colonic strips were incubated for 30–60 min with the antagonists prior to addition of agonists. In most cases, one of three preparations served as control and the two others were used for the study of 5-HT in the presence of a set concentration of antagonist. The effects of 5-HT (30 nM) or α-methyl-5-HT (1 μM) on exogenous substance P (0.15–0.3 nM)-induced contractions were evaluated by comparing the response before and after the addition of agonists in the same preparation at 40 min intervals.

### *Analysis of data*

The effects of agonists and antagonists on the EFS-induced contractions were expressed as a percentage of the mean of the 3 consecutive control responses prior to drug addition. The percentage effects of the agonists were plotted as mean values to obtain log concentration-response curves. $pEC_{50}$ (−log molar concentration of the agonist that enhances the response to EFS by 50% of the maximal effect that can be reached with that agonist) of 5-HT and α-methyl-5-HT was determined from each curve according to the method of Van Rossum (1963). Means ± s.e.mean of $n$ experiments are given throughout the paper. Antagonist effects were compared with control experiments and significance assessed by Student's $t$ tests. In some cases Dunnet's test were used. Results were considered significant if $P<0.05$.

### *Drugs*

The following drugs were used: atropine sulphate, hexamethonium chloride dihydrate, histamine dihydrochloride (Wako, Osaka, Japan); tetrodotoxin (Sankyo, Tokyo, Japan); substance P, spantide (Peptide Institute, Osaka, Japan); 5-HT creatine sulphate (Merck, Darmstadt, Germany); indomethacin (Sigma, St. Louis, MO, U.S.A.); α-methyl-5-HT maleate, ketaserin tartrate, metitepine maleate (Research Biochemicals Inc, Natick, U.S.A.); ondansetron (gift from Glaxo Research Group, Ware, U.K.); DAU 6285 hydrochloride (endo-6-methoxy-8-methyl-8-azabicyclo [3.2.1] oct 3-yl-2, 3-dihydro-2-oxo-1H-benzimidazole-1 carboxylate hydrochloride, a gift from Dr C.A. Rizzi, Boehringer Ingelheim, Italy). All drugs were initially dissolved in saline with the following exceptions: indomethacin was dissolved in distilled water containing equimolar concentrations of $Na_2CO_3$, and 5-HT or α-methyl-5-HT were dissolved in saline containing ascorbic acid (0.12 mM). All subsequent dilutions of the drugs were made with saline, except the 5-HT or α-methyl-5-HT solutions, which always contained ascorbic acid (0.12 mM). The reported concentrations are the final bath concentrations.

## Results

### *Response to electrical field stimulation (EFS)*

In the presence of atropine (0.2 μM) and indomethacin (2 μM), EFS (1 Hz, 0.3 ms, 160 mA, for 10 s) of longitudinal muscle of the guinea-pig proximal colon produced a phasic contraction (the amplitude of which was 29.6 ± 0.6% of the histamine (10 μM, $n=24$)-induced maximal contraction) within a few seconds after the 10 s stimulation period. The submaximal contractions evoked by EFS were completely abolished by tetrodotoxin (300 nM, $n=4$, Figure 1a). Moreover, the contractions evoked by EFS were significantly reduced ($P<0.05$) by both hexamethonium (100 μM), a nicotinic receptor antagonist, and spantide (10 μM), a tachykinin receptor antagonist (76.2 ± 3.1%, $n=6$ and 72.8 ± 5.7%, $n=6$ respectively, compared to pre-drug control).

### *Effects of 5-HT*

The administration of 5-HT (30 nM) caused a sustained increase in electrically evoked contractions ($P<0.001$, Figure 1b). The enhancing action of 5-HT was rapid in onset and lasted for as long as the amine remained in the bath, but on washing for 40 min the effects were reversed (Figure 1b). 5-HT (1–100 nM) caused an increase in electrically evoked contractions in a concentration-dependent manner to a maximal effect of 219.9 ± 11.7% at 30 nM, resulting in a $pEC_{50}$ value of 8.20 ± 0.11 ($n=6$, Figure 2). 5-HT (30 nM) also evoked a transient contraction in some tissues (4 out of 6 strips), amounting to 12.7 ± 3.6% of the effect of histamine 10 μM ($n=6$). The enhancing effects of 5-HT were mimicked by α-methyl-5-HT (0.1–3 μM, $n=6$, Figure 2). In all strips the maximal effects of agonists at each concentration were reached within 5 min after drug administration. The maximal enhancement by α-methyl-5-HT was close to that produced by 5-HT (198.0 ± 9.9% at 1 μM), resulting in a $pEC_{50}$ value of 6.59 ± 0.05 ($n=6$). α-methyl-5-HT (1 μM) also evoked a transient contraction in some tissues (3 out of 6 strips), amounting to 15.4 ± 1.6% of the effect of histamine 10 μM ($n=6$).

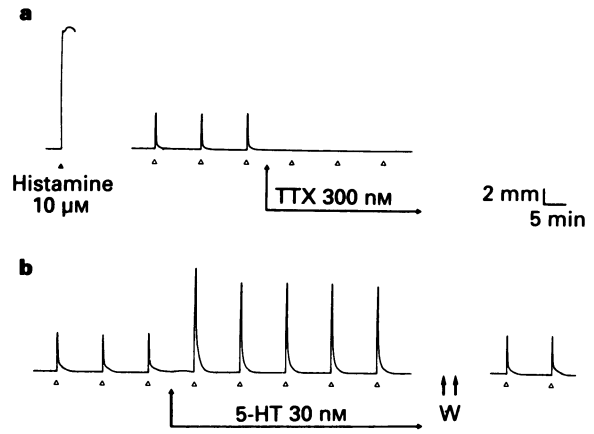


**Figure 1** Typical tracing showing the effects of (a) tetrodotoxin (TTX; 300 nM) and (b) 5-hydroxytryptamine (5-HT; 30 nM) on submaximal electrically evoked (Δ; 1 Hz, 0.3 ms, 160 mA, for 10 s) contractions of the longitudinal muscle of guinea-pig isolated proximal colon. The effects of 5-HT were removed by wash out (W = wash out for 40 min). Vertical calibration shows 2 mm shortening of the tissue; horizontal calibration shows 5 min. Atropine (0.2 μM) and indomethacin (2 μM) were present throughout experiments.

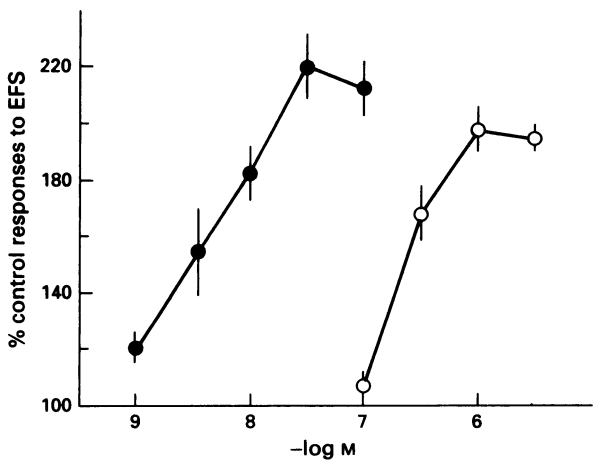


**Figure 2** Concentration-response curves for the enhancing effect of 5-hydroxytryptamine (5-HT, ●) and α-methyl-5-HT (○) on electrically evoked contractions of the guinea-pig isolated proximal colon. The enhancement of neurogenic contractions are expressed as a percentage of the control response to electrical stimulation prior to the drug addition. Each point represents the mean ± s.e.mean of 6 preparations. Atropine (0.2 μM) and indomethacin (2 μM) were present throughout experiments.

### *Effects of antagonists*

To evaluate the participation of ganglionic transmission or of endogenous tachykinins in the enhancing action of 5-HT, hexamethonium and spantide were studied. When the tissue was preincubated with hexamethonium (100 μM) for 30–40 min, the enhancement of the electrically evoked contractions induced by 5-HT (30 nM) was reduced (Figure 3). Similarly, 30–40 min preincubation of the tissue with spantide (10 μM) reduced the 5-HT-induced enhancement of the electrically evoked contractions (Figure 3).

None of the 5-HT receptor antagonists investigated had any significant influence on the amplitude of the submaximal contractions evoked by EFS (Table 1). Moreover, as shown in Table 1, metitepine (0.03 μM), a 5-$HT_1$/5-$HT_2$ receptor antagonist, ketanserin (0.01 μM), a 5-$HT_2$ receptor antagonist and ondansetron (1 μM), a 5-$HT_3$ receptor antagonist, did not affect the enhancement of electrically evoked contractions induced by 5-HT. However, DAU 6285 (0.3–3 μM), a 5-$HT_4$ receptor antagonist, significantly inhibited the enhancement of EFS-induced responses by 5-HT in a concentration-dependent manner. At the higher concentration used, DAU 6285 (3 μM) abolished the enhancing action of 5-HT. DAU 6285 (3 μM) also abolished the enhancement of EFS-induced responses by α-methyl-5-HT (1 μM, $n = 6$, 94.2 ± 6.0% compared to pre-drug control).

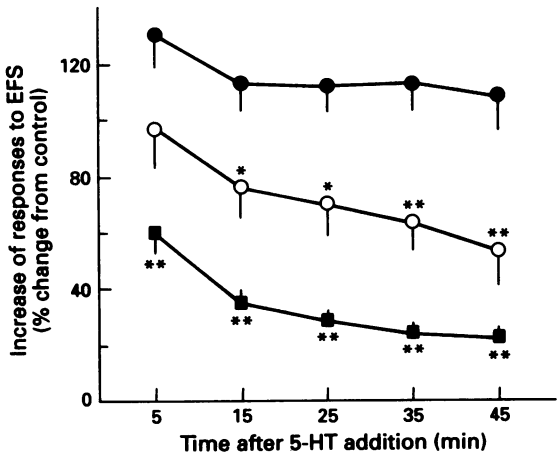


**Figure 3** Time course of the enhancing effect of 5-hydroxytryptamine (5-HT, 30 nM) on electrically evoked contractions of the longitudinal muscle of guinea-pig proximal colon in the absence (●) and presence of hexamethonium (100 μM, ○) or spantide (10 μM, ■). All results are expressed as the % change from control responses before the addition of 5-HT. Each point represents the mean ± s.e.mean of 6 preparations. *$P<0.05$ and **$P<0.01$ as compared with drug-treatment groups versus control groups using Dunnett's multiple comparison test. Atropine (0.2 μM) and indomethacin (2 μM) were present throughout the experiments.

### *Effects of 5-HT and α-methyl-5-HT on substance P-induced contractions*

Substance P (SP, 0.15–0.3 nM) produced transient contraction of the colonic strip in a concentration-dependent manner. The SP (0.3 nM)-induced contraction was abolished by preincubation of the tissue with spantide (10 μM, $n = 5$), but was unaffected by tetrodotoxin (0.3 μM, $n = 5$). As shown in Table 2, both 5-HT (30 nM) and α-methyl-5-HT (1 μM) were without affect on contractions evoked by exogenous SP (0.15 or 0.3 nM).

## Discussion

In the presence of atropine and indomethacin, the EFS-induced contraction of the guinea-pig proximal colon is probably mediated via non-cholinergic excitatory neurones, because tetrodotoxin abolished the contractions. Moreover, the EFS-induced contraction was partially hexamethonium-sensitive, suggesting that the response is, at least in part, due to stimulation of intramural preganglionic cholinergic neurones. In the present study, a tachykinin receptor antagonist, spantide also significantly reduced the EFS-induced contractions. The result is consistent with previous work indicating that endogenous tachykinins are involved in the atropine-resistant and hexamethonium-sensitive excitatory responses of the guinea-pig proximal colon *in vivo* (Giuliani *et al.*, 1993). In the present study, we found that the atropine-resistant contraction of the colonic strip evoked by EFS was enhanced in the presence of 5-HT. The nicotinic receptor antagonist, hexamethonium significantly reduced the enhancement of EFS-induced responses by 5-HT, suggesting that the enhancing action of the amine is mediated in part by the facilitation of nicotinic cholinergic transmission and that 5-HT may activate preganglionic parasympathetic nerve ter-

**Table 2** Effect of 5-HT (30 nM) and α-methyl-5-HT (1 μM) on substance P (SP)-induced contractions of the guinea-pig isolated proximal colon

| *Compounds* | *Response to SP* (% of 10 μM histamine) | n |
|---|---|---|
| Control | | |
| SP (0.15 nM) | 23.9 ± 2.0 | 13 |
| SP (0.3 nM) | 43.2 ± 2.2 | |
| +5-HT 30 nM | | 13 |
| SP (0.15 nM) | 25.7 ± 2.4 | 8 |
| SP (0.3 nM) | 42.9 ± 3.1 | 8 |
| +α-methyl-5-HT 1 μM | | |
| SP (0.15 nM) | 24.6 ± 5.0 | 5 |
| SP (0.3 nM) | 39.8 ± 3.4 | 5 |

5-HT and α-methyl-5-HT were given 5–10 min prior to the addition of SP. Each value represents the mean ± s.e.mean. 5-HT and α-methyl-5-HT did not affect SP-induced contractions ($P>0.05$, Student's $t$ test).

**Table 1** Effect of 5-HT receptor antagonists on electrically evoked contractions and on the enhancing effect of 5-HT (30 nM) in the guinea-pig isolated proximal colon

| *Pretreatment* | n | *EFS-induced contractions* (% of control) *Absence of 5-HT* | *Presence of 5-HT* |
|---|---|---|---|
| None | 16 | 100 | 212.6 ± 8.3[a] |
| Metitepine (0.03 μM) | 6 | 97.2 ± 1.8 | 205.9 ± 13.7 |
| Ketanserin (0.01 μM) | 6 | 98.0 ± 2.1 | 185.6 ± 5.9 |
| Ondansetron (1 μM) | 6 | 96.1 ± 5.1 | 223.8 ± 12.2 |
| DAU 6285 (0.3 μM) | 6 | 100.9 ± 3.8 | 131.1 ± 7.4* |
| (3 μM) | 6 | 100.0 ± 7.1 | 100.4 ± 6.5* |

Each value represents the mean ± s.e.mean
*$P<0.001$ in comparison with the control (None) value[a] by Student's $t$ test

minals or cholinergic interneurones to release acetylcholine. Recently, Mizutani *et al.* (1992) have shown that 5-HT induced an ascending contraction through an enteric excitatory pathway formed by a series of cholinergic interneurones and final cholinergic motor neurones in canine small intestine. Spantide also inhibited the enhancement of EFS-induced responses by 5-HT, suggesting that an increase in tachykininergic neurotransmission is involved in this enhancement. 5-HT is known to potentiate the contractile responsiveness of vascular smooth muscle to noradrenaline, histamine or angiotensin II (Moreland *et al.*, 1985; Van Nueten *et al.*, 1982). However, the fact that 5-HT or α-methyl-5-HT examined in the present study did not affect the contractile response to exogenous substance P, one of the tachykinin family, suggests that the enhancement of EFS-induced contractions by 5-HT was not the result of enhanced smooth muscle responsiveness to tachykinins. This suggests that the enhancement of non-cholinergically mediated contractions by 5-HT may involve a prejunctional rather than a postjunctional mechanism, such as facilitation of tachykinin release from non-cholinergic neurones. In contrast to the present results, Galligan (1992) has previously shown that non-cholinergically mediated contractions of the guinea-pig ileum were inhibited by 5-HT in a concentration-dependent manner. Although the cause of this discrepancy is not clear, the different role of 5-HT in different regions of the intestine may be involved.

According to the current classification, four main types of 5-HT receptors, termed 5-$HT_1$, 5-$HT_2$, 5-$HT_3$, 5-$HT_4$, can be distinguished (Bradley *et al.*, 1986; Clarke *et al.*, 1989). Studies with antagonists have given some insight into the types of 5-HT receptor responsible for the enhancing effects of 5-HT on EFS-induced contractions. Metitepine, a 5-$HT_1$/5-$HT_2$ receptor antagonist, ketanserin, a 5-$HT_2$ receptor antagonist, and ondansetron, a 5-$HT_3$ receptor antagonist, failed to block the enhancing effects of the amine, suggesting that the neuronal 5-HT receptor does not belong to either the 5-$HT_1$, 5-$HT_2$ or 5-$HT_3$ receptor class. On the other hand a novel 5-$HT_4$ receptor antagonist, DAU 6285 (Dumuis *et al.*, 1992), abolished the enhancing action of 5-HT, suggesting an involvement of the 5-$HT_4$ receptor subtype. α-Methyl-5-HT, a 5-$HT_2$ receptor agonist, produced a similar enhancement of EFS-induced contractions, but was 10 to 30 times less potent than 5-HT. This agonist appears to interact with a 5-$HT_4$ receptor in the guinea-pig proximal colon, since the enhancing action of α-methyl-5-HT was abolished by DAU 6285. Similar results have also been reported in the rat oesophageal tunica muscularis mucosae (Baxter *et al.*, 1991) or piglet isolated right atrium (Medhurst & Kaumann, 1993). The inhibitory effects of DAU 6285 on the enhancing action of these agonists appear not to be due to a non-specific effect, since DAU 6285 did not affect EFS-evoked contractions or contractions evoked by carbachol (data not shown). Thus, these results support the hypothesis that the activation of 5-$HT_4$ receptors can augment the atropine-resistant contractions evoked by EFS through the facilitation of intramural nicotinic cholinergic and tachykininergic neurotransmissions of the guinea-pig proximal colon.

In conclusion, our data indicate that in the guinea-pig proximal colon, 5-HT produced an enhancement of atropine-resistant neurogenic contractions induced by EFS through prejunctional mechanisms and that the enhancement is mediated by the stimulation of 5-$HT_4$ receptors located on intramural preganglionic cholinergic neurones and tachykininergic neurones.

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# Reduction of carrageenin oedema and the associated c-Fos expression in the rat lumbar spinal cord by nitric oxide synthase inhibitor

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**1** Three hours after intraplantar carrageenin (6 mg/150μl of saline) Fos-like immunoreactivity (Fos-LI) was mainly observed in L4 and L5 segments of the dorsal horn. Both superficial (I–II) and deep laminae (V–VI) neurones were labelled.

**2** We have studied the effect of systemic administration of a nitric oxide synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME) on carrageenin evoked c-Fos expression and thus the contribution of nitric oxide to this expression.

**3** Pre-administration of L-NAME (10, 25, 50, 100 mg $kg^{-1}$, i.v.) dose-dependently reduced the number of superficial and deep laminae Fos-LI neurones, 100 mg $kg^{-1}$ produced a 63 ± 2% and 72 ± 4% reduction of Fos-LI neurones respectively, $P<0.0001$ for both superficial and deep neurones.

**4** Pre-administered L-NAME dose-relatedly reduced the carrageenin-evoked paw and ankle oedema, with 100 mg $kg^{-1}$ of L-NAME resulting in a 74 ± 2% and 103 ± 2% reduction respectively.

**5** Post-administration of L-NAME (10 mg $kg^{-1}$, i.v.) reduced the number of superficial and deep laminae Fos-LI neurones (65 ± 7% and 53 ± 8% reduction respectively, $P<0.01$ for both superficial and deep neurones).

**6** Post-administered L-NAME reduced both the paw and ankle oedema (52 ± 8% and 62 ± 10% reduction respectively, $P<0.0001$ for both paw and ankle).

**7** Pre-administered D-NAME (100 mg $kg^{-1}$, i.v.), the inactive isomer of L-NAME, produced a weak reduction of the number of superficial laminae Fos-LI neurones (26 ± 8% reduction, $P<0.05$), without influencing the deep Fos-LI neurones (5 ± 8% enhancement) or the oedema.

**8** Systemic L-arginine (1200 mg $kg^{-1}$) did not reverse the reduction of the total number of Fos-LI neurones induced by 100 mg $kg^{-1}$ of L-NAME, or the effect of L-NAME on the paw and ankle oedema.

**9** Intraplantar L-arginine (30 mg) did not reverse the effect of L-NAME (100 mg $kg^{-1}$) on the total number of Fos-LI neurones. However, the inhibitory effects of L-NAME on the paw and ankle oedema were partially reversed by intraplantar L-Arginine (34 ± 9% and 45 ± 11% reduction of carrageenin oedema respectively) with these effects being significant as compared to the effect of L-NAME alone ($P<0.05$ for both).

**10** There is a strong correlation between the reduction of the number of Fos-LI neurones and the oedema by L-NAME, clearly demonstrating a predominant role of peripheral NO in the development of one of the signs of carrageenin inflammation.

**Keywords:** Carrageenin; c-Fos; nitric oxide synthase inhibitor; spinal cord of rat

## Introduction

A recent study, using the purified polyclonal antibody against rat cerebellar nitric oxide synthase, has demonstrated nitric oxide synthase (NOS) activity in laminae I–IV and X of the rat spinal cord, with the majority of NOS positive neurones also staining for NADPH diaphorase (Saito *et al.*, 1994), thus confirming previous studies of spinal NADPH diaphorase (Zhang *et al.*, 1993; Herdegen *et al.*, 1994; see references in Traub *et al.*, 1994). NADPH diaphorase immunoreactive neurones have also been demonstrated in the sensory ganglion of the rat spinal cord (Aimi *et al.*, 1991; Zhang *et al.*, 1993). The mechanisms of the production and roles of nitric oxide (NO) during nociceptive transmission have recently been reviewed (see references in Meller & Gebhart 1993). Previous behavioural and electrophysiological studies have indirectly evaluated the role of NO during nociception using NOS inhibitors, such as $N^G$-nitro-L-arginine methyl ester hydrochloride (L-NAME); in general NOS inhibitors did not greatly influence noxious electrically evoked spinal neuronal responses (Haley *et al.*, 1992) and noxious thermal behavioural responses (Malmberg & Yaksh 1993). However, systemic (Moore *et al.*, 1993) and intrathecal (Haley *et al.*, 1992; Malmberg & Yaksh 1993; Yamamoto *et al.*, 1993) NOS inhibitors have been shown to reduce noxious responses to formalin. In addition, carrageenin induced hyperalgesia has been shown to be reduced by intrathecal L-NAME (Meller *et al.*, 1994). Collectively, these studies provide evidence for a spinal contribution of NO during nociceptive transmission of more prolonged pain states. Furthermore during hyperalgesia, interactions between NO and N-methyl-D-aspartate (NMDA) receptor-mediated events have been demonstrated (see references in Meller & Gebhart, 1993; Malmberg & Yaksh, 1993; Radhakrishnan & Henry, 1993).

In addition to the spinal role of NO, there is evidence that endogenous NO may have both pro- and anti-inflammatory effects depending on the experimental conditions (see references in Nathan, 1992). Peripheral proinflammatory roles of nitric oxide has been shown in models of inflammation (Lippe *et al.*, 1993; Vane *et al.*, 1994) including carrageenin

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inflammation (Ialenti *et al.*, 1992). In contrast, L-NAME has been shown not to modulate formalin-induced oedema in mice (Moore *et al.*, 1991). Although the mechanisms of the NO contribution to inflammation are not fully elucidated, NOS inhibitors have been shown to reduce bradykinin (Hughes *et al.*, 1990; Teixeira *et al.*, 1993), phospholipase $A_2$ and substance P (Hughes *et al.*, 1990)-evoked oedema formation. In addition, inhibition of NOS has been shown to reduce bradykinin-evoked inflammation, both in terms of vasodilatation and plasma extravasation (Khalil & Helme, 1992). Since NO has been shown to have both a peripheral and spinal role during nociceptive activation and/or transmission, with NOS inhibitors being antinociceptive, it has been proposed that NOS inhibitors may be putative analgesics.

The aim of our study was to evaluate simultaneously the effects of the well established NOS inhibitor, L-NAME, on the peripheral signs of inflammation induced by carrageenin and the subsequent spinal expression of c-Fos (Draisci & Iadarola 1989; Noguchi *et al.*, 1991; 1992; Honoré *et al.*, 1994a). Intraplantar carrageenin (Winter *et al.*, 1962) produces a classical inflammation (see references in Kocher *et al.*, 1987) and a heat and mechanical hyperalgesia (Joris *et al.*, 1990; see references in Dubner & Ruda, 1992; Ferreira *et al.*, 1993). Intrathecal L-NAME has been shown to reduce the development and maintenance of thermal hyperalgesia produced by carrageenin (Meller *et al.*, 1994). Furthermore, a recent study has suggested a functional relationship between the spinal expression of immediate early genes and nitric oxide (Herdegen *et al.*, 1994).

In this series of experiments we have assessed the role of NO during nociceptive transmission by studying the effects of the NOS inhibitor, L-NAME, on carrageenin inflammation and the expression of spinal c-Fos. In addition the effect of the inactive isomer of L-NAME, $N^G$-nitro-D-arginine methyl ester hydrochloride (D-NAME) alone, and administration of systemic and intraplantar L-arginine, before systemic L-NAME, on carrageenin inflammation and the expression of c-Fos were studied. Finally, the effect of systemic L-NAME, administered after the injection of carrageenin, on carrageenin inflammation and the expression of c-Fos was also studied.

(A preliminary account of this work has been published in Buritova *et al.*, 1994).

## Methods

### *Experimental animals*

Experiments were performed on 74 adult male albino Sprague-Dawley rats (Charles River, France), weighing 225–250 g. Two groups of experiments were performed with statistical comparisons made within each group of experiments but not between groups of experiments.

In the first series of experiments, the effects of varying doses of L-NAME (injectable solution, Sigma, in saline) on c-Fos expression 3 h after carrageenin was studied. L-NAME (10, 25, 50 or 100 mg $kg^{-1}$, $n = 8$ for each group) was injected intravenously 30 min prior to carrageenin administration (6 mg 150 $\mu l^{-1}$, in saline). A control group of rats received an equal volume of intravenous saline ($n = 8$). Rats were perfused 3 h post carrageenin injection since we have previously shown c-Fos labelling to be maximal, with an equal distribution in the superficial and deep dorsal horn neurones at this time point (Honoré *et al.*, 1994a).

In the second series of experiments, under the same experimental conditions, the effect of intravenous D-NAME (injectable solution, Sigma in saline) on c-Fos expression 3 h after carrageenin was studied. D-NAME (100 mg $kg^{-1}$, $n = 5$) was injected 30 min before carrageenin injection (6 mg 150 $\mu l^{-1}$, in saline). The effects of a pre-administered versus post-administered L-NAME, injected 30 min before ($n = 5$) or 30 min after ($n = 5$) carrageenin, on carrageenin evoked c-Fos expression was studied. Finally the effects of systemic L-arginine (injectable solution, in saline; Sigma, 1200 mg $kg^{-1}$- i.v., $n = 5$) and intraplantar L-arginine (30 mg/intraplantar, $n = 5$), administered 10 min before L-NAME (100 mg/$kg^{-1}$ i.v.), which was administered 30 min before carrageenin, on c-Fos expression 3 h after carrageenin was studied. A control group of rats received an equal volume of intravenous saline ($n = 5$).

### *Immunohistochemistry*

Three hours after the carrageenin injection, the animals were deeply anaesthetized with pentobarbitone (55 mg $kg^{-1}$, i.p.; Sanofi) and perfused intracardially with 200 ml of phosphate buffered saline 0.1 M (PBS) folowed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The spinal cord was then removed and post-fixed for 4 h in the same fixative, and cryoprotected overnight in 30% sucrose in PB. Frontal frozen section 40 μm thick were cut and collected in PB to be processed immunohistochemically as free floating sections. The serial sections from the lumbar segment were immunostained for Fos-like protein according to the avidin-biotin-peroxidase method (Hsu *et al.*, 1981). The tissue sections were incubated for 30 min at room temperature in a blocking solution of 3% normal goat serum in PBS with 0.3% Triton-X (NGST). The sections were then incubated overnight at 4°C in the primary antiserum directed against the c-Fos protein (Oncogene Science Inc.). The Fos antibody, a rabbit polyclonal antibody directed against residues 4–17 of the N-terminal region of the peptide, was used at 1:4,000. The incubated sections were washed 3 times in 1% NGST and incubated in biotinylated rabbit anti-sheep IgG for 1 h at room temperature, washed twice in 1% NGST and incubated for 1 h in Avidin-Biotin-Peroxidase complex (Vectastain, Vector Laboratories). Finally, the sections were washed 3 times in PBS and developed in 1-naphtol ammonium carbonate solution (89.5 ml 0.1 M PB, 10 ml ammonium carbonate (1% in distilled water), 0.5 ml 1-naphtol (N-199-2 Aldrich, 10% in absolute alcohol) and 0.1 ml hydrogen peroxide) for 5 min, and were then washed 3 times in PB to stop the staining reaction. The sections were mounted on gelatine-subbed slides and air dried for the stain to be intensified and made alcohol-resistant through basic dye enhancement in 0.025% crystal violet (42555 Aldrich) in PB for 3 min. After 2 short PB rinses to take off the excess stain, sections were differentiated in 70% alcohol and the differentiation time was evaluated under the microscope. After being air dried, the slides were coverslipped. To test the specificity of the primary antibody, controls were performed; preabsorption with the corresponding synthetic peptide or omission of any stage in the protocol abolished the staining.

### *Counting of Fos labelled cells*

Tissue sections were first examined by darkfield microscopy to determine the segmental level according to Molander *et al.* (1984), as well as the gray matter landmarks. The sections were then examined under lightfield microscopy at × 10 to localize Fos-LI neurones. Labelled nuclei were counted using a camera lucida attachment. To study the laminar distribution 4 regions were defined: superficial dorsal horn (laminae I–II; superficial), nucleus proprius (laminae III–IV; nucleus proprius), neck of the dorsal horn (laminae V–VI; neck) and the ventral gray (laminae VII–X; ventral).

We have previously shown that the most numerous Fos positive neurones were localized in the L4–L5 segments, two counts per slice were made: (1) the total number of Fos-LI neurones in the gray matter for 10 sections through L4–L5 segments, and (2) the number of Fos-LI neurones per specific laminar region of the spinal gray matter in these 10 sections.

### *Inflammatory observations*

In order to asses the development of the inflammation, we considered one of the signs of inflammation, the peripheral oedema, at the time the animals were killed. The diameters of the ankle and of the paw, both ipsilateral and contralateral to the stimulation were measured. Both the study of Fos-LI and the assessment of inflammatory parameters were performed in the same rats.

### *Statistical tests*

Statistical analysis with analysis of variance (ANOVA) was made to compare the total number of labelled cells, using 1-way ANOVA for the different groups of animals, and 2-way ANOVA for the different groups of animals and the laminar region. For comparisons of the ankle or the paw diameters we used 1-way ANOVA for the different groups of animals. The Fishers' PLSD test was used for multiple comparisons and simple regressions were performed to establish dose-dependent effects. The investigator responsible for plotting and counting the Fos-LI neurones was 'blind' to the experimental situation of each animal.

## Results

### *Expression of spinal Fos-LI by intraplantar carrageenin*

Fos-LI neurones were virtually absent (less than 5 neurones per section) in the lumbar spinal gray matter of non-stimulated freely moving rats. In contrast, in carrageenin-stimulated animals, Fos-LI neurones appeared as round structures, stained to a variable degree. To quantify the number of Fos-LI neurones, we took into account all labelled neurones without considering the intensity of the staining. In agreement with our previous study on carrageenin evoked c-Fos expression (Honoré *et al.*, 1994a), Fos-LI neurones were observed in lumbar segments L2–L6, with maximal labelling observed in segments L4 and L5. In the two series of experiments, the total number of Fos-LI neurones after carrageenin were 136 ± 2 and 109 ± 7 per section, respectively. For both series of experiments the Fos-LI neurones were equally distributed in the superficial laminae (I–II) and deep laminae (V–VI) of the dorsal horn (first series: 37 ± 1% and 39 ± 2% respectively, second series: 45 ± 3% and 38 ± 3% respectively), with a small amount of labelling in the ventral horn.

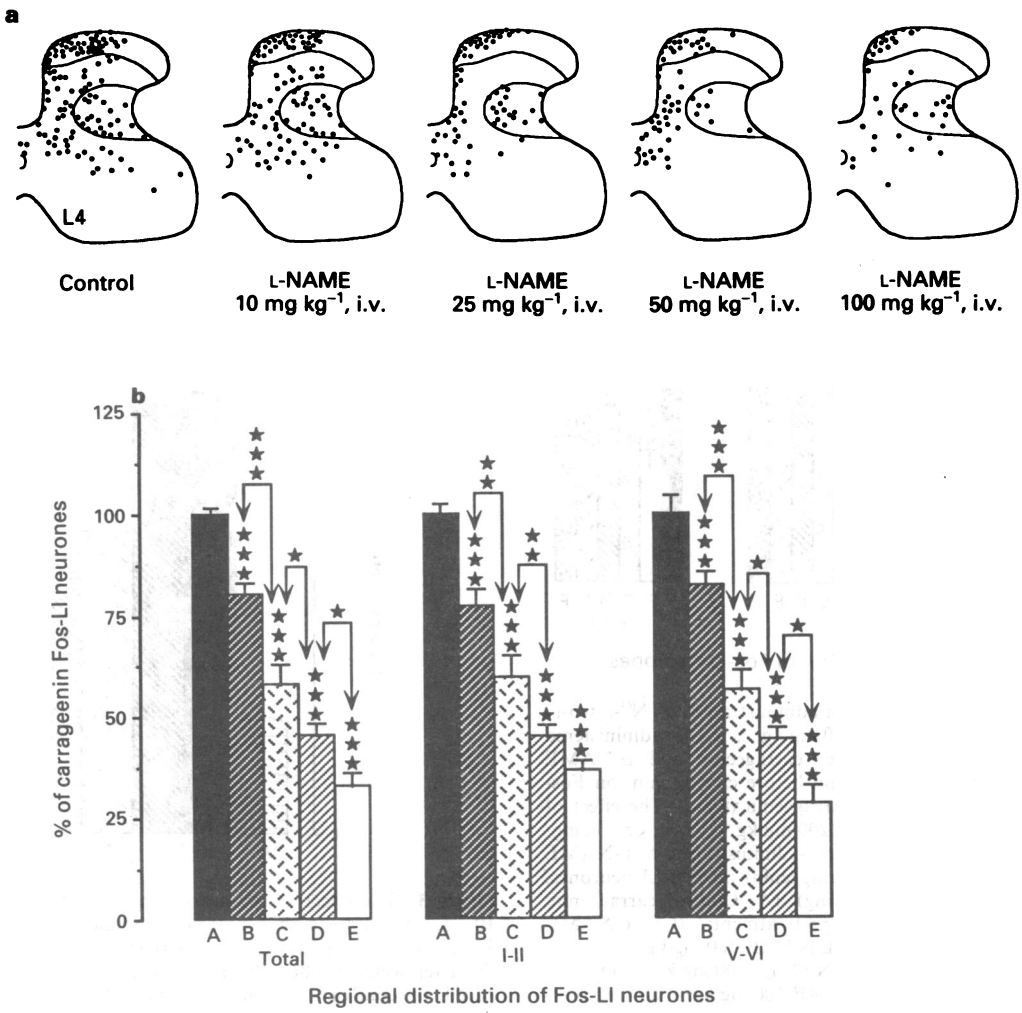


**Figure 1** Camera lucida drawings and histograms showing the effect of systemic $N^G$-nitro-L-arginine methyl ester (L-NAME, 10, 25, 50 or 100 mg kg$^{-1}$, i.v.) on Fos-like immunoreactivity (Fos-LI neurones), 3 h after intraplantar carrageenin (6 mg). L-NAME was administered 30 min prior to carrageenin. The control group received systemic saline (a) Each diagram includes all labelled neurones in one 40 μm section; each dot represents one labelled neurone. The boundaries of the superficial laminae and of the reticular part of the neck of the dorsal horn are outlined. (b) Systemic pre-administration of L-NAME dose-dependently decreased the number of Fos-LI neurones after carrageenin, in both the superficial and deep laminae. (A) Control carrageenin; (B) L-NAME 10 mg kg$^{-1}$, i.v.; (C) L-NAME 25 mg kg$^{-1}$, i.v.; (D) L-NAME 50 mg kg$^{-1}$ i.v.; (E) L-NAME 100 mg kg$^{-1}$ i.v. Results are expressed as percentages of the mean number Fos-LI neurones after carrageenin alone ± s.e.mean in all laminae of the L4–L5 segments (Total), in superficial laminae and in the neck of the dorsal horn in the L4–L5 segments. Significance as compared to carrageenin alone was performed by ANOVA, PLSD Fisher's test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

*L-NAME reduces the number of spinal Fos-LI neurones*

In the first series of experiments, pre-administered systemic L-NAME (10, 25, 50 and 100 mg/kg) significantly reduced the total number of Fos-LI neurones as compared to the total number of Fos-LI neurones after carrageenin alone ($20 \pm 3\%$, $P<0.001$, $42 \pm 5\%$, $P<0.0001$, $55 \pm 3\%$, $P<0.0001$ and $77 \pm 3\%$ reduction, $P<0.0001$, respectively, Figure 1). This effect of L-NAME was dose-dependent ($r^2 = 0.643$, $P<0.0001$).

Both the number of superficial (laminae I–II) and deep (laminae V–VI) Fos-LI neurones were reduced to a similar extent by L-NAME (Figure 1), with the effect of L-NAME on both populations of Fos-LI neurones being dose-dependent (superficial laminae, $r^2 = 0.575$, $P<0.0001$ and deep laminae, $r^2 = 0.654$, $P<0.0001$). There was no significant difference between the effect of L-NAME on the superifical and deep Fos-LI neurones.

*The effect of L-arginine and L-NAME on the number of spinal Fos-LI neurones*

In the second series of experiments, there was a tendency towards a reversal of the effect of 100 mg kg$^{-1}$ of L-NAME on the total number of FLI-neurones by an earlier pre-administration of systemic L-arginine ($38 \pm 8\%$ reduction, Figure 2), however this effect was not significantly different from the effect of L-NAME alone ($57 \pm 8\%$ reduction). In addition, there was a tendency towards a reversal of the effect of 100 mg kg$^{-1}$ of L-NAME on the superficial and deep Fos-LI neurones by systemic L-arginine; again this effect was not significantly different from the effect of the highest dose of L-NAME alone (Figure 2).

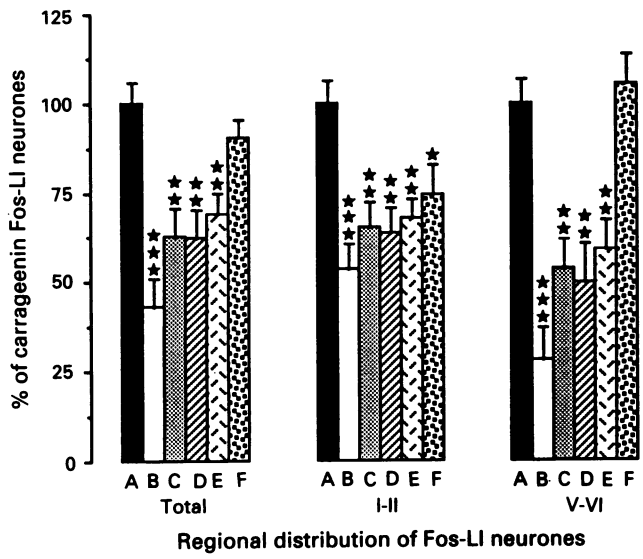


**Figure 2** The effects of pre- and post-administration of N$^G$-nitro-L-arginine methylester (L-NAME, 100 mg kg$^{-1}$, i.v.), administered either 30 min before or 30 min after carrageenin and D-NAME (100 mg kg$^{-1}$, i.v.), administered 30 min before carrageenin, on Fos-LI neurones, 3 h after intraplantar carrageenin (6 mg). The effect of pre-administration of L-arginine (1200 mg kg$^{-1}$, i.v. or 30 mg/intraplantar) given 10 min before the pre-administration of L-NAME (100 mg kg$^{-1}$, i.v., 30 min prior to carrageenin) on Fos-LI neurones, 3 h after intraplantar carrageenin (6 mg). (A) Control carrageenin; (B) L-NAME 100 mg kg$^{-1}$, i.v. pretreatment; (C) L-NAME 100 mg kg$^{-1}$, i.v. post-treatment; (D) L-NAME 100 mg kg$^{-1}$ i.v. + L-arginine 1200 mg kg$^{-1}$, i.v.; (E) L-NAME 100 mg kg$^{-1}$ i.v. + L-arginine 30 mg intraplantar; (F) D-NAME 100 mg kg$^{-1}$, i.v. pretreatment. Pre- and post-administered L-NAME reduced the number of Fos-LI neurones to a similar extent. L-Arginine did not reverse the effects of L-NAME on the Fos-LI neurones. D-NAME did not significantly influence the total number of Fos-LI neurones or the number of deep Fos-LI neurones, but weakly reduced the superficial Fos-LI neurones. Results are expressed as percentages of the mean number Fos-LI neurones after carrageenin alone ± s.e.mean in all laminae of the L4–L5 segments (Total), in superficial laminae and in the neck of the dorsal horn in the L4–L5 segments. Significance as compared to carrageenin alone was performed by ANOVA, PLSD Fisher's test $*P<0.05$, $**P<0.01$, $***P<0.001$).

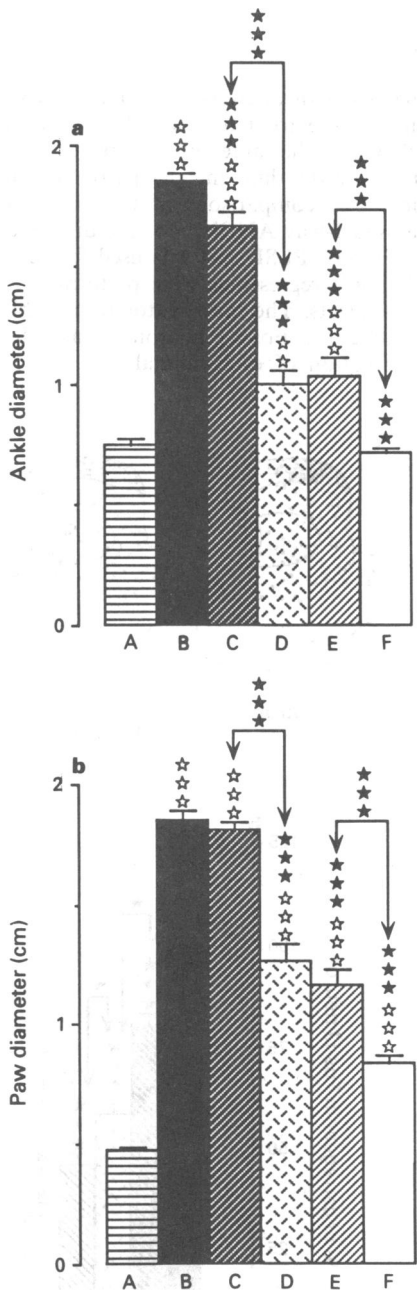


**Figure 3** The effect of N$^G$-nitro-L-arginine methyl ester (L-NAME, 10, 25, 50 or 100 mg kg$^{-1}$, i.v.) on the ankle (a) and paw (b) oedema, 3 h after intraplantar carrageenin (6 mg). Both the paw and ankle diameter were significantly increased 3 h after carrageenin as compared to control non-stimulated rats. Pre-administered L-NAME dose-relatedly reduced the carrageenin induced paw and ankle oedema. With the highest dose of L-NAME the diameter of the ankle was not different from non-stimulated rats. (A) Non stimulated rat; (B) control carrageenin; (C) L-NAME 10 mg kg$^{-1}$, i.v.; (D) L-NAME 25 mg kg$^{-1}$, i.v.; (E) L-NAME 50 mg kg$^{-1}$, i.v.; (F) L-NAME 100 mg kg$^{-1}$, i.v. Results are presented as absolute values of paw and ankle diameters ± s.e.mean (centimetres). Significance compared to carrageenin alone and compared to the non-stimulated rats was performed by ANOVA, PLSD Fisher's test, solid and open stars respectively ($**P<0.01$, $***P<0.001$). Significant differences between different doses of L-NAME are indicated by arrows.

With a prior administration of intraplantar L-arginine (30 mg 50 $\mu l^{-1}$), there was a tendency towards a reversal of the effect of 100 mg $kg^{-1}$ of L-NAME on the total number of Fos-LI neurones (31 ± 5% reduction, Figure 2). However, the effect of intraplantar L-arginine plus L-NAME was not significantly different from the effect of L-NAME alone (57 ± 8% reduction). Again, similar effects of intraplantar L-arginine plus L-NAME on the superficial and deep Fos-LI neurones were observed, with these effects not being significantly different from those observed with L-NAME alone (Figure 2).

### *Post-administered L-NAME reduces the number of spinal Fos-LI neurones*

In the second series of experiments, post administered L-NAME (100 mg $kg^{-1}$, i.v.) reduced the total number of Fos-LI neurones (37 ± 7% reduction, $P<0.05$ as compared to carrageenin alone, Figure 2). In addition, the number of superficial and deep Fos-LI neurones were significantly reduced (35 ± 7% and 46 ± 8% reduction respectively, $P<0.05$ for both, as compared to carrageenin alone). Both pre- and post-administered L-NAME reduced the total number of Fos-LI neurones, in addition to the superficial and deep Fos-LI neurones, to a similar extent.

### *The effect of D-NAME on the number of spinal Fos-LI neurones*

Systemic D-NAME (100 mg $kg^{-1}$) produced no significant effect on the total number of Fos-LI neurones and the deep Fos-LI neurones (10 ± 5% reduction and 5 ± 8% enhancement respectively, Figure 2). However, a weak depressive effect of D-NAME on the superficial Fos-LI neurones was observed (26 ± 8% reduction, $P<0.05$ as compared to carrageenin alone), but this effect was smaller than that observed with the same dose of L-NAME (46 ± 3% reduction, Figure 2).

### *Carrageenin oedema*

The effects of L-NAME (10, 25, 50 and 100 mg $kg^{-1}$) on one of the signs of carrageenin inflammation, paw and ankle oedema, were studied simultaneously with the expression of c-Fos in all rats. The inflammatory oedema was measured as the increases in the ankle and paw diameters. Three hours after the carrageenin injection, the mean ankle diameter and paw diameter were considerably larger than those of the control non-stimulated rats (281 ± 9% and 357 ± 17% increase respectively, Figure 3).

Pre-administered L-NAME reduced both the ankle and paw oedema in a dose-dependent manner ($r^2 = 0.565$, $P<0.0001$ and $r^2 = 0.687$, $P<0.0001$ respectively, Figure 3). With the highest dose of L-NAME (100 mg $kg^{-1}$), the oedema of the paw and ankle were greatly reduced (55 ± 4% and 84 ± 10% reduction, respectively, Figure 3). The effects of L-NAME on the ankle and paw oedema and spinal expression of c-Fos were strongly correlated ($r^2 = 0.73$, $P<0.0001$ and $r^2 = 0.625$, $P<0.0001$, respectively). Post-administration of the highest dose of L-NAME (100 mg $kg^{-1}$, i.v.) reduced the paw and ankle diameter (52 ± 8% and 62 ± 10% reduction, respectively), with these effects not being significantly different from those observed with pre-administration of 100 mg $kg^{-1}$ of L-NAME (Figure 4).

The effects of L-NAME on the paw and ankle diameters were not reversed by systemic L-arginine (Figure 4). However, the effects of L-NAME on the paw and ankle diameters (55 ± 4% and 84 ± 10% reduction respectively), were partly reversed by intraplantar L-arginine (34 ± 9% and 45 ± 11% reduction of the control paw and ankle oedema respectively, Figure 4). Systemic D-NAME did not reduce the oedema of the paw and the ankle (1 ± 5% and 14 ± 8% facilitation of the control paw and ankle oedema respectively, Figure 4).

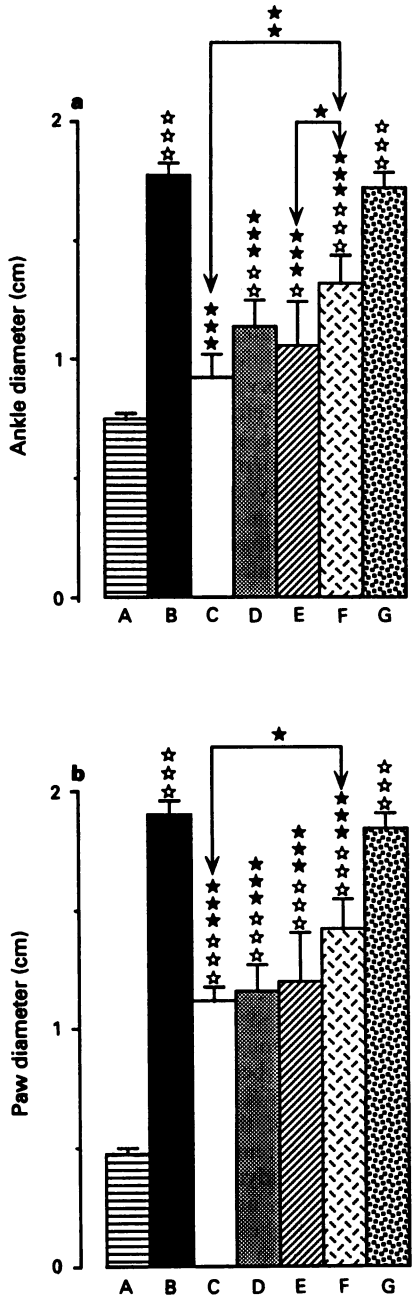


**Figure 4** The effects of pre- and post-administration of $N^G$-nitro-L-NAME, 100 mg $kg^{-1}$ i.v.), pre-administration of D-NAME (100 mg $kg^{-1}$, i.v.) and pre-administration of L-arginine (1200 mg $kg^{-1}$, i.v. or 30 mg/intraplantar) plus L-NAME (100 mg $kg^{-1}$, i.v.), on the ankle (a) and paw (b) oedema, 3 h after intraplantar carrageenin (6 mg). With co-administration of L-arginine and L-NAME, L-arginine was administered 10 min before L-NAME. (A) Non stimulated rat; (B) control carrageenin; (C) L-NAME 100 mg $kg^{-1}$, i.v. pretreatment; (D) L-NAME 100 mg $kg^{-1}$, i.v. post-treatment; (E) L-NAME 100 mg $kg^{-1}$, i.v. + L-arginine 1200 mg $kg^{-1}$, i.v.; (F) L-NAME 100 mg $kg^{-1}$, i.v. + L-arginine 30 mg intraplantar; (G) D-NAME 100 mg $kg^{-1}$, i.v. pretreatment. Both the paw and ankle diameter were significantly increased 3 h after carrageenin as compared to control non-stimulated rats. Pre and post-administered L-NAME reduced the paw and ankle diameters to an equal extent. Systemic L-arginine did not reverse the effect of L-NAME on either the paw or ankle diameter. However, intraplantar L-arginine partially reversed the effect of L-NAME on the paw and ankle diameter. D-NAME did not influence the increased paw and ankle diameters. Results are presented as absolute values of paw and ankle diameters ± s.e.mean (centimetres). Significance compared to carrageenin alone and compared to the non-stimulated rats was performed by ANOVA, PLSD Fisher's test, solid and open stars respectively (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Significant differences between pharmacological groups are indicated by arrows

## Discussion

Three hours after intraplantar injection of carrageenin, numerous Fos-LI neurones were observed in the superficial laminae (I–II) and deep laminae (V–VI) of the dorsal horn, and to a lesser extent in ventral horn, of lumbar L4–L5 segments of the spinal cord, thus confirming our previous study (Honoré *et al.*, 1994a). Due to experimental constraints, including the necessity to study a large number of experimental groups for a complete pharmacological study, it was not possible to perform all experiments simultaneously and therefore two series of experiments were performed. The c-Fos technique is associated with an inherent variability as illustrated by the small difference between the total number of Fos-LI neurones observed after carrageenin for the two series of experiments, therefore comparisons between the two series of experiments are not statistically valid. However, within each series of experiments the expression of Fos-LI neurones was remarkably consistent, as illustrated by the virtually equal distribution of Fos-LI neurones between the superficial and deep laminae.

Pre-administered systemic L-NAME dose-dependently reduced the number of Fos-LI neurones observed in the superficial and deep laminae of the dorsal horn after carrageenin. In addition, the highest dose of L-NAME studied, given 30 min after intraplantar carrageenin, also significantly reduced the number of Fos-LI neurones in the superficial and deep laminae. There was no significant difference between the effect of the pre and the post-administration of 100 mg $kg^{-1}$ of L-NAME in any of the areas studied. The ability of post-administered L-NAME to reduce carrageenin-evoked c-Fos expression is expected in view of the time course of the associated inflammation, increases in paw temperature and hyperalgesia (Joris *et al.*, 1990, see refs. In Dubner & Ruda, 1992). It is likely that the timing of the post-administered L-NAME, before peak development of inflammation and hyperalgesia, enables it to reduce these events effectively and consequently reduce the expression of c-Fos in a manner similar to pre-administered L-NAME. However our interpretation has to be cautious since a significant increase in spinal c-fos mRNA has been observed as early as 30 min after carrageenin (Draisci & Iadarola, 1989). Nevertheless in this model, the timing of the expression of c-Fos after the increase in c-fos mRNA remains to be determined and more extensive experiments studying the effect of L-NAME given at even later time points are required.

The effects of the highest dose of L-NAME on Fos-LI neurones were not reversed by either systemic or intraplantar L-arginine. Previous studies have indicated that high doses of L-arginine are required to reduce the effects of L-NAME (Malmberg & Yaksh, 1993). In our study L-arginine, at a dose 12 times greater than the highest dose of L-NAME, and given prior to the L-NAME, did not block the effect of L-NAME on c-Fos expression. This lack of effect of L-arginine probably reflects differences in the pharmacokinetics of L-NAME and L-arginine, since L-arginine acts competively with L-NAME for NOS, it is more advantageous for L-arginine to be at the site of action prior to L-NAME. Although in our study L-arginine was administered before L-NAME it is not possible to predict the relative concentrations of the two compounds at their sites of action. Our results with systemic L-arginine are in keeping with an electrophysiological study in which intrathecal L-arginine did not reverse the depressive effects of intrathecal L-NAME on dorsal horn neuronal responses to peripheral formalin (Haley *et al.*, 1992).

The inactive isomer of L-NAME, D-NAME (100 mg kg−1) did not influence the total number of Fos-LI neurones or the deep Fos-LI neurones; this is in clear contrast to the reduction of Fos-LI neurones by the equivalent dose of L-NAME. Overall, our results with D-NAME indicate that the effect of L-NAME on spinal Fos-LI neurones is specific, due to the inhibition of the nitric oxide synthase enzyme, and are not due to non specific effects. However, a weak reduction of the superficial Fos-LI neurones by D-NAME was observed. Although this effect of D-NAME may be interpretated as evidence for L-NAME acting via mechanisms other than NOS inhibition, the dose of D-NAME studied was high and the effect extremely weak as compared to that observed with the same dose of L-NAME. Furthermore, D-NAME did not influence the deep Fos-LI neurones which were strongly reduced by the same dose of L-NAME.

Since spinal c-Fos expression is strongly associated with noxious spinal transmission (see references in Herdegen *et al.*, 1991; Abbadie *et al.*, 1994; Zieglgänsberger & Tölle, 1993), the ability of L-NAME to reduce such an expression strongly implies a pro-nociceptive role of NO. In this respect it must be emphasised that L-NAME reduced c-Fos expression in the superficial and deep laminae neurones of the dorsal horn, the same laminae in which noxiously evoked neurones are located (Besson & Chaouch, 1987; Willis & Coggeshall, 1991). Furthermore, the effects of L-NAME on c-Fos are reminiscent of the effects of paracetamol, aspirin (Honoré *et al.*, 1994b) and indomethacin (Honoré *et al.*, 1994a) in the same model of inflammatory pain, thus providing additional indirect evidence for a pro-nociceptive role of NO. Our results are in strong agreement with previous studies which have shown L-NAME to reduce both dorsal horn neuronal responses (Haley *et al.*, 1992) and behavioural responses (Malmberg & Yaksh, 1993) to formalin, in addition to reducing carrageenin-evoked hyperalgesia (Meller *et al.*, 1994), suggesting L-NAME diminishes more prolonged nociceptive transmission associated with the development of central hypersensitivity which is associated with inflammatory processing (Dubner & Ruda, 1992).

Three hours after intraplantar injection of carrageenin, both the paw and ankle diameters were considerably larger than control non stimulated rats, the peripheral oedema associated with carrageean indicates the development of inflammation. Both pre and post-administration of systemic L-NAME dramatically reduced the carrageenin oedema, in a clear dose-related manner. These results strongly support a peripheral role of NO in the development of carrageenin-evoked oedema. Our results are in keeping with previous studies which have reported a peripheral role of NO in the development of inflammation (Ialenti *et al.*, 1992; Lippe *et al.*, 1993; Vane *et al.*, 1994) and a previous electrophysiological study in which peripherally administered L-NAME reduced the second phase response, resulting from peripheral inflammation, of dorsal horn neurones to peripheral formalin (Haley *et al.*, 1992).

To substantiate further the role of NO during carrageenin oedema, the effect of L-arginine administered prior to L-NAME on the oedema was studied. The reduction of oedema by the highest dose of L-NAME was not reversed by the prior administration of systemic L-arginine. As discussed above, this lack of effect may reflect the timing of the L-arginine administration or the dose studied. In contrast, the reduction of the oedema by L-NAME was partially attenuated by intraplantar L-arginine. These results suggest that the systemic dose of L-arginine studied was either too low or had poor access to the inflammatory site. Importantly the oedema was not influenced by systemic D-NAME, suggesting the effect of L-NAME on the carrageenin oedema is not due to non specific effects of the drug.

L-NAME has been shown to induce increases in blood pressure and a reduction in blood flow in different organs (Gardiner *et al.*, 1990), and the contribution of these effects to the antinociceptive effect of L-NAME is an important consideration. Since methods for recording blood pressure in freely moving animals are limited, it was not feasible for us to monitor the contribution of changes in blood pressure to the effects of L-NAME on the oedema and c-Fos expression. In addition, any invasive surgical procedures, or even the use of a pneumatic sensory cuff, may influence the spinal c-Fos

expression. However, 7-nitro indazole (7-NI), an NOS inhibitor has been reported to be anti-nociceptive, without influencing blood pressure (Moore *et al.*, 1993). Thus future studies are necessary to determine the effect of 7-NI on carrageenin inflammation and c-Fos expression, in the absence of any possible cardiovascular effects.

In conclusion, L-NAME greatly reduced carrageenin oedema, implicating an important contribution of NO to the development of inflammation. The reduction of the carrageenin oedema by L-NAME was strongly correlated with the decreased spinal expression of c-Fos. These results suggest that the reduction of spinal c-Fos by L-NAME is due to a diminished spinal input and predominantly reflects a peripheral action of systemic L-NAME. However, in view of the results of previous studies using intrathecal L-NAME (Haley *et al.*, 1992; Malmberg & Yaksh, 1993; Meller *et al.*, 1994), our results cannot exclude an additional central effect of systemic L-NAME and thus a spinal contribution of NO to c-Fos expression as previously described (Lee *et al.*, 1992).

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# Interaction between gallopamil and cardiac ryanodine receptors

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**1** In a sarcoplasmic reticulum fraction obtained from rat hearts, the analysis of equilibrium [$^3$H]-ryanodine binding showed high and low affinity sites ($K_D$ = 1.3 nM and 2.8 μM, $B_{max}$ = 2.2 pmol mg$^{-1}$ and 27.8 pmol mg$^{-1}$). The dissociation rate constant increased at 1 μM vs 4 nM [$^3$H]-ryanodine concentration, and micromolar ryanodine slowed the dissociation of nanomolar ryanodine.

**2** The binding of 4 nM [$^3$H]-ryanodine was not affected by gallopamil, while the binding of 100 nM to 18 μM [$^3$H]-ryanodine was partly displaced. Data analysis suggested that gallopamil inhibited low affinity [$^3$H]-ryanodine binding, with $IC_{50}$ in the micromolar range.

**3** Gallopamil decreased the dissociation rate constant of 1 μM [$^3$H]-ryanodine. While gallopamil alone did not affect the dissociation of 4 nM [$^3$H]-ryanodine, gallopamil and micromolar ryanodine slowed it to a greater extent than micromolar ryanodine alone.

**4** Our results are consistent with the hypothesis that the ryanodine receptor is a negatively cooperative oligomer, which undergoes a sequential alteration after ryanodine binding. Gallopamil has complex actions: it inhibits ryanodine binding to its low affinity site(s), and probably modulates the cooperativity of ryanodine binding and/or the transition to a receptor state characterized by slow ryanodine dissociation. These molecular actions could account for the previously reported effect of gallopamil on the sarcoplasmic reticulum calcium release channel.

**Keywords:** Gallopamil; calcium antagonists; heart; sarcoplasmic reticulum; ryanodine; calcium channels

## Introduction

Calcium (Ca) antagonists are a group of drugs which are currently used in the treatment of angina pectoris, arterial hypertension and arrhythmias. These compounds are able to inhibit the slow inward Ca current in cardiac and smooth muscle cells, by acting on sarcolemmal L-type voltage channels (Nayler, 1988). However, intracellular targets of Ca antagonists have been described (Zernig, 1990). In particular, it has been suggested that some Ca antagonists may interfere with the sarcoplasmic reticulum (SR) Ca release channel, and reduce the probability of channel opening and/or channel conductivity (Hirata *et al.*, 1983; Fernandez-Belda & Gomez-Fernandez, 1987; Valdivia *et al*, 1990; Ohkusa *et al.*, 1991; Zucchi *et al.*, 1992).

The SR Ca channel binds the alkaloid ryanodine (Ry) and has been identified as the Ry receptor (Inui *et al.*, 1987; Imagawa *et al.*, 1987). In the absence of Ry, the channel undergoes spontaneous openings and closures. Ry has a biphasic effect: at low concentrations it locks the channel in a low-conductivity configuration; at higher concentrations it determines channel blockade (Meissner, 1986). This biphasic action has been related to the presence of multiple Ry binding sites. Interaction with the high affinity site induces the formation of the low-conductivity state; interaction with the low affinity site(s) leads to channel inactivation (McGrew *et al.*, 1989; Pessah & Zimanyi, 1991). Comparative analysis of Ry binding and SR channel function after different interventions [changes in electrolyte and nucleotide concentrations, addition of anthraquinone drugs, phosphorylation by different kinases (Pessah *et al.*, 1985; Meissner & Henderson, 1987; Michalak *et al.*, 1988; Abramson *et al.*, 1988; Holmberg & Williams, 1990; Chu *et al.*, 1990; Takasago *et al.*, 1991; Mack *et al.*, 1992)], has shown that interventions which affect SR Ca efflux also affect Ry binding.

In this paper we investigated the effect of gallopamil (also known as methoxyverapamil or D-600), a phenylalkylamine Ca antagonist, on Ry binding in rat cardiac SR.

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## Methods

### *Preparation of cardiac SR*

Sprague-Dawley rats, fed with standard diet (275–300 g body weight), were anaesthetized with a mixture of ether and air. After injection of 1000 iu sodium heparin in the femoral vein, the heart was quickly excised and perfused retrogradely through the aorta at a perfusion pressure of 80 mmHg. The perfusion buffer was equilibrated with a mixture of oxygen (95%) and carbon dioxide (5%), and contained (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11. The temperature was kept between 37°C and 37.4°C, and the pH was 7.4.

After 10 min of perfusion, in order to allow complete washout of blood from the coronary vessels and stabilization of the contractile activity, the hearts were quickly removed from the perfusion apparatus and washed in isotonic saline. The ventricles were separated from the atria and great vessels, finely minced, and homogenized in five volumes of 300 mM sucrose and 10 mM imidazole (pH 7.0 at 4°C), by 15 + 15 passes in a Potter-Elvejheim homogenizer set at 800 r.p.m. and kept in a cold room at 4°C. The homogenates obtained from 3–4 hearts were pooled and used to prepare a microsomal fraction enriched in SR, according to the procedure described by Harigaya & Schwartz (1969), with minor modifications. The homogenate was centrifuged at 800 *g* for 10 min, in order to remove unfractionated cells and nuclei. The supernatant was then centrifuged at 8700 *g* for 20 min. The resulting supernatant was re-centrifuged at 8700 *g* for 20 min, yielding another supernatant fraction which was centrifuged at 40000 *g* for 30 min. The precipitate was resuspended in a glass homogenizer with 5 volumes of 600 mM KCl and 10 mM imidazole (pH 7.0 at 4°C). This suspension

was centrifuged at 40000 *g* for 25 min to remove solubilized actomyosin, and the precipitate, representing the microsomal fraction enriched in SR, was resuspended in homogenization buffer.

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin used as standard.

### *Assay of [$^3$H]-Ry equilibrium binding*

High affinity Ry binding was determined as described by Holmberg & Williams (1990), with minor changes. Unless otherwise specified, membranes (protein concentration 0.1–0.3 mg ml$^{-1}$) were incubated at 37°C for 120 min in a buffered medium (final volume 0.5 ml) containing 25 mM imidazole (pH 7.4 at 37°C), 1 M KCl, 0.2–50 nM [$^3$H]-Ry (6 Ci mmol$^{-1}$), 0.950 mM EGTA, 1.013 mM $CaCl_2$, and different concentrations of gallopamil where appropriate. Free Ca concentration was 20 μM. In some experiments, the $CaCl_2$ concentration was adjusted in order to obtain free Ca concentrations ranging from 0.1 μM to 1 mM.

In pilot experiments we observed that [$^3$H]-Ry binding was not modified by the inclusion of protease inhibitors in the binding buffer (5 μM leupeptin, 2 μM pepstatin, 0.25 μM aprotinin, 200 μM phenylmethyl sulphonyl fluoride, 2 mM iodacetamide, and 2 mM benzamide). Therefore, in subsequent assays such inhibitors were usually omitted.

The binding reaction was terminated by filtration through cellulose nitrate filters with pores of 0.45 μM (Sartorius, Göttingen, Germany) presoaked in 25 mM imidazole (pH 7.4) and 1 M KCl (washing buffer). The filters were washed with 2 × 5 ml aliquots of washing buffer, and shaken overnight in 8 ml of scintillation fluid (Optiphase II, LKB). Radioactivity was then measured at 50% efficiency in a LKB Wallac 1214 scintillation counter. Each vial was counted for 10 min. Incubations were performed in duplicate, and non-specific binding was determined in the presence of 10 μM Ry. The difference between the counts of duplicate samples was <10% in all cases.

In order to assess low affinity Ry binding, [$^3$H]-Ry was used at concentration ranging from 0.4 μM to 20 μM (120 Ci mol$^{-1}$), and non-specific binding was determined in the presence of 2 mM Ry. Filters were washed with 2 × 5 ml aliquots of washing buffer and 5 ml of 10% (W/V) ethanol. Careful analysis of filter binding in the absence of added membranes was performed at each concentration of [$^3$H]-Ry. Since we observed that filter bound [$^3$]-Ry was partly displaced by unlabelled Ry, specific binding was calculated as: ([$^3$H]-Ry bound in the presence of membranes − [$^3$H]-Ry bound in the presence of membranes and 2 mM unlabelled Ry) − ([$^3$H]-Ry bound in the absence of membranes − [$^3$H]-Ry bound in the absence of membranes and in the presence of 2 mM unlabelled Ry).

### *Measurement of association and dissociation kinetics*

[$^3$H]-Ry association was determined by incubating rat SR vesicles (0.15 mg/ml$^{-1}$) at 37°C with 4 nM or 1 μM [$^3$H]-Ry. The binding buffer was the same as described above. After appropriate incubation times (1–360 min), aliquots (50 μl) of reaction medium were filtered, washed, and counted as described above. The measurements were performed in the presence or absence of 2000 fold excess of unlabelled Ry, to measure non-specific binding, and/or different concentrations of gallopamil.

In dissociation studies, rat SR vesicles (1 mg ml$^{-1}$) were preincubated at 37°C for 120 min with 4 nM or 1 μM [$^3$H]-Ry in binding buffer. At the end of this period, 20 μl of the medium were filtered and counted to measure equilibrium binding. Dissociation was then initiated by adding 100 fold excess of Ry-free binding buffer. Aliquots (1 ml) were filtered at different time intervals (30 s to 360 min), and counted to assess residual binding. Where appropriate, unlabelled Ry and/or gallopamil were included in the buffer used to induce dissociation. Correction for non-specific binding was performed by repeating each experiment after the inclusion of 2000 fold excess unlabelled Ry during the 120 min of preincubation.

Association and dissociation experiments were repeated in the absence of SR vesicles to correct for filter binding. Such correction was performed both for total and for non-specific binding, i.e. both in the absence and in the presence of unlabelled Ry.

### *Chemicals and radionuclides*

[9,21-$^3$H(N)]-ryanodine (60 Ci mmol$^{-1}$) was obtained from New England Nuclear-Du Pont (Milan, Italy), and diluted to its final specific activity (see below) with unlabelled Ry. Unlabelled Ry was purchased from Calbiochem (San Diego, CA, U.S.A.), as a mixture of Ry and didehydroryanodine, and its concentration was checked on the basis of the absorbance at 268 nm, using an extinction coefficient of $1.45 \times 10^4$ M$^{-1}$. Such extinction coefficient was calculated by the manufacturer for the specific lot of Ry used in these experiments. EGTA was obtained from Sigma (St. Louis, MO, U.S.A.) and its alleged purity was 97%. Gallopamil hydrochloride was obtained from Knoll AG (Ludwigshaven, Germany). Gallopamil was initially dissolved in ethanol at 50 mM concentration. Control experiments showed that the amount of ethanol so introduced (final concentration: 2% with 1 mM gallopamil, <0.2% in all other cases) did not affect [$^3$H]-Ry binding. All other reagents were of analytical grade.

Free Ca concentration was calculated according to Fabiato & Fabiato (1979), using a computer programme which included an empirical correction for the ionic strength of the buffer.

### *Data analysis*

Equilibrium binding data were analyzed through the SCAFIT (LIGAND) programme (Munson, 1983). The analysis of kinetic experiments was performed as described by Weiland & Molinoff (1981). The observed association rate constant ($K_{obs}$) was calculated as the slope of the pseudo-first order plot of $\ln[B_e/(B_e-B)]$ vs time, where $B_e$ is the amount of radioligand bound at equilibrium. The pseudo-first order association rate constant ($K_1$) was calculated from the equation $K_{obs} = K_1(L \times B_{max}/B_e)$, where L is the concentration of free radioligand and $B_{max}$ is the concentration of binding sites, derived from equilibrium studies. The dissociation rate constant ($K_{-1}$) was calculated as the slope of the plot of $\ln(B/B_0)$ vs time, where $B_0$ is the amount of radioligand bound at time zero. Regression analysis and the comparison of regression coefficients were performed as described by Armitage (1971).

Dissociation curves were also interpolated by a double exponential (two site model), i.e. by the equation $B/B_0 = \alpha e^{-K't} + (1-\alpha) e^{-K't}$, where $K$ and $K'$ are the dissociation rate constants for the two sites, while $\alpha$ and $(1-\alpha)$ are their fractional densities. To assess whether the double exponential model provided a significantly better fit than the single exponential model, we used the 'extra sum of squares' principle (Draper & Smith, 1981; Munson, 1983).

Unless otherwise specified, when experiments were performed in duplicate, the difference between individual measurements was <10%.

## Results

### *Equilibrium studies: characterization of Ry binding*

The Scatchard plots derived from [$^3$H]-Ry saturation binding curves in the high (0.2–50 nM) and low (0.4–20 μM) affinity

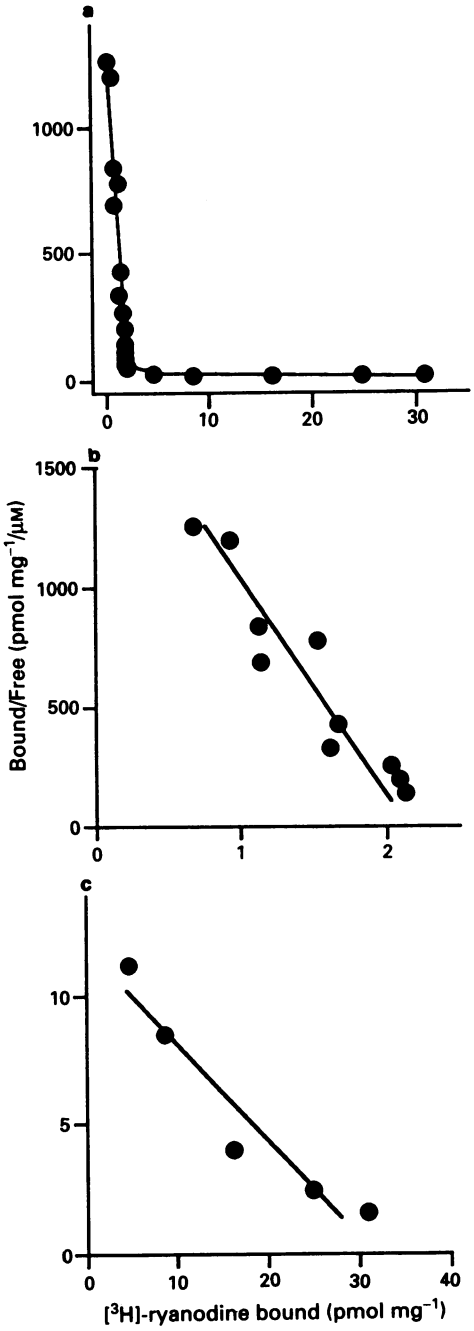


**Figure 1** Scatchard plots of [$^3$H]-ryanodine ([$^3$H]-Ry) saturation binding determined at pCa = 4.7 (free Ca = 20 μM). Data represent specific binding. [$^3$H]-Ry was used at concentrations ranging from 0.2 nM to 20 μM. See Methods for further details. (a) Overall view; (b) the nanomolar, ([$^3$H]-Ry = 0.2–50 nM) and (c) the micromolar ([$^3$H]-Ry = 0.4–20 μM) portions of the plot are reproduced on different scales. Data analysis gave the following estimates of the binding parameters: $K_{D1}$ = 1.3 nM, $K_{D2}$ = 2.3 μM, $B_{max1}$ = 2.3 pmol mg$^{-1}$, $B_{max2}$ = 32.4 pmol mg$^{-1}$. Note that in this experiment the incubation time was 60 min, and on the basis of the results of our association experiments (see below) binding may have been underestimated by 5–10%.

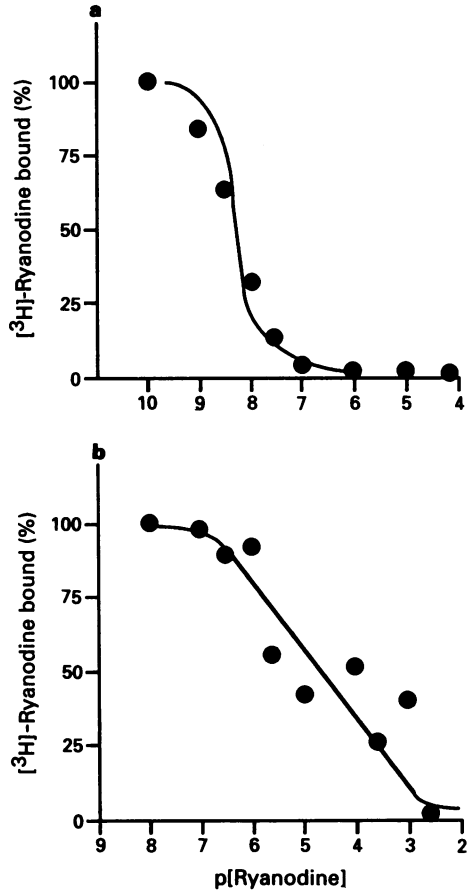


**Figure 2** Displacement of 4 nM (a) and 1 μM (b) [$^3$H]-ryanodine ([$^3$H]-Ry) by unlabelled Ry. Binding represents specific binding and is expressed as percentage of equilibrium binding. Data are the mean of two different experiments. Data analysis by the SCAFIT (LIGAND) programme identified two sites for [$^3$H]-Ry, characterized by $K_{i1}$ = 2.8 nM, $K_{i2}$ = 3.0 μM, $B_{max1}$ = 1.7 pmol mg$^{-1}$, $B_{max2}$ = 28.0 pmol mg$^{-1}$. The Hill numbers for the two sites were 0.96 and 0.36 respectively.

range are shown in Figure 1. Displacement curves of 4 nM (Figure 2a) and 1 μM [$^3$H]-Ry (Figure 2b) by unlabelled Ry are shown. In the nanomolar range, specific binding was >85% of total binding; about two thirds of non-specific binding were due to filter binding, and filter-bound radioactivity was not displaced by unlabelled Ry (displacement was <2%). In the micromolar range, specific binding was <50% of total binding (it was as low as 20–25% of total binding in the high micromolar range), and filter binding accounted for up to 40% of total binding. Both saturation and displacement experiments were best fitted by a two site model ($P$<0.001 vs the single site model). The combined analysis of equilibrium and displacement data by the SCAFIT (LIGAND) programme gave the following estimates: $K_{D1}$ = 1.3 ± 0.1 nM, $K_{D2}$ = 2.8 ± 0.5 μM, $B_{max1}$ = 2.2 ± 0.1 pmol mg$^{-1}$, $B_{max2}$ = 27.8 ± 3.2 pmol mg$^{-1}$. Models including more than 2 binding sites provided no further improvement in data fitting. The Hill number of high affinity binding was close to 1 (0.96 ± 0.05), while the Hill number of low affinity binding was significantly <1 (0.35 ± 0.02).

High affinity [$^3$H]-Ry binding was Ca-dependent: it was negligible at pCa ≥ 7, reached a maximum at pCa = 4.7 and decreased slightly at 3 ≤ pCa ≤ 4. No Ca-dependence was observed for low affinity binding. The latter was not affected by changes in Mg concentration (0.5 to 50 mM) or by the non-hydrolysable ATP analogue, β-γ methylene ATP in the range of 0 to 10 mM (data not shown).

### *Equilibrium studies: effect of gallopamil*

Gallopamil had no major effect on 4 nM [$^3$H]-Ry binding (Figure 3, triangles). This result was obtained at two different free Ca concentrations (pCa = 6 and 4.7). On the contrary, the binding of 100 nM to 3 μM [$^3$H]-Ry was displaced by gallopamil. The maximum displacement ranged from 40 to 65% (Figure 3a). The residual binding decreased at increas-

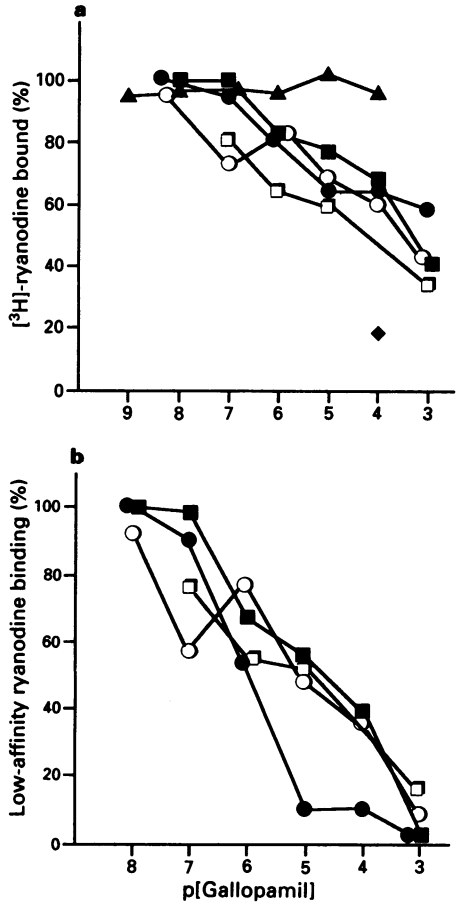


**Figure 3** Effect of gallopamil on [$^3$H]-ryanodine ([$^3$H]-Ry) binding. The experiments were performed with pCa = 4.7 at the following [$^3$H]-Ry concentrations: 4 nM (▲), 100 nM (●), 500 nM (■), 1 μM (○), 3 μM (□), 18 μM (◆). The effect of gallopamil on 4 nM [$^3$H]-Ry binding was also determined at pCa = 6, and no displacement was produced (data not shown). Each curve represents the mean of two different experiments, while the point obtained with 18 μM [$^3$H]-Ry was derived from a single experiment: (a) represents total specific binding, and (b) represents low affinity binding, i.e. residual binding after subtraction of the high affinity component, as estimated on the basis of saturation binding experiments. $IC_{50}$ values were: 1.4 μM, 15.9 μM, 5.0 μM, and 4.3 μM for the displacement of 100 nM, 500 nM, 1 μM, and 3 μM [$^3$H]-Ry respectively. The corresponding Hill number values were: 0.67, 0.27, 0.35, and 0.29 respectively.

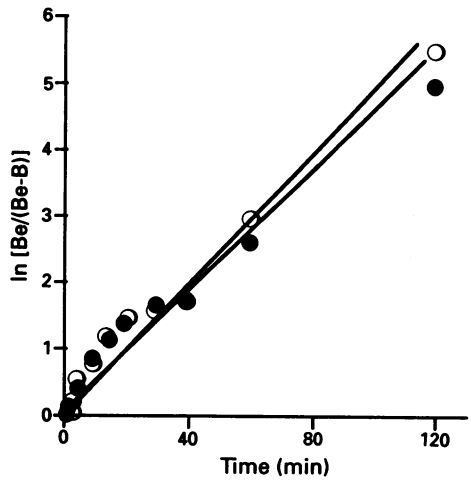


**Figure 4** Association of 4 nM [$^3$H]-ryanodine ([$^3$H]-Ry): pseudo-first order plot. (●) Conrol conditions; (○) results obtained after the addition of 100 μM gallopamil to the association buffer. The data were best fitted by single exponential, and the average values of the $K_{obs}$ and $K_1$ were $0.0393 \pm 0.0019\ min^{-1}$ and $0.00716 \pm 0.0004\ min^{-1}\ nM^{-1}$ respectively, without significant changes in the presence of gallopamil.

ing [$^3$H]-Ry concentrations and is likely to represent, at least in part, the high affinity component of [$^3$H]-Ry binding. This interpretation is supported by the observation that, in an additional experiment, 100 μM gallopamil produced 82% displacement of 18 μM [$^3$H]-Ry. In Figure 3b, displacement data were plotted after subtraction of high affinity [$^3$H]-Ry binding. Data analysis yielded a Hill number <1, and an $IC_{50}$ in the low micromolar range. There was no clear linear relationship between $IC_{50}$ and [$^3$H]-Ry concentration, so that no $K_{0.5}$ value could be calculated (Weiland & Molinoff, 1981).

### *Kinetic studies*

The association and dissociation of 4 nM [$^3$H]-Ry are shown in Figure 4 and 5. The analysis of association data (Figure 4, ●) yielded $K_{obs} = 0.0393\ min^{-1}$ and $K_1 = 0.00716\ min^{-1}\ nM^{-1}$, while the $K_{-1}$ derived from the analysis of Ry dissociation (Figure 5, ●) averaged $0.00978\ min^{-1}$. The kinetic $K_D$, calculated as $K_{-1}/K_1$, averaged 1.36 nM, in good agreement with the equilibrium $K_D$ obtained from the Scatchard analysis. Gallopamil (100 μM) did not affect either the association or the dissociation of 4 nM [$^3$H]-Ry (see Figures 4 and 5, ○).

As previously reported (McGrew *et al.*, 1989; Chu *et al.*, 1990; Pessah & Zimanyi, 1991), in the presence of 10 μM unlabelled Ry the dissociation of 4 nM [$^3$H]-Ry became biphasic, and a slow component ($K_{-1} = 0.00164\ min^{-1}$) appeared after a lag of about 60 min (Figure 5, ■). This effect of micromolar unlabelled Ry persisted in the presence of gallopamil, and the $K_{-1}$ of the slow component was further reduced in the presence of gallopamil (Figure 5, ▲), although dose-effect studies showed (Figure 6) that a significant action required high gallopamil concentration (100 μM).

The association and dissociation of 1 μM [$^3$H]-Ry are shown in Figure 7. Association data were best interpolated by a single exponential (Figure 7a), with $K_{obs} = 0.0297\ min^{-1}$. Owing to the existence of binding cooperativity and of two-step ligand-receptor interactions (see below), it was impossible to obtain a reliable estimate of the $K_1$ (Weiland & Molinoff, 1981).

Dissociation was not complete, and residual binding averaging 20–25% of total binding persisted even after 4 h. Such residual binding represented high affinity Ry binding, since the 100 fold dilution used to induce dissociation left a residual [$^3$H]-Ry concentration of 10 nM. Single-exponential analysis of the data after subtraction of the residual binding yielded a $K_{-1}$ of $0.155\ min^{-1}$. If the dissociation was followed with high temporal resolution in the first few minutes (up to 8 experimental points within the first 3 min) a very fast component could be identified (Figure 7b, ●), since a significantly better fit ($P<0.01$) was obtained with a double exponential model, whose dissociation rate constants averaged $2.267\ min^{-1}$ and $0.132\ min^{-1}$. The calculated site ratio was close to 1 (1:1.38).

In the presence of gallopamil, the dissociation of 1 μM [$^3$H]-Ry was delayed, although the residual binding was virtually unchanged. A plot of single-exponential $K_{-1}$ versus gallopamil concentration is shown in Figure 8. The $K_{-1}$ was significantly reduced at gallopamil concentration $\geq 1$ μM, and the effect was clearly dose-dependent. In an additional experiment (Figure 7b, ○), the dissociation of 1 μM [$^3$H]-Ry was followed at high temporal resolution in the presence of a saturating concentration of gallopamil (100 μM). Two components could still be observed in the dissociation curve ($P<0.01$ for the double-exponential versus the single-expo-

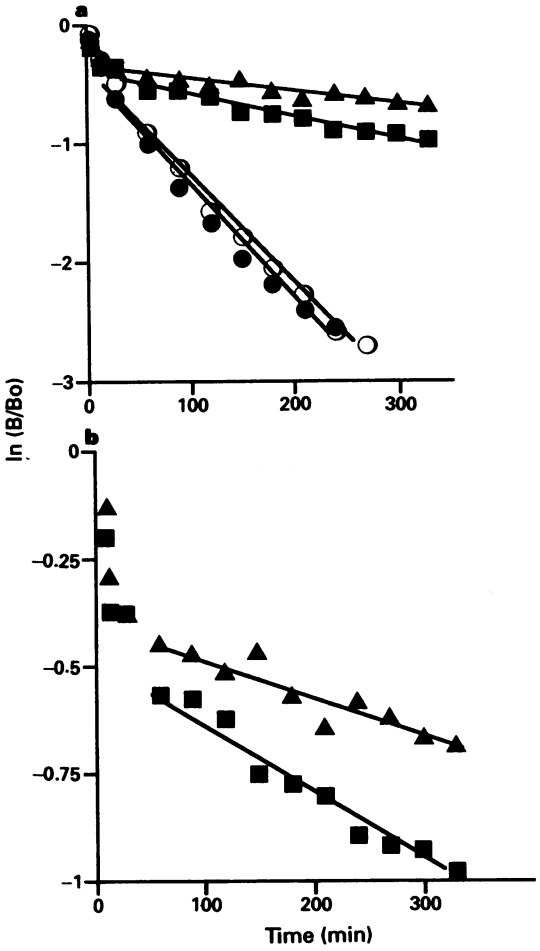


**Figure 5** (a) Dissociation of 4 nM [$^3$H]-ryanodine ([$^3$H]-Ry): first order plot. Each point is the mean of measurements performed in two different preparations: (●) control conditions, i.e. dissociation was induced by 100 fold dilution with Ry-free binding buffer; (○) data obtained after the addition of 100 μM gallopamil to the dissociation buffer; (■) dissociation was induced by 100 fold dilution with binding buffer containing 10 μM unlabelled Ry; (▲) dissociation was induced by 100 fold dilution with binding buffer containing 10 μM unlabelled Ry and 100 μM gallopamil. The calculated values of the $K_{-1}$ were $0.00978 \pm 0.00055\ min^{-1}$ in control conditions, and $0.00954 \pm 0.00039\ min^{-1}$ in the presence of 100 μM gallopamil. In the presence of 10 μM Ry a slow component appeared ($K_{-1} = 0.00164 \pm 0.00011\ min^{-1}$), and when 100 μM gallopamil was added to 10 μM Ry the $K_{-1}$ of this component decreased to $0.000898 \pm 0.00012\ min^{-1}$. Part of the figure is reproduced on a different scale in (b), in order to show the different slope observed with 10 μM Ry vs 10 μM Ry and 100 μM gallopamil.

nential fit), and both dissociation constants were reduced by gallopamil ($1.660\ min^{-1}$ and $0.047\ min^{-1}$).

## Discussion

Several lines of evidence suggest that phenylalkylamine Ca antagonists may interfere with SR Ca channels. In skeletal muscle, gallopamil inhibited excitation-contraction coupling after conditioning $K^+$ contracture, and this action was indepenent from sarcolemmal Ca channel blockade (Eisenberg *et al.*, 1983; Berwe *et al.*, 1987). Inhibition of SR Ca release by verapamil was observed in skeletal muscle SR (Fernandez-Belda & Gomez-Fernandez, 1987; Valdivia *et al.*, 1990). In heart muscle, we observed that gallopamil increased SR Ca uptake (Zucchi *et al.*, 1992); this action was not addictive with the stimulation produced by 300 μM Ry, and

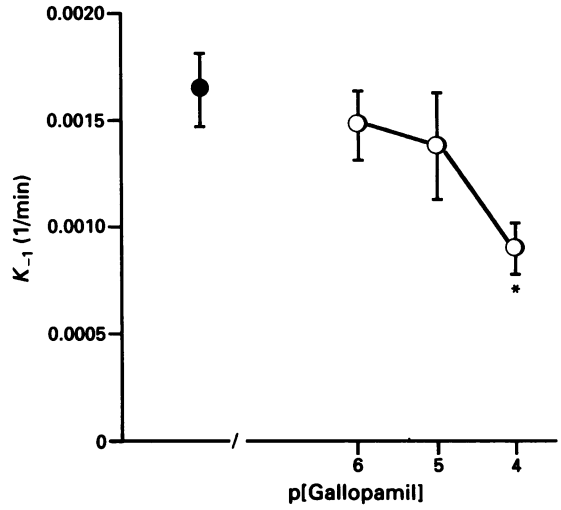


**Figure 6** Effect of gallopamil on the slow component of ryanodine (Ry) dissociation from its high affinity site ([$^3$H]-Ry concentration = 4 nM). The $K_{-1}$ of the slow component of the dissociation (see Figure 5) is plotted vs gallopamil concentration: (●) control condition, i.e. dissociation performed in the presence of 10 μM unlabelled Ry; (○) dissociation performed in the presence of 10 μM unlabelled and 1 to 100 μM gallopamil. S.e.mean was calculated by regression analysis (Armitage, 1971). $^*P<0.01$ vs control.

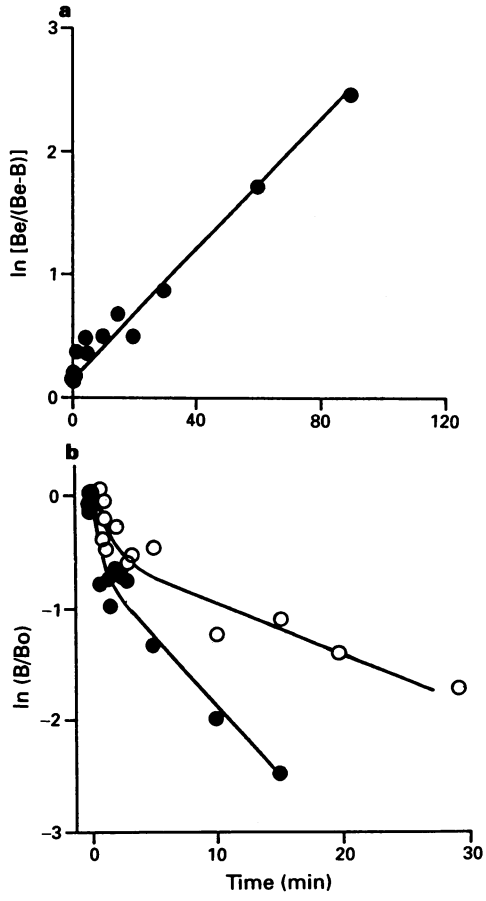


**Figure 7** (a) Pseudo-first order plot of 1 μM [$^3$H]-ryanodine ([$^3$H]-Ry) association. The $K_{obs}$ averaged $0.0297 \pm 0016\ min^{-1}$. (b) First order plot of 1 μM [$^3$H]-Ry dissociation. Each point is the mean of measurements performed in two different preparations: (●) control conditions; (○) data obtained after the addition of 100 μM gallopamil to the dissociation buffer. Single exponential analysis yielded $K_{-1} = 0.155\ min^{-1}$ in control conditions, and $K_{-1} = 0.057\ min^{-1}$ in the presence of gallopamil. A significantly better fit was obtained through a double exponential model, in which dissociation rate constants averaged: $2.267\ min^{-1}$ and $0.132\ min^{-1}$ in control conditions; $1.660\ min^{-1}$ and $0.047\ min^{-1}$ in the presence of gallopamil.

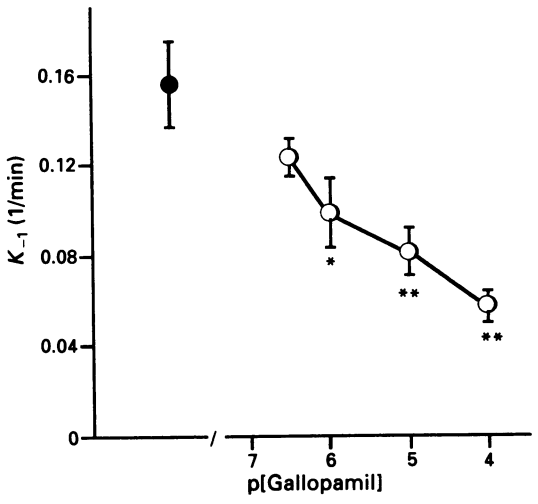


**Figure 8** Effect of gallopamil on the dissociation of ryanodine (Ry) from its low affinity site. The $K_{-1}$ of 1 μM [$^3$H]-Ry dissociation is plotted vs gallopamil concentration: (●) control conditions; (○) dissociation performed in the presence of 0.3 to 100 μM gallopamil. The $K_{-1}$ was calculated by single exponential analysis. These experiments were not projected to identify the very fast component of 1 μM [$^3$H]-Ry dissociation, which required high temporal resolution. High resolution data were obtained only in control conditions and in the presence of 100 μM gallopamil, and are shown in Figure 7. S.e.mean was calculated by regression analysis (Armitage, 1971). $^*P<0.05$, $^{**}P<0.01$ vs control.

Ca-ATPase activity was not affected, suggesting that the target of gallopamil was the Ca release channel.

The SR Ca channel corresponds to the Ry receptor (Inui *et al.*, 1987; Imagawa *et al.*, 1987), and pharmacological interventions able to modify channel function affect Ry binding (Pessah *et al.*, 1985; Meissner & Henderson, 1987; Michalak *et al.*, 1988; Abramson *et al.*, 1988; Holmberg & Williams, 1990; Chu *et al.*, 1990; Takasago *et al.*, 1991; Mack *et al.*, 1992). For these reasons we determined the effect of gallopamil on Ry binding in cardiac SR.

The standard model of the Ry receptor assumes that it includes 4 negatively cooperative oligomers, and that it undergoes a sequential alteration as a consequence of Ry binding. From the functional point of view, it is thought that Ry initially binds to the open state of the channel, and that the sequential alteration corresponds to channel blockade (Pessah & Zimanyi, 1991; Buck *et al.*, 1992; Zimanyi *et al.*, 1992).

In the equilibrium studies, we obtained a curvilinear Scatchard plot, and identified two apparent binding sites, with $K_D$ in the order of 1 nM and 2 μM respectively. The Hill number calculated from displacement studies was close to 1 for the high affinity site, and <1 for the low affinity site. These findings are in agreement with previous reports (Inui *et al.*, 1987; 1988; Lai *et al.*, 1989; McGrew *et al.*, 1989; Pessah & Zimanyi, 1991), and are consistent with negative allosteric coupling between identical binding sites. As already reported by Lai *et al.* (1989) in the skeletal muscle, $Ca^{2+}$ concentration appeared to affect high but not low affinity binding.

Pessah & Zimanyi (1991) were able to detect 4 different binding sites. In our experiments no improvement in data fitting was obtained with models including more than two binding sites. However, the existence of additional sites with intermediate affinity cannot be excluded, since we could determine only a limited number of binding points, which were selected to identify the highest and lowest affinity sites. It has been argued that up to 50 binding points may be necessary for a reliable comparison of two and three site models (Molinoff *et al.*, 1981). The biexponential character of 1 μM Ry dissociation and the low Hill number calculated from 1 μM Ry displacement actually suggest the existence of at least two low affinity sites.

Although the tetramer model of the Ry receptor predicts a value of 3 for the ratio of low to high affinity sites (Lai *et al.*, 1988; 1989), values in the range of 10 to 50 were obtained in this study as well as by most other investigators, both in heart (Inui *et al.*, 1987; 1988; McGrew *et al.*, 1989; Pessah & Zimanyi, 1991) and in skeletal muscle (McGrew *et al.*, 1989; Pessah & Zimanyi, 1991; Buck *et al.*, 1992). It must be stressed that the accuracy of any estimate of low affinity site density is rather low, owing to the technical problems related to the binding assay.

Negative cooperativity and/or the existence of a two-step reaction can account for the increase in $K_{-1}$ and the decrease in $K_{obs}$ observed with micromolar vs nanomolar Ry. In particular, negative cooperativity is strongly suggested by the biexponential nature of the dissociation of 1 μM Ry, both dissociation constants being higher than the $K_{-1}$ obtained with 4 nM Ry. On the other side, a two-step reaction mechanism is necessary to explain the appearance of a slow component in the dissociation of 4 nM Ry when micromolar Ry was included in the dissociation buffer (Pessah & Zimanyi, 1991; Zimanyi *et al.*, 1992).

The kinetic constants which we obtained with 4 nM Ry are in close agreement with those reported in heart tissue by McGrew *et al.* (1989) and by Pessah & Zimanyi (1991), who used 9 nM and 1 nM Ry respectively. The $K_{-1}$ and $K_{obs}$ measured with 1 μM Ry are much different (respectively 55 times higher and 20 times lower) from the values determined by Pessah & Zimanyi in the skeletal muscle, although values of $K_{obs}$ close to ours were obtained by Buck *et al.* (1992). This discrepancy might be due to differences in the sequential alteration of the Ry receptor between skeletal muscle and cardiac tissue. In particular, Pessah & Ziymani obtained a lower $K_{-1}$ with 1 μM vs 50 nM Ry, contrary to the predictions of their negative cooperative model, and speculated that under their experimental conditions (which included 3 h of incubation) sequential alteration of the Ry receptor might have occurred with 1 μM Ry.

Gallopamil did not affect high affinity Ry binding to cardiac SR, consistent with previous results (Pessah *et al.*, 1985). Valdivia *et al.* (1990) reported that in skeletal muscle SR, verapamil was able to displace 5 nM Ry. However, significant displacement was observed only under peculiar conditions, i.e. alkaline pH (8.5) and ionic strength much lower than required for optimal binding (150 mM NaCl). The pH of the buffer was critical, and at neutral pH Ry displacement by gallopamil did not exceed 20%.

The effect of Ca antagonists on low affinity Ry binding had never been investigated. Our results showed that gallopamil displaced Ry from its low affinity site(s). The Hill number calculated from the displacement experiments was <1, and no clear linear relationship was observed between [$^3$H]-Ry concentration and $IC_{50}$, suggesting that the action of gallopamil is complex, and cannot be accounted for by simple competitive inhibition.

Kinetic studies showed that the dissociation rate constants of 1 μM Ry decreased in the presence of gallopamil, while the dissociation of 4 nM Ry was not affected. It is not easy to understand how gallopamil can displace Ry and yet slow its dissociation. A possible interpretation holds that the negative cooperativity of Ry binding may be reduced in the presence of gallopamil. However, the action of gallopamil must be more complex, since we also observed that gallopamil and 10 μM Ry were more effective that 10 μM Ry alone in introducing a slow component in the dissociation of 4 nM Ry, suggesting that gallopamil might also influence the transition to a receptor state characterized by slow Ry dissociation.

In summary, the interaction of gallopamil with the Ry receptor might produce multiple effects: (a) partial displacement of Ry from its low affinity site(s); (b) modulation of the cooperativity of Ry binding and of the sequential alteration of the receptor produced by Ry binding.

The precise site of action of gallopamil remains to be determined. Gallopamil might interact directly with the Ry receptor (Valdivia *et al.*, 1990), but it cannot be excluded that its effect may be mediated by proteins closely coupled to the receptor, such as the FK605 binding protein (Jayaraman *et al.*, 1992), or even the dyhydropyridine receptor itself (Block *et al.*, 1988). Evidence of an interaction between Ry and the dyhydropyridine receptor was reported by Valdivia & Coronado (1989), who observed that dyhydropyridine binding to skeletal muscle membranes was displaced by high concentrations of Ry ($IC_{50} = 95\,\mu M$).

In any case, it seems likely that this is the molecular basis for the pharmacological actions which have been observed in cardiac and skeletal muscle SR, i.e. inhibition of SR Ca release (Fernandez-Belda & Gomez-Fernandez, 1987; Valdivia *et al.*, 1990; Zucchi *et al.*, 1992). The comparison of dose-effect relationships obtained in binding and Ca-flux studies is not easy. In the present work, the half-effective gallopamil concentration, as calculated from equilibrium and dissociation experiments, was in the micromolar range. In the Ca-flux studies, phenylalkylamines were found to be effective at concentrations ranging from $10^{-7}$ to $10^{-5}$ M, which could be consistent with the binding data, but the dose-response relationship was bell-shaped. In particular, in bovine cardiac SR the maximum effect was observed with $0.1\,\mu M$ gallopamil and concentrations $> 1\,\mu M$ were ineffective (Zucchi *et al.*, 1992), while in frog skeletal muscle Berwe *et al.* (1987) reported that micromolar gallopamil inhibited excitation-contraction coupling, but $250\,\mu M$ gallopamil had the opposite effect.

Our results give no hint to explain such findings. It should be remembered that, in general, the pharmacological action of a channel modulator cannot be predicted on the basis of its effect on ligand binding. As a tentative interpretation of the above results, we might speculate that low concentrations of gallopamil stabilize an inactive state of the SR channel, while higher concentrations stabilize the active state as well (Berwe *et al.*, 1987). However, the evaluation of this hypothesis requires further investigation.

The mechanism of action of gallopamil is different from that of the SR channel blockers ruthenium red, neomycin or FLA 365. While all these substances inhibit high affinity Ry binding (Mack *et al.*, 1992), gallopamil displaced Ry only from its low affinity sites(s).

Since gallopamil can cross the sarcolemma (Hescheler *et al.*, 1982) and displaces Ry at concentrations which lie within the range that can be achieved in human plasma (Nayler, 1988), its action on the Ry receptor might have clinical importance. In particular, it is though that Ca release from the SR is a crucial issue in the pathogenesis of irreversible myocardial injury during ischaemia and reperfusion. Drugs able to interfere with this structure might therefore open new possibilities in the treatment of ischaemic heart disease.

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# Acute versus chronic administration of phosphodiesterase inhibitors on allergen-induced pulmonary cell influx in sensitized guinea-pigs

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**1** The aims of this study were to determine which phosphodiesterase (PDE) isoenzymes are involved in the control of eosinophil accumulation in the airways of ovalbumin (OVA)-immunized guinea-pigs by the use of isoenzyme selective inhibitors and to compare the effects of acute versus chronic administration of PDE isozyme inhibitors on pulmonary cell influx in ovalbumin-immunized guinea-pigs.

**2** Guinea-pigs were sensitized and subsequently challenged with aerosolized OVA. Twenty four hours later bronchoalveolar lavage (BAL) was performed to permit assessment of inflammatory cell accumulation. A significant increase in the number of eosinophils was observed in the lavage fluid from OVA-immunized ($13.6 \pm 1.4 \times 10^4$ ml$^{-1}$ in acute experiments and $10.1 \pm 1.4 \times 10^4$ ml$^{-1}$ in chronic experiments) animals compared with sham-treated controls ($5.6 \pm 0.6 \times 10^4$ ml$^{-1}$ in acute experiments and $5.1 \pm 0.6 \times 10^4$ ml$^{-1}$ in chronic experiments). There was no difference in neutrophil, mononuclear cell or total cell numbers between the two groups.

**3** Acute administration of a high dose of selective and non-selective PDE inhibitors by the i.p. route had no significant effect on eosinophil accumulation in the airways.

**4** Chronic administration of a low dose (3 mg kg$^{-1}$, i.p., twice daily for 7 days) of the type IV PDE inhibitor, RO 20-1724, and the PDE III/IV inhibitor, zardaverine, produced a significant inhibition of eosinophil accumulation (46% and 59% respectively).

**5** These results suggest that the type IV PDE isoenzyme plays a role in the control of allergen-induced eosinophil infiltration into the airways, but indicate that a period of low dose chronic treatment with a type IV or mixed type III/IV PDE inhibitor is necessary for eosinophil accumulation in the airways to be reduced.

**Keywords:** Sensitized guinea-pig; cyclic nucleotide phosphodiesterase isoenzymes, isoenzyme selective inhibitors

## Introduction

Xanthines have been used in the treatment of bronchial asthma for many years with their primary therapeutic activity initially considered to be through bronchodilatation (Weinburger, 1980; Persson, 1986). Recent evidence suggests that theophylline, the most widely prescribed xanthine, may derive its therapeutic activity in asthma from anti-inflammatory effects (Pauwels, 1987; Ward *et al.*, 1993; Sullivan *et al.*, 1994). The second messengers adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) regulate the function of several cell types involved in the pathophysiology of asthma, including inflammatory cells, and there is much evidence to suggest that elevation of cyclic AMP levels suppresses the activities of these cells (Torphy & Undem, 1991; Giembycz & Dent, 1992). Therefore, the cellular mechanism underlying the putative anti-inflammatory activity of theophylline may be related to the ability of this drug to inhibit the cyclic AMP and cyclic GMP regulatory enzyme, phosphodiesterase.

Currently there are at least seven different PDE isoenzyme families with specific and selective inhibitors (Nicholson *et al.*, 1991; Giembycz, 1994). Specific PDE isoenzymes are differentially regulated and expressed (Dent *et al.*, 1991); the PDE IV isoenzyme is the major isoenzyme in many inflammatory cells although PDE III predominates in platelets and is also found together with PDE IV in T-lymphocytes (Robicsek *et al.*, 1991). Drugs capable of selectively inhibiting specific PDE isoenzymes can be expected to be more useful therapeutically than non-selective PDE inhibitors because of a lower expected systemic side effect profile.

Eosinophils are the predominant inflammatory cell type in the airways of asthmatics and there is strong evidence that they play a critical role in the pathogenesis of bronchial asthma (Wardlaw & Kay, 1987). The number of eosinophils found in the airways of asthmatics correlates with the disease severity (Horn *et al.*, 1975). Activated eosinophils are able to synthesize and release toxic and pro-inflammatory mediators in response to specific stimuli, (Frigas & Gleich, 1986) and thus one possible therapeutic strategy for the therapy of asthma would be to target the mechanisms involved in the control of eosinophil accumulation and activation in the airways. There is evidence that PDE and cyclic AMP play a pivotal role in the accumulation and activation of eosinophils (Yukawa *et al.*, 1989).

Acute administration of theophylline and selective PDE isoenzyme inhibitors has been reported to reduce eosinophil infiltration induced by allergen challenge in sensitized guinea-pigs (Gristwood *et al.*, 1991; Schudt *et al.*, 1991; Manzini *et al.*, 1993; Underwood *et al.*, 1993). However, the majority of studies which have examined the effect of theophylline and selective isoenzyme inhibitors on eosinophil accumulation are not relevant to the clinical situation for two reasons; firstly the doses which have been used in these studies have been given as one dose acutely, prior to antigen challenge, and secondly because the doses which have been employed are much higher than those used clinically.

The aims of the present study have been firstly to try to determine which PDE isoenzymes are important in regulating eosinophil accumulation in the airways by utilising selective isoenzyme inhibitors, and secondly to compare the effects of acute high dose administration of isoenzyme selective inhibitors with that of more clinically relevant chronic low dose treatment for 7 days.

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Part of this work has been presented to the British Pharmacological Society (Banner & Page, 1994a).

## Methods

### *Sensitization and challenge procedure*

Male Dunkin Hartley guinea-pigs (Charles River) (200–250 g) were injected i.p. with ovalbumin (OVA) (0.5 ml per animal; 20 μg OVA in $Al(OH)_3$ (moist gel)); this preparation produced an injectable stable suspension containing excess $Al(OH)_3$. Sham animals were injected with 0.5 ml $Al(OH)_3$ alone. After a period of 18–21 days, animals were exposed to an aerosol of OVA (100 μg $ml^{-1}$) for 1 h in an exposure chamber. The rate of flow of ovalbumin in the chamber was approximately 10 ml $h^{-1}$.

### *Bronchoalveolar lavage*

Animals were anaesthetized, 24 h after aerosol exposure, with urethane (25%, w/v, 7 ml $kg^{-1}$, i.p.) and the trachea cannulated. BAL was performed by instilling 5 ml sterile saline into the lungs via the tracheal cannula and the fluid was immediately removed. The same fluid was then re-injected and the procedure repeated 5 times in total. This procedure resulted in a 40–60% recovery of BAL fluid from the lungs of the guinea-pig. Total cell counts were performed on the resultant BAL fluid using an improved Neubauer haemocytometer. Cytospin preparations were prepared in a Shandon Cytospin 2 centrifuge: 100 μl of BAL fluid was added to each cytospin cup and the samples were centrifuged for 1 min at 1300 r.p.m. Slides were fixed in acetone and stained with haematoxylin and carbol chromotrope according to the method described by Lendrum (1944). Differential cell counts were performed on each slide by counting 200 cells at random, the cell types were classified as neutrophils, eosinophils and mononuclear cells according to standard morphological criteria. Cells were counted 'blind'. The results are expressed as the number of neutrophils, eosinophils and mononuclear cells per ml of BAL fluid. The remaining BAL fluid was centrifuged (10 min, 1000 *g*) and the resultant cells and cell free supernatants were divided into aliquots and frozen for later assays.

### *Acute dosing protocol*

Eighteen to twenty one days after immunization, guinea-pigs were given one i.p. injection of a PDE inhibitor [vinpocetine (PDE I inhibitor), SK&F 94836 (PDE III inhibitor), RO 20-1724 (PDE IV inhibitor), zardaverine (mixed type III/IV inhibitor), zaprinast (PDE V inhibitor), 3-isobutyl-1-methylxanthine (IBMX) and theophylline (non-selective PDE inhibitors)] or the appropriate vehicles 1 h prior to OVA exposure in the chamber. In a similar manner sham-treated animals were given a single i.p. injection of the appropriate vehicle 1 h prior to antigen, OVA, challenge.

All drugs were dissolved in dimethylsulphoxide (DMSO) except IBMX which was dissolved in saline. PDE inhibitors were administered at a dose of 30 mg $kg^{-1}$ except for IBMX which was given at doses of 250 μg $kg^{-1}$, 500 μg $kg^{-1}$, 1 mg $kg^{-1}$ and 2 mg $kg^{-1}$ due to the toxicity of this drug at higher doses. Bronchoalveolar lavage was performed 18–24 h later after exposure to OVA according to the method described above. Total and differential cell counts were then performed on BAL fluid.

### *Multiple dosing protocol*

Eleven to fourteen days after immunization animals were injected i.p. twice daily with 3 mg $kg^{-1}$ of one of the PDE inhibitors used in the acute studies or the appropriate vehicle, which was DMSO, for 7 days. This dosing regime approximates to recent clinical studies demonstrating anti-inflammatory activity of theophylline in asthmatics. A group of sham-treated animals were injected with the vehicle, DMSO, in the same manner. On day 7, 1 h after a final injection, all animals were exposed to an aerosol of OVA as described above in an exposure chamber for 1 h. BAL was performed 18–24 h later and total and differential cell counts conducted on the BAL fluid recovered.

### *Statistical tests*

Data are expressed as mean ± s.e.mean. Each group of animals treated with a specific drug was compared with a time-matched control group by unpaired *t* test. For simplicity, control groups have been combined for purposes of data presentation.

### *Drugs*

The following drugs were used: vinpocetine (Gedeon Richter Ltd, Budapest, Hungary), SK&F 94836 (6-[4-(N2-methyl-N3-cyanoguanidino) phenyl]-4,5-dihydro-5-methylpyridazin-3 (2H)-one) (SmithKline Beecham Pharmaceuticals Inc., King of Prussia, PA, U.S.A), RO 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) (Calbiochem), zardaverine (B 842-90) (Forschungslaboratorien Byk Gulden, Germany), zaprinast (M&B 22,948) (Rhone-Poulenc Rorer, Dagenham, Essex), 3-isobutyl-1-methyl xanthine (IBMX), theophylline, chicken egg ovalbumin (Grade V), chromotrope 2R, Erhlichs Haematoxylin, Brilliant blue, urethane (Sigma, Poole, Dorset), aluminium hydroxide gel (BDH Chemicals, Poole, Dorset).

## Results

### *Acute experiments*

The results from the acute studies are shown in Table 1. Total and differential cell counts were performed on the BAL fluid recovered from animals post antigen, OVA, challenge. There was no significant difference between the total numbers of cells $ml^{-1}$ found in the BAL fluid of sham-treated and OVA-immunized animals ($35.5 \pm 2.5 \times 10^4$ cells $ml^{-1}$ and $42.3 \pm 2.6 \times 10^4$ cells $ml^{-1}$ respectively). Of the total cells counted the number of neutrophils and mononuclear cells recovered from immunized animals ($1.4 \pm 0.2 \times 10^4$ cells $ml^{-1}$ and $27.3 \pm 2.2 \times 10^4$ cells $ml^{-1}$ respectively) was not significantly different from the numbers of neutrophils and mononuclear cells ($2.4 \pm 0.6 \times 10^4$ cells $ml^{-1}$ and $27.5 \pm 2.2 \times 10^4$ cells $ml^{-1}$ respectively) counted in the BAL fluid from sham-treated animals. However a significant increase in the number of eosinophils ($P < 0.05$) in the BAL fluid from OVA immunized animals ($13.6 \pm 1.4 \times 10^4$ cells $ml^{-1}$) was seen compared with the number of eosinophils recovered from the BAL fluid from sham-treated animals ($5.6 \pm 0.6 \times 10^4$ cells $ml^{-1}$).

None of the PDE inhibitors examined had any significant effect on the total numbers of cell $ml^{-1}$ recovered from BAL fluid, post challenge, compared with the appropriate time-matched immunized vehicle-treated group of animals (Table 1). There was also no significant difference in the relative numbers of eosinophils (Figure 1), neutrophils or mononuclear cells obtained from the drug-treated animals compared with the immunized vehicle-treated group.

### *Chronic experiments*

The results from the chronic studies are shown in Table 2. Total and differential cell counts were performed on the BAL fluid recovered from animals post antigen challenge. There was no significant difference in the total number of cells $ml^{-1}$ found in the BAL fluid of sham-treated and OVA-immunized

**Table 1** Effect of PDE inhibitors administered 1 h before challenge on inflammatory cell composition in bronchoalveolar lavage fluid

| *Treatment* | n | *Total cells* | *Neutrophils* | *Eosinophils* | *Mononuclear cells* |
|---|---|---|---|---|---|
| Sham | 28 | 35.5 ± 2.5 | 2.4 ± 0.6 | 5.6 ± 0.6 | 27.5 ± 2.2 |
| Control | 33 | 42.3 ± 2.6 | 1.4 ± 0.2 | 13.6 ± 1.4 | 27.3 ± 2.2 |
| Vinpocetine | 12 | 48.0 ± 4.3 | 1.5 ± 0.3 | 16.0 ± 3.2 | 30.5 ± 3.2 |
| SK&F 94836 | 10 | 28.5 ± 1.8 | 0.8 ± 0.4 | 11.4 ± 1.8 | 16.3 ± 0.9 |
| RO 20-1742 | 11 | 53.1 ± 7.9 | 3.3 ± 1.6 | 20.4 ± 2.5 | 29.4 ± 5.1 |
| Zardaverine | 9 | 41.2 ± 6.1 | 6.7 ± 5.1 | 12.5 ± 3.3 | 22.1 ± 2.3 |
| Zaprinast | 11 | 44.5 ± 4.9 | 2.7 ± 0.7 | 14.5 ± 3.6 | 27.3 ± 4.5 |
| Theophylline | 13 | 53.2 ± 5.7 | 1.8 ± 0.6 | 11.7 ± 2.6 | 39.7 ± 3.9 |
| Sham saline | 7 | 33.9 ± 6.7 | 2.6 ± 1.1 | 4.0 ± 1.2 | 27.3 ± 5.6 |
| Control saline | 10 | 39.2 ± 4.1 | 2.3 ± 0.7 | 14.3 ± 3.8 | 22.6 ± 4.5 |
| IBMX (2 mg kg$^{-1}$) | 7 | 19.6 ± 2.5 | 0.9 ± 0.3 | 9.0 ± 2.1 | 9.7 ± 1.0 |
| IBMX (1 mg kg$^{-1}$) | 8 | 42.8 ± 4.3 | 2.4 ± 1.1 | 9.9 ± 1.2 | 30.5 ± 4.5 |
| IBMX (500 μg kg$^{-1}$) | 10 | 44.5 ± 7.9 | 2.0 ± 0.5 | 12.8 ± 3.2 | 29.7 ± 5.6 |
| IBMX (250 μg kg$^{-1}$) | 6 | 57.3 ± 9.4 | 2.2 ± 0.8 | 17.3 ± 5.7 | 37.8 ± 5.6 |

Values are expressed as $10^4$ cells ml$^{-1}$ (mean ± s.e.mean).
Doses of PDE inhibitors are 30 mg kg$^{-1}$ except IBMX which was administered at the doses indicated.

**Table 2** Effect of chronic treatment with PDE inhibitors for 7 days on inflammatory cell composition in the airways

| *Treatment* | n | *Total cells* | *Neutrophils* | *Eosinophils* | *Mononuclear cells* |
|---|---|---|---|---|---|
| Sham | 31 | 38.4 ± 2.0 | 2.0 ± 0.5 | 5.1 ± 0.6 | 31.3 ± 1.8 |
| Control | 33 | 43.7 ± 3.1 | 3.4 ± 1.2 | 10.1 ± 1.4 | 30.2 ± 2.0 |
| Vinpocetine | 9 | 40.0 ± 4.2 | 2.1 ± 0.8 | 10.2 ± 1.5 | 27.7 ± 3.8 |
| SK&F 94836 | 11 | 38.3 ± 5.0 | 4.3 ± 2.3 | 8.0 ± 1.6 | 26.0 ± 3.0 |
| RO-20 1724 | 9 | 37.4 ± 4.7 | 2.3 ± 1.9 | *5.5 ± 1.3 | 29.6 ± 4.0 |
| Zardaverine | 12 | *23.7 ± 3.0 | 2.1 ± 0.7 | *4.1 ± 1.4 | *17.5 ± 3.2 |
| Zaprinast | 10 | 45.3 ± 7.6 | 2.8 ± 0.8 | 10.2 ± 3.0 | 32.3 ± 4.8 |
| Theophylline | 13 | 36.2 ± 5.4 | 2.5 ± 1.4 | 6.5 ± 1.5 | 27.2 ± 5.6 |
| IBMX | 12 | 43.9 ± 4.3 | 1.5 ± 0.5 | 9.2 ± 1.9 | 33.2 ± 4.1 |

*$P < 0.05$ versus control.
Values are expressed as $10^4$ cells ml$^{-1}$ (mean ± s.e.mean). PDE inhibitors were administered at a dose of 3 mg kg$^{-1}$ i.p. twice daily for 7 days.

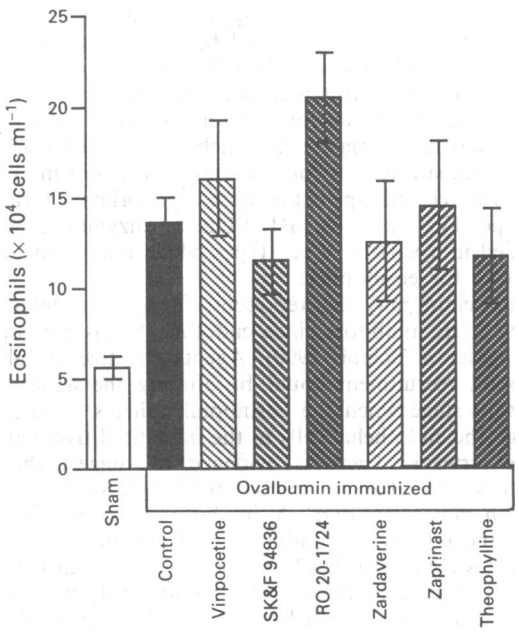


**Figure 1** Effect of acute treatment with PDE inhibitors on antigen-induced eosinophil accumulation in the bronchoalveolar lavage (BAL) fluid of immunized guinea-pigs. Values are mean ± s.e.mean. Shading of histograms is designed to correspond with drug specificity of action on PDE isoenzymes. *$P < 0.05$ vs time-matched controls (note that all time matched control groups have been combined for simplicity of graphical presentation).

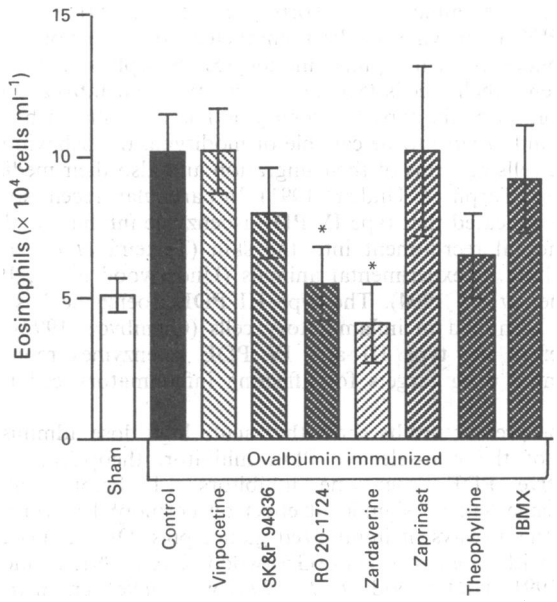


**Figure 2** Effect of chronic treatment with PDE inhibitors on antigen-induced eosinophil accumulation in the bronchoalveolar lavage (BAL) fluid of immunized guinea-pig. Values are mean ± s.e.mean. Shading of histograms is designed to correspond with drug specificity of action on PDE isoenzymes. *$P < 0.05$ vs time-matched controls (note that all time matched control groups have been combined for simplicity of graphical presentation).

animals ($38.4 \pm 2.0 \times 10^4$ cells $ml^{-1}$ and $43.7 \pm 3.1 \times 10^4$ cells $ml^{-1}$ respectively). Of the total cells counted the numbers of neutrophils ($3.4 \pm 1.2 \times 10^4$ cells $ml^{-1}$) and mononuclear cells ($30.2 \pm 2.0 \times 10^4$ cells $ml^{-1}$) in the BAL fluid from immunized animals post challenge was not significantly different from the numbers of neutrophils ($2.0 \pm 0.5 \times 10^4$ cells $ml^{-1}$) and mononuclear cells ($31.3 \pm 1.8 \times 10^4$ cells $ml^{-1}$) recovered from the BAL fluid from sham-treated animals. However, a significant increase ($P<0.05$) in the number of eosinophils in BAL fluid of OVA-sensitized animals ($10.1 \pm 1.4 \times 10^4$ cells $ml^{-1}$) was seen compared with the number of eosinophils found in the BAL fluid from sham-treated animals ($5.1 \pm 0.6 \times 10^4$ cells $ml^{-1}$).

In contrast to the results obtained from the acute experiments, twice daily treatment with the type IV PDE inhibitor, RO 20-1724 (3 mg $kg^{-1}$) for 7 days resulted in a significant reduction in the numbers of eosinophils recovered ($5.5 \pm 1.3 \times 10^4$ cells $ml^{-1}$) compared with the immunized vehicle-treated group (Figure 2).

Also, in contrast to the effect of zardaverine administered acutely at a dose of 30 mg $kg^{-1}$, this mixed type III/IV PDE inhibitor when given twice daily for 7 days, at a dose of 3 mg $kg^{-1}$, produced a significant inhibition in the total number of cells recovered ($23.7 \pm 3.0 \times 10^4$ cells $ml^{-1}$). This inhibition in cell numbers was reflected in a significant reduction of eosinophils ($4.1 \pm 1.4 \times 10^4$ cells $ml^{-1}$) (Figure 2) and mononuclear cells ($17.5 \pm 3.2 \times 10^4$ cells $ml^{-1}$) whilst there was no change in the numbers of neutrophils in the BAL fluid compared with the immunized vehicle-treated animals.

None of the other PDE inhibitors examined had any significant effect on either the total numbers of cells $ml^{-1}$ or on the numbers of neutrophils, eosinophils and mononuclear cells recovered from BAL fluid compared with their time-matched immunized vehicle-treated groups.

## Discussion

Elevation of cellular cyclic nucleotide levels via the inhibition of PDE can inhibit both the activation and chemotaxis of a variety of immune and inflammatory cells including lymphocytes, monocytes and eosinophils (Thompson *et al.*, 1976; Averill & Kammer, 1985; Torphy & Undem, 1991). The type IV PDE isoenzyme has been characterized in eosinophils, T-lymphocytes, neutrophils, monocytes, basophils, mast cells and endothelial cells (Souness *et al.*, 1991; Giembycz, 1992). Inhibition of the type IV isoenzyme has been shown both *in vivo* and *in vitro* to be capable of modifying the behaviour of these cells in terms of their migration and also their mediator release (Torphy & Undem, 1991). In particular, recent studies have indicated that type IV PDE isoenzyme inhibitors inhibit eosinophil recruitment into the skin (Teixeira *et al.*, 1994) and lung of experimental animals (Underwood *et al.*, 1993; Turner *et al.*, 1994). The type III PDE isoenzyme has also been identified in inflammatory cells (Giembycz, 1992) and therefore the type III and IV PDE isoenzymes represent potential drug targets for affecting inflammatory cell function.

The present results show that acute high dose administration of the non-selective PDE inhibitor, theophylline and selective PDE isoenzyme inhibitors 1 h before antigen challenge had no significant effect on eosinophil recruitment into the airways of immunized guinea-pigs. Our results contrast with those of others (Gristwood *et al.*, 1991; Schudt *et al.*, 1991; Underwood *et al.*, 1993) who have demonstrated inhibitory effects on eosinophil accumulation by acute administration of PDE inhibitors. However, such differences may be attributable to differences in methodology between these studies and ours. Some of these studies have used higher doses than those used in our study and have also given PDE inhibitors prior to and following antigen challenge. For example the study conducted by Gristwood and co-workers (1991) gave two injections of theophylline (50 mg $kg^{-1}$) orally, the first 2 h before and the second 6 h after antigen challenge. Different routes of administration have also been employed by other groups and Underwood and co-workers (1993) found that rolipram administered at a dose of 10 mg $kg^{-1}$ i.g. was effective in inhibiting eosinophil numbers in BAL fluid. However, we are not aware of any studies which have used the same immunization schedule as we have and given a single i.p. injection of a PDE IV inhibitor only 1 h before antigen challenge which have been able to demonstrate an inhibitory effect on eosinophil accumulation in the airways.

In contrast to the findings with acute administration of PDE inhibitors, zardaverine and RO 20-1724 when administered at a low dose chronically produced a significant inhibition of eosinophil accumulation in the airways. Zardaverine also reduced the numbers of mononuclear cells present in the airways. However, the type III PDE isoenzyme inhibitor, SK&F 94836, failed to inhibit eosinophil infiltration.

Zardaverine inhibits the type III and IV PDE isoenzymes and RO 20-1724 is a selective inhibitor of the type IV PDE isoenzyme (Nicholson & Shahid, 1994). These results therefore suggest that inhibition of the type IV PDE isoenzyme is most probably required for inhibition of eosinophil accumulation, and that if zardaverine and RO 20-1724 are acting through inhibition of the phosphodiesterase enzyme, cyclic nucleotide levels may need to be elevated chronically to achieve this effect.

If PDE IV inhibition is responsible for the effects of zardaverine and RO 20-1724, then there are several possible cellular targets for this action since a series of events lead to eosinophil accumulation in the airways. Theoretically the drugs could be acting by at least three ways to reduce eosinophil numbers in the airways: (i) by inhibiting eosinopoesis, (ii) by decreasing eosinophil survival or (iii) by redistributing or preventing eosinophils from moving from the blood to the airways. Evidence to support the first mechanism of action is provided by the finding that seven days of treatment with the PDE IV isoenzyme inhibitor, rolipram, decreased the number of circulating eosinophils in parasite-infected mice (DeBrito *et al.*, 1991) by inhibiting the development of eosinophil progenitor cells.

These effects may be achieved by actions on different cell targets: (i) by inhibition of the activation of eosinophils and their subsequent recruitment into the airways (ii) an action on the eosinophil precursors and/or (iii) other cell types such as lymphocytes or mast cells which release mediators which act as chemoattractants for eosinophils. It is not immediately obvious which cell type is the most likely primary target since the type IV and type III PDE isoenzymes have been identified in a variety of cell types which release chemotactic mediators for eosinophils.

Endothelium may also represent a drug target for modulation by PDE inhibitors since endothelial cells contain PDE IV (Lugnier & Schini, 1990). Agents effective at inhibiting eosinophil recruitment could be altering the adhesion and migratory stage when the eosinophil adheres to and moves through the endothelial cells to the interstitial tissue and then to the airways. There is evidence to suggest that PDE inhibitors are able to inhibit the expression of specific adhesion molecules. PDE IV inhibitors have been shown to inhibit the release of tumour necrosis factor (TNF-$\alpha$) from monocytes (Maschler & Christensen, 1991) which is a potent inducer of the expression of the adhesion molecules, ICAM-1 and ELAM-1, on endothelial cells (Wellicome *et al.*, 1990). Since endothelial cells contain PDE IV (Lugnier & Schini, 1990) it is therefore conceivable that inhibition of the PDE IV enzyme could down regulate the expression of adhesion molecules, thus preventing eosinophil binding to endothelial cells. Finally, it is also possible that PDE inhibitors may be affecting the permeability of endothelial cells. Type III and IV inhibitors have been shown to reduce endothelial cell

permeability *in vitro* (Seiburt *et al.*, 1992; Suttorp *et al.*, 1993) and it has been demonstrated *in vivo* that rolipram can inhibit PAF-induced plasma extravasation in guinea-pig airways (Raeburn & Karlsson, 1992).

Monocytes and T-lymphocytes are a possible primary target. Theophylline and IBMX have been shown to inhibit the generation of potent eosinophil chemoattractants such as PAF and complement C2 from human monocytes (Lappin *et al.*, 1984). The activation and proliferation of T-lymphocytes which are believed to play an important role in eosinophil recruitment into the airways (Corrigan & Kay, 1989) can also be inhibited by mixed type III/IV PDE inhibitors (Robicsek *et al.*, 1991; Schudt *et al.*, 1992) and by type IV PDE inhibitors (Averill & Kammer, 1985; Banner & Page, 1994b).

Mast cells are another possible target. However, it is unlikely that the inhibitory effect of RO 20-1724 and zardaverine is on this cell type in asthma since the PDE IV isoenzyme inhibitor, rolipram, can prevent ovalbumin-induced eosinophil influx even when administered after antigen challenge (Sturm *et al.*, 1990) and hence after mast cell degranulation has already occurred. Furthermore, Turner and co-workers (1994) also found that rolipram had no effect on acute antigen-induced bronchospasm in atopic monkeys.

In conclusion these results suggest that the type IV PDE isoenzyme may be involved in the control of eosinophil accumulation in the airways of immunized guinea-pigs and indicate that a period of chronic treatment with type IV or type III/IV inhibitors is necessary before inhibition is observed.

K.H.B. is a Medical Research Council Scholar.

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# Cytochrome P450-dependent effects of bradykinin in the rat heart

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1 Vasodilator responses to bradykinin (BK) in the rat heart are reported to be independent of NO and cyclo-oxygenase/lipoxygenase products of arachidonic acid (AA).

2 We verified that inhibition of NO synthase with L-nitroarginine (50 μM) and cyclo-oxygenase with indomethacin (2.8 μM) were without effect on vasodilator responses to BK (10–1000 ng) in the Langendorff rat heart preparation.

3 L-Nitroarginine elevated perfusion pressure, signifying a crucial role of NO in the maintenance of basal vasculature tone.

4 In hearts treated with L-nitroarginine to eliminate NO and elevate perfusion pressure, vasodilator responses were reduced by inhibitors of cytochrome P450 (P450), clotrimazole (1 μM) and 7-ethoxyresorufin (1 μM). 17-Octadecynoic acid (17-ODYA 2 μM), a mechanism based inhibitor of P450-dependent metabolism of fatty acids, also reduced vasodilator responses to BK.

5 These results confirm that NO and prostaglandins do not mediate vasodilator responses to BK in the rat heart but suggest a major role for a P450-dependent mechanism via AA metabolism.

**Keywords:** Rat perfused heart; vasodilatation to bradykinin; nitric oxide; prostaglandins; cytochrome P450

## Introduction

Bradykinin (BK) is a nonapeptide that elicits endothelium-dependent vasodilatation, an effect generally attributed to its ability to stimulate nitric oxide (NO) synthesis (Vane *et al.*, 1990). However, there are also numerous reports of NO-independent vasodilator responses to BK, particularly in coronary vessels (Richard *et al.*, 1990; Cocks & Angus, 1991; Tschudi *et al.*, 1991). Thus, there is evidence that BK can stimulate the formation/release of, a yet to be characterized, hyperpolarizing factor (Beny & Brunet, 1988; Flavahan *et al.*, 1989; Nagao *et al.*, 1992). Moreover, BK can also stimulate phospholipases to release arachidonic acid (AA) which can be metabolized by cyclo-oxygenase, lipoxygenase and cytochrome P450 (P450) to products that may contribute to the vascular response. In skeletal muscle the vasodilator effect of BK is mediated by prostaglandins (Messina *et al.*, 1975) whereas in cerebral vessels free radicals, derived from cyclo-oxygenase metabolism of AA, appear to mediate the response (Kontos *et al.*, 1984). Recently, we reported that a major component of the vasodilator response to BK in the isolated, perfused kidney of the rat was dependent on P450 (Fulton *et al.*, 1992). Baydoun & Woodward (1991) also found that the vasodilator response to BK in the isolated heart of the rat was independent of NO and cyclo-oxygenase and lipoxygenase metabolites of AA. However, a role for P450-derived AA metabolites was not addressed. As the coronary vasculature is heavily invested with P450 (Pinto *et al.*, 1987) we used the isolated heart to assess the contribution of P450 to the coronary vasodilator effect of BK.

## Methods

Male Wistar rats (350–400 g) were anaesthetized with pentobarbitone (65 mg $kg^{-1}$, i.p.) and given heparin (1000 u $kg^{-1}$, i.v.). Following thoracotomy, the heart was excised and perfused, via an aortic cannula, at constant flow with oxygenated Krebs buffer at 37°C according to the method of Langendorff (1895) as modified by Broadley (1979). Flow was adjusted to 8–8.5 ml $min^{-1}$ which resulted in a basal perfusion pressure of 25–40 mmHg which increased and stabilised at approximately 80 mmHg after 15–20 min. To amplify vasodilator responses, vascular tone was further elevated with phenylephrine (0.75–1 μM), U46619 (8–14 nM) or nitroarginine (50 μM).

In the first series of experiments the contribution of NO to the coronary vasodilator response to BK was assessed by comparing changes in perfusion pressure in hearts constricted with either phenylephrine or U46619 and those in which NO synthesis was inhibited with L-nitroarginine which alone was sufficient to raise perfusion pressure. Thus, phenylephrine was relatively ineffective in causing constriction compared to L-nitroarginine, elevating perfusion pressure to 96 mmHg versus 136 mmHg. Therefore, experiments were also conducted in hearts treated with U46619 to achieve a similar perfusion pressure to that obtained with L-nitroarginine. In all subsequent experiments, L-nitroarginine (50 μM) was used to eliminate NO and to elevate vascular tone. Thus, the control group except for those experiments shown in Figures 1 and 2, consisted of L-nitroarginine-treated hearts and BK responses were compared to those obtained in hearts treated with L-nitroarginine and inhibitors of cyclo-oxygenase or cytochrome P450 which were added to the perfusate from the start of the experiment. Indomethacin (2.8 μM) was used to inhibit cyclo-oxygenase whereas clotrimazole (1 μM), 7-ethoxyresorufin (1 μM) or 17-octadecynoic acid (17-ODYA, 2 μM) were used to inhibit cytochrome P450. We have previously shown that these concentrations of P450 inhibitors do not influence renal vasodilator or aortic vasorelaxant responses to acetylcholine or sodium nitroprusside (Oyekan *et al.*, 1991, 1994). After a dose-response curve to BK was obtained, the vasodilator response to 1000 ng nitroprusside was determined to assess any non-specific effects of the inhibitors used.

Data from the various treatment and control groups were compared by analysis of variance and individual data points compared by Fisher's test using the Stat View statistical programme (Brain Power Inc, California, U.S.A.). Differences were considered significant when $P < 0.05$.

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### *Materials*

17-ODYA, U46619 (11, 9 epoxy methano-prostaglandin $H_2$), was obtained from Biomol, Plymouth Meeting, PA, U.S.A. and stored in ethanol (10 mg ml$^{-1}$) at −20°C. 7-Ethoxyresorufin was obtained from Pierce Laboratories, Rockford, Il, U.S.A., and stored at −20°C in ethanol (1 mg ml$^{-1}$). Clotrimazole, L-nitroarginine, phenylephrine and sodium nitroprusside were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Clotrimazole was dissolved in ethanol and L-nitroarginine, phenylephrine and sodium nitroprusside in water. Indomethacin which was dissolved in 4% $NaHCO_3$ was a gift from Merck.

## Results

Phenylephrine elevated coronary perfusion pressure to 96 ± 11 mmHg and BK produced dose-dependent reductions in perfusion pressure (Figure 1). Inhibition of NO synthesis with L-nitroarginine, which resulted in a perfusion pressure of 136 ± 4 mmHg, did not reduce the vasodilator responses to BK (Figure 1) but rather enhanced them, attaining significance at the 1000 ng dose (61 ± 6 mmHg vs 38 ± 5 mmHg in the phenylphrine-treated group). The vasodilator response to nitroprusside was slightly, but insignificantly, increased in the presence of L-nitroarginine (41 ± 8 vs 30 ± 5 mmHg). As there were substantial differences in perfusion pressure between the two groups, we also compared responses to BK in hearts preconstricted with U46619 to elevate perfusion pressure to the same level as that attained with L-nitroarginine in the presence of indomethacin. Thus, U46619 resulted in a perfusion pressure of 129 ± 7 mmHg, comparable to that achieved with L-nitroarginine. However, vasodilator responses to BK were not different in L-nitroarginine plus indomethacin and U46619-treated hearts (Figure 2) although responses to nitroprusside were greatly enhanced by the combination of L-nitroarginine and indomethacin.

Inhibition of cyclo-oxygenase with indomethacin or cytochrome P450 with clotrimazole, 7-ethoxyresorufin or 17-ODYA did not affect the perfusion pressure attained with L-nitroarginine. Indomethacin was without effect on the coronary vasodilator responses to all doses of BK in hearts preconstricted with L-nitroarginine (data not shown), confirming the results reported by Baydoun & Woodward (1991). In contrast, clotrimazole markedly reduced the vasodilator responses to BK (Figure 3) in L-nitroarginine-treated hearts but did not modify that to nitroprusside. Thus, responses to BK were inhibited by up to 80% in the presence of clotrimazole ($P < 0.01$). 7-Ethoxyresorufin was less effective than clotrimazole in inhibiting responses to BK, a significant reduction (39%) being obtained only at the highest dose of BK (Figure 4). 7-Ethoxyresorufin, like clotrimazole, was without effect on the coronary vasodilator response to nitroprusside.

As inhibition of cytochrome P450 with clotrimazole or 7-ethoxyresorufin reduced coronary vasodilator responses to

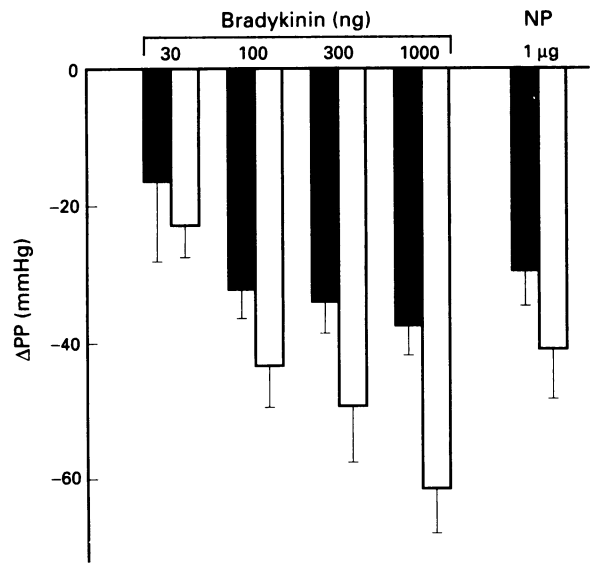


**Figure 1** Perfusion pressure responses to bradykinin and nitroprusside (NP) in preconstricted rat hearts: effect of NO synthesis inhibition with L-nitroarginine (50 μM): solid column, phenylephrine 1 μM ($n = 5$); open columns, L-nitroarginine 50 μM ($n = 9$).

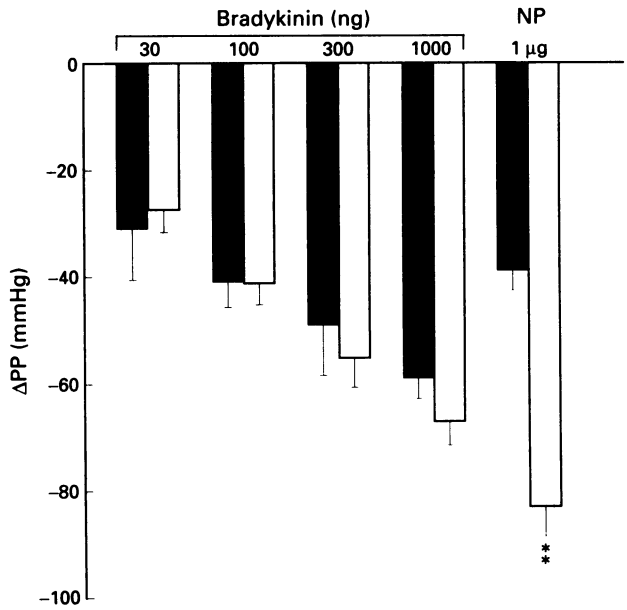


**Figure 2** Comparison of perfusion pressure responses to bradykinin in rat hearts constricted with L-nitroarginine to inhibit NO synthesis and those constricted with U46619: solid column, U46619 2–5 ng ml$^{-1}$ ($n = 3$); open columns, L-nitroarginine 50 μM + indomethacin 2.8 μM ($n = 9$). $^{**}P < 0.01$.

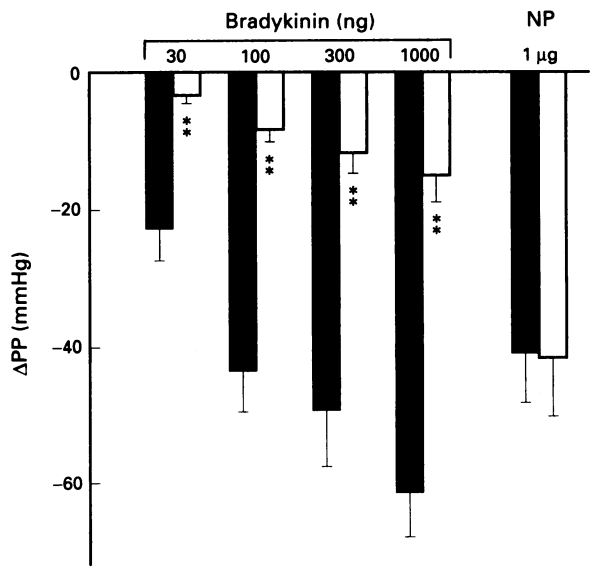


**Figure 3** Effect of inhibition of P450 with clotrimazole on vasodilator responses to bradykinin and nitroprusside in rat isolated hearts in which L-nitroarginine was used to eliminate NO synthesis and raise perfusion pressure: solid columns, L-nitroarginine 50 μM ($n = 9$); open columns, L-nitroarginine 50 μM + clotrimazole 1 μM ($n = 9$). $^{**}P < 0.01$.

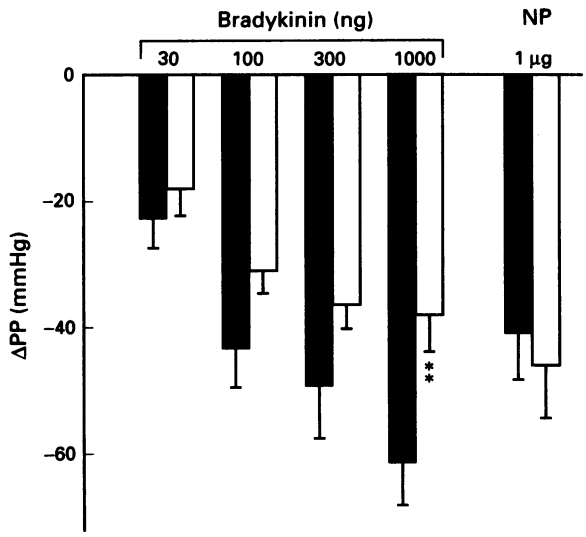


**Figure 4** Bradykinin-induced changes in the perfusion pressure of rat isolated hearts preconstricted with L-nitroarginine in the presence and absence of the cytochrome P450 inhibitor, 7-ethoxyresorufin: solid column, L-nitroarginine 50 μM ($n = 9$); open columns, L-nitroarginine 50 μM + 7-ethoxyresorufin 1 μM ($n = 5$). $^{**}P < 0.01$.

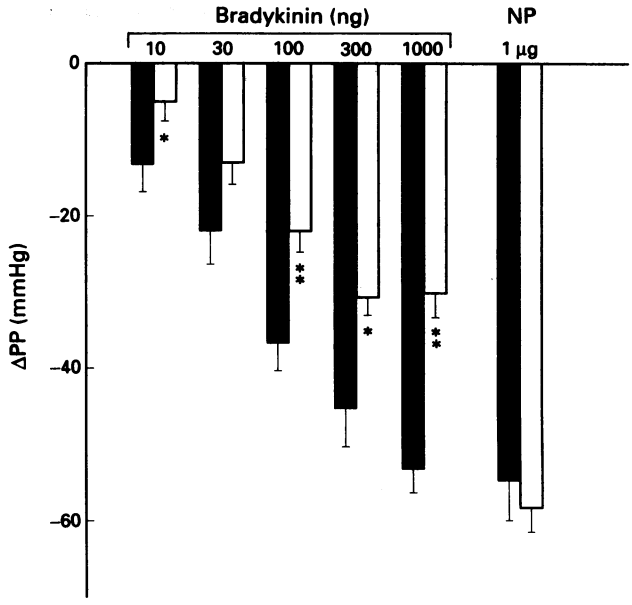


**Figure 5** Vasodilator responses to bradykinin and nitroprusside in rat isolated perfused hearts preconstricted with L-nitroarginine (50 μM) in the absence and presence of 17-ODYA to inhibit cytochrome P450 metabolism of fatty acids: solid columns, L-nitroarginine 50 μM ($n = 5$); open columns, L-nitroarginine 50 μM + 17-ODYA 2 μM ($n = 5$). $^{*}P < 0.05$; $^{**}P < 0.01$.

BK, we next tested the effects of 17-ODYA in order to investigate the possibility that metabolites of AA, generated via cytochrome P450, were involved in the response to BK. As 17-ODYA is a suicide-substrate inhibitor of cytochrome P450 isozymes that metabolize long-chain fatty acids (Zou *et al.*, 1994b), it possesses a specificity for mono-oxygenases that metabolize AA that is not demonstrated by other inhibitors. 17-ODYA reduced responses to all doses of BK in L-nitroarginine-treated hearts, the greatest inhibition (43%) occurring with the highest dose of BK (Figure 5), but did not modify the vasodilator response to nitroprusside.

## Discussion

The results of this study demonstrate that, in the isolated perfused heart of the rat, vasodilator responses to BK are not mediated by NO as inhibition of synthesis with L-nitroarginine did not reduce the responses, confirming the findings of Baydoun & Woodward (1991). Although we did not verify that the concentration of L-nitroarginine (50 μM) used by us abolished synthesis of NO, there are reasons for believing this to be the case. This concentration was shown by Cachofeiro & Nasjletti (1991) to prevent stimulated increases in urinary cyclic GMP from the rat kidney; L-nitroarginine produced a marked increase in coronary perfusion pressure, greater than could be achieved with phenylephrine, suggesting removal of an endogenous vasodilator system that is crucial to the maintenance of basal vascular tone; responses to nitroprusside were enhanced, albeit insignificantly, in the presence of L-nitroarginine consistent with reduction of background levels of NO. However, the two groups used to test the contribution of NO, i.e., those treated with phenylephrine vs those treated with L-nitroarginine, exhibited different perfusion pressures, 96 vs 135 mmHg and, therefore, the maximum responses to BK in the control group may have been limited by the level of tone. Consequently, we also compared responses in hearts constricted with U46619 to attain a similar perfusion pressure to that achieved with L-nitroarginine. As no differences were observed in the vasodilator response to BK we can exclude a role of NO. Similarly, in porcine and canine isolated coronary arteries vasorelaxant responses to BK were minimally affected or unaffected by interventions aimed at reducing the synthesis or action of NO (Richard *et al.*, 1990; Cocks & Angus, 1991; Tschudi *et al.*, 1991). Consequently, mediators other than NO, are required to explain the coronary vasodilator effect of BK. The results of this study and those of a previous study of the rat heart (Baydoun & Woodward, 1991) provide evidence that BK utilizes a P450-dependent mechanism in certain vascular tissues. Thus, clotrimazole and 7-ethoxyresorufin attenuated responses to BK in the rat heart but did not affect dilator responses to nitroprusside indicating that these agents were specific for P450. These results are qualitatively similar to those reported by Pinto *et al.* (1987) for canine coronary arteries in which a role for cyclo-oxygenase and P450 in the vasorelaxant response to BK was suggested, based on the use of indomethacin and SKF525A. In the present study we also showed that 17-ODYA, an inhibitor of P450 metabolism of fatty acids, attenuated the coronary vasodilator response to BK, providing evidence for P450-dependent metabolism of AA which is released in response to BK. The observations that three structurally distinct inhibitors of P450 reduce BK responses provide strong evidence for a role of this system. Furthermore, as these agents differ in their capacity to inhibit the various P450 isozymes that metabolize AA, epoxygenase versus ω and ω-1 hydroxylase, their effects provide insights as to the identity of the putative mediator. Thus, clotrimazole, which was the most effective inhibitor of the BK response, is more specific for the epoxygenase pathway of P450 metabolism of AA whereas 17-ODYA and 7-ethoxyresorufin, which produced less attenuation of the response, exhibit no differential inhibitory activity towards epoxygenase vs ω-hydroxylase (Zou *et al.*, 1994a). Therefore, these results are consistent with an epoxygenase product as the mediator of P450-dependent coronary vasodilator responses to BK. Moreover, preliminary results of GC-MS analysis of coronary perfusates indicate that the ω-hydroxylase pathway of AA metabolism is negligible in the rat heart whereas epoxide released can be demonstrated.

In summary, this study suggests that a P450-dependent mechanism via metabolism of AA is the major mediator of the coronary vasodilator effect of BK in the rat and that the contribution of NO is minimal or absent. These results are consistent with the ability of BK to release AA and its

subsequent conversion by vascular P450, the highest levels of which are found in the coronary vasculature (Pinto *et al.*, 1987). However, the identity of the mediator and its mechanism of vasodilatation remain to be elucidated.

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# Involvement of $B_2$ receptors in the bradykinin-induced relaxation of guinea-pig isolated trachea

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**1** The aim of this study was to determine the receptor type and involvement of arachidonic acid metabolites in bradykinin-induced relaxation of the guinea-pig isolated trachea.

**2** In the resting tracheal preparation, bradykinin (0.1 nM–30 μM induced a concentration-related contractile response ($pD_2 = 8.8 \pm 0.3$). The maximal tension (1056 ± 321 mg) was observed at 0.3 μM bradykinin. In contrast, when tracheal preparations were pre-contracted with histamine (30 μM leading to a half-maximum response), a concentration-related relaxation was observed with bradykinin. At the highest concentration of bradykinin used (3 μM), a reversal of 63 ± 13% of the contractile response to histamine was observed. Both effects of bradykinin were inhibited by the cyclo-oxygenase inhibitor, indomethacin (1 μM). In concentration-response curves, melittin (10 nM–1 μM), a direct activator of phospholipase $A_2$, mimicked both effects of bradykinin. The highest concentration of melittin used (1 μM), induced a tension of 813 ± 120 mg and led to the reversal of 41 ± 8% of the contractile response to histamine. The contractile effect of melittin was inhibited in the presence of both indomethacin (1 μM) and AA861 (1 μM), a 5-lipoxygenase inhibitor.

**3** [Des Arg$^9$]-bradykinin (1 nM–3 μM), a $B_1$-receptor agonist, was unable to relax precontracted guinea-pig tracheal preparations. The relaxation induced by bradykinin was antagonized by the $B_2$ receptor antagonists, Hoe 140 (D-Arg$^0$[Hyp$^3$,Thi$^5$,D-Tic$^7$,Oic$^8$]bradykinin) and NPC 17761 (D-Arg$^0$[Hyp$^3$,D-HypE(*trans*-thiophenyl)$^7$,Oic$^8$]bradykinin). Hoe 140 (0.1 μM to 0.6 μM) behaved as a non-competitive antagonist with an apparent $pA_2 = 7.2 \pm 0.4$, whereas NPC 17761 (0.3 to 1 μM) competitively antagonized bradykinin-induced relaxation with a $pK_B = 7.3 \pm 0.2$. The Schild regression slope did not differ from unity, $0.96 \pm 0.20$, $P < 0.05$.

**4** These data demonstrate that bradykinin-induced relaxation of guinea-pig trachea occurs via the activation of bradykinin $B_2$-receptors. The stimulation of $B_2$-bradykinin receptors induces the activation of the cyclo-oxygenase pathway, leading either to contraction or relaxation depending on the tone of the trachea.

**Keywords:** Guinea-pig trachea; bradykinin; melittin; contraction; relaxation; bradykinin $B_2$-receptors; phospholipase $A_2$; Hoe 140; NPC 17761

## Introduction

Bradykinin is a potent mediator of inflammation and is implicated in asthma (for a review see Barnes, 1992 and Trifilieff *et al.*, 1993b). On the basis of agonist potencies, the major airway receptors for bradykinin causing bronchoconstriction in asthmatic subjects belong to the $B_2$ type (Polosa & Holgate, 1990). In the human airway, the contraction of smooth muscle is mediated via cholinergic (Fuller *et al.*, 1987) and peptidergic mechanisms (Dixon & Barnes, 1989; Ichinose *et al.*, 1992). Inhibition of the cyclo-oxygenase pathway did not modify the bronchoconstrictor effect of inhaled bradykinin in human subjects (Fuller *et al.*, 1987; Polosa & Holgate, 1990).

In guinea-pig airways, the effect and the mechanism of action of bradykinin are complex and controversial. Bradykinin can produce both contraction and relaxation of the guinea-pig isolated trachea (Mizrahi *et al.*, 1982; Rhaleb *et al.*, 1988; Bramley *et al.*, 1990; Frossard *et al.*, 1990). The contractile response was first described as insensitive to early available $B_2$-receptor antagonists (Farmer *et al.*, 1989). Recently, the availability of new $B_2$-receptor antagonists such as Hoe 140 (Hock *et al.*, 1991; Wirth *et al.*, 1991) and NPC 17731 led to the demonstration of the involvement of $B_2$-receptors in the contractile response of bradykinin both *in vivo* (Wirth *et al.*, 1991) and *in vitro* (Farmer *et al.*, 1991a; Field *et al.*, 1992; Rhaleb *et al.*, 1992; Trifilieff *et al.*, 1992; 1993a). Bradykinin can also induce relaxation of guinea-pig trachea. A role for the epithelium has been suggested in this effect (Bramley *et al.*, 1990; Frossard *et al.*, 1990; Schlemper & Calixto, 1994). However, Bramley *et al.* (1990) noted that intact preparations (with epithelium) gained appreciable tone during the equilibration period, whereas rubbed trachea (without epithelium) developed no visible tone. We have therefore investigated whether the effects induced by bradykinin were related to the presence of epithelium or were dependent on the tone of the organ as has been described for prostaglandin $E_2$ ($PGE_2$) and arachidonic acid-induced responses (Braunstein *et al.*, 1988; Nijkamp & Folkerts, 1987; Tschirhart *et al.*, 1987). The relaxant effect of bradykinin has been observed in the guinea-pig isolated trachea precontracted with histamine (Mizrahi *et al.*, 1982). This relaxant effect was considered to be dependent on prostaglandins, without the involvement of $B_1$ or $B_2$ receptors, due to the inefficiency of bradykinin antagonists (Mizrahi *et al.*, 1982; Rhaleb *et al.*, 1988; Bramley *et al.*, 1989). A direct activation of one of the enzymes of the arachidonic acid cascade by bradykinin has been proposed (Rhaleb *et al.*, 1988). In the present study, the effects of new $B_2$-receptor antagonists (Hoe 140 and NPC 17761) on bradykinin-induced relaxation were investigated. The response to melittin, a direct activator of phospholipase $A_2$ (Shier *et al.*, 1979), was compared with that of bradykinin. The present study shows that the bradykinin-induced relaxation is mediated via a bradykinin $B_2$-receptor coupled with the activation of the arachidonic acid cascade. Thus, the products of the cyclo-oxygenase reaction can induce contraction or relaxation depending on the tone of the guinea-pig isolated trachea.

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## Methods

Male albino Dunkin Hartley guinea-pigs (Elevage Saint-Antoine, Pleudaniel, France) weighing 250–350 g were anaesthetized with sodium pentobarbitone, 45 mg $kg^{-1}$ i.p. The trachea was rapidly dissected out and immersed in a modified Krebs physiological solution (composition mM: NaCl 120, KCl 4.75, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $KH_2PO_4$ 1.15, $NaHCO_3$ 25, glucose 10). The trachea was cleaned of connective tissue and opened longitudinally through the cartilage, opposite the smooth muscle. Fragments corresponding to four cartilaginous rings were cut from each trachea, discarding the upper and the lower part of the tissue. To prepare the epithelium-free tracheal preparations, the luminal surface was rubbed gently with a cotton swab as described previously (Da Silva *et al.*, 1992). Tracheal fragments were suspended in 10 ml organ baths containing the modified Krebs physiological solution at 37°C gassed with a mixture of $O_2$ (95%) and $CO_2$ (5%), pH = 7.4. Variations in smooth muscle tone were measured isometrically with Narco F60 Myographs connected to Narco MKIII Physiograph (Narco Bio-Systems, Inc, Houston, TX, U.S.A.) after washing (three times at 15 min intervals) and equilibration for 30 min at a tension of 2 g, which was found to be optimal for measuring changes in tension. The effects of melittin, bradykinin and two bradykinin-receptor antagonists (Hoe 140 and NPC 17761) in isolated tissues may be influenced by endogenous peptidases enzymes. Although many peptidases are able to hydrolyse kinins, endopeptidase 24.11 was found to be the most important modulator of bradykinin-induced effects in airways (Dusser *et al.*, 1988; Da Silva *et al.*, 1992). Therefore, all experiments were conducted in the presence of an inhibitor of endopeptidase 24.11, DL-thiorphan (10 μM). Inhibitors were added 30 min prior to bradykinin or melittin. Bradykinin receptor-antagonists were added 10 min after the addition of thiorphan to the organ bath. Concentration-response curves were constructed by the cumulative addition of bradykinin or melittin. We studied the effects of bradykinin and melittin on guinea-pig tracheal preparations set at 2 g tension (resting preparations) or set at 2 g tension and then precontracted by the addition of 30 μM histamine, which corresponds to the $EC_{50}$ for this agonist (precontracted preparations). In all experiments, only one cumulative concentration-response curve was constructed in the presence or absence of a single concentration of bradykinin receptor-antagonist or enzyme inhibitor.

### *Reagents*

Bradykinin and melittin (Sigma Chemical Co., St Louis, MO, U.S.A.) were prepared as 4 mM stock solutions and aliquots were stored at −20°C. DL-Thiorphan (Bachem, Bubbendorf, Switzerland), indomethacin (Merck, Sharp & Dohme, Riom, France) and AA861 (2-(12-hydroxydodeca-5,10-cliynyl)-3,5,6-trimethyl-1,4-benzoquinone) (Takeda Chemicals Industries Ltd, Japan) were dissolved in absolute ethanol at 20, 2 and 2 mM, respectively. D-Arg$^0$[Hyp$^3$,Thi$^5$,D-Tic$^7$,Oic$^8$]-bradykinin (Hoe 140) was a gift from Hoechst AG (Frankfurt, Germany). D-Arg$^0$[Hyp$^3$,D-HypE(trans-thiophenyl)$^7$,Oic$^8$]-bradykinin (NPC 17761) was a gift from Scios Nova Inc. (Baltimore, MD, U.S.A.). Hoe 140 and NPC 17761 were prepared as 0.1 mM stock solutions in distilled water. Additional dilutions were performed in modified Krebs physiological solution.

### *Data analysis*

$pD_2$ values (negative $\log_{10}$ of the molar concentration of agonist required to elicit 50% of the maximal response) were calculated (Van Rossum, 1963). Dose-ratios were calculated from the equi-effective molar concentration of bradykinin in the presence and absence of antagonist that led to 30% to the reversal of the contractile response to histamine. $pA_2$ and $pK_B$ values were obtained by linear regression of 'Schild plots' derived from concentration-response curves (Arunlakshana & Schild, 1959). Differences between groups were assessed by Student's paired or unpaired $t$ test and considered significant when $P<0.05$.

## Results

Bradykinin (0.1 nM–30 μM) induced a concentration-related contraction in the resting tracheal preparations (Figure 1a). This response was prevented by 1 μM indomethacin, a cyclooxygenase inhibitor. A slight contractile effect remained in the presence of indomethacin with bradykinin concentrations greater than 0.1 μM (Figure 1a). A maximal tension of 1056 ± 321 mg was induced by 0.3 μM bradykinin. The estimated $pD_2$ for bradykinin was 8.8 ± 0.3 ($n$ = 5). The same effect was observed in tracheal preparations without epithelium, the $pD_2$ and maximal tension being 7.4 ± 0.1 and

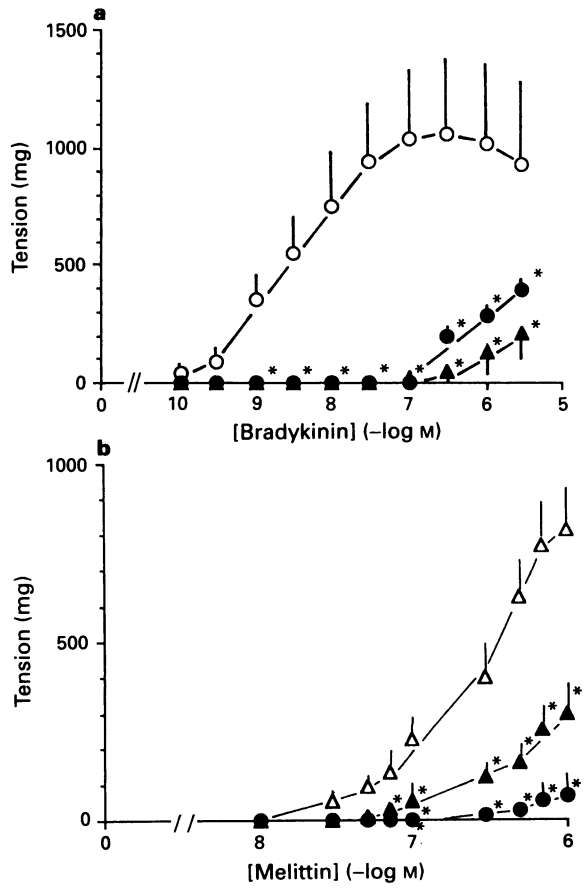


**Figure 1** (a) Log concentration-response curves for bradykinin in guinea-pig tracheal preparations in the presence of thiorphan (10 μM). Tracheal preparations were suspended under 2 g tension before starting the concentration-response curve for bradykinin (○). Values are means ± s.e.mean for 5 experiments. Experiments were performed in the absence (○), in the presence of 1 μM indomethacin (▲) or in the presence of 1 μM indomethacin plus 1 μM AA861 (●) added 30 min before the first concentration of bradykinin. *Values significantly different between control preparations and preparations in the presence of indomethacin: $P<0.05$. (b) Log concentration-response curves for melittin in guinea-pig tracheal preparations in the presence of thiorphan 10 μM. Tracheal preparations were suspended under 2 g tension before starting the concentration-response curve for melittin (△). Values are means ± s.e.mean for 6 experiments. Experiments were performed in the absence (△), or in the presence of 1 μM indomethacin (▲) or in the presence of 1 μM indomethacin plus 1 μM AA861 (●) added 30 min before the first concentration of melittin. *Values significantly different between control preparations and preparations in the presence of indomethacin or with indomethacin plus AA861: $P<0.05$.

830 ± 77 mg, respectively ($n = 5$). AA861 (1 μM), a 5-lipoxygenase inhibitor (Ashida *et al.*, 1983), did not modify the responses to bradykinin in the presence or absence of indomethacin (Figure 1a). A contractile response of resting tracheal preparations was observed at concentrations from 30 nM to 1 μM melittin which was partly prevented by 1 μM indomethacin (Figure 1b) or 1 μM AA861 (not shown). Complete inhibition of the contractile response was observed in the combined presence of indomethacin and AA861 (Figure 1b).

A relaxant effect of bradykinin was observed with 3 nM of the peptide, following a pre-contraction of tracheal preparations with histamine (Figure 2a). The highest bradykinin concentration used (3 μM) led to the reversal of 63 ± 13% of the contractile response to histamine. In tracheal preparations without epithelium, the highest bradykinin concentration used (3 μM) led to the reversal of 42 ± 4% ($n = 5$) of the histamine-induced contraction. Indomethacin (1 μM) fully prevented this relaxant effect of bradykinin but did not significantly modify the precontraction by histamine (Figure 2a). The selective $B_1$-receptor agonist [des-Arg$_9$]-bradykinin did not reverse the histamine-induced tone (Figure 2a). Melittin relaxed the precontracted guinea-pig isolated trachea at concentrations greater than from 30 nM (Figure 2b). The highest melittin concentration used (1 μM) reversed 41 ± 8% ($n = 6$) of the histamine-induced contraction. This effect of melittin was entirely prevented by 1 μM indomethacin (Figure 2b).

The effect of selective $B_2$-receptor antagonists on the relaxant response of precontracted tracheal preparations to bradykinin is shown on Figure 3. Hoe 140 decreased the bradykinin-dependent relaxation, but is effect was insurmountable even on increasing the concentration of bradykinin. The apparent $pA_2$ was determined as 7.2 ± 0.4. In contrast, NPC 17761 led to a shift to the right of the bradykinin concentration-response curve (Figure 3). The corresponding $pK_B$ value was 7.3 ± 0.2 with a Schild's plot slope of 0.96 ± 0.20 which did not differ from unity, $P < 0.05$ (Figure 3). The responses to melittin were unaffected by bradykinin antagonists (Figure 4).

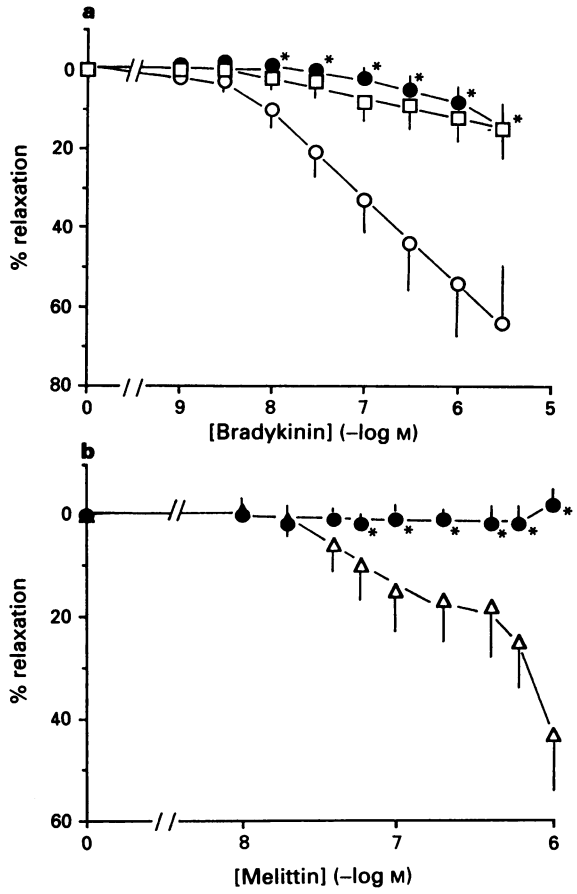


**Figure 2** (a) Log concentration-response curves for bradykinin and [des-Arg$^9$]-bradykinin in precontracted guinea-pig tracheal preparations in the presence of thiorphan (10 μM). Tracheal preparations were suspended under 2 g tension then stimulated with histamine (30 μM) before starting the concentration-response curve for bradykinin (○) or [des-Arg$^9$]-bradykinin (□). Experiments with bradykinin were performed in the absence (○) or in the presence of 1 μM indomethacin (●) added 30 min before the first concentration of bradykinin. The tension induced by histamine was 1420 ± 174 mg. Values are means ± s.e.mean for 5 experiments. *Values significantly different between control preparations stimulated with bradykinin and preparations stimulated with bradykinin in the presence of indomethacin: $P < 0.05$. (b) Log concentration-response curves for melittin in precontracted guinea-pig tracheal preparations in the presence of thiorphan (10 μM). Tracheal preparations were suspended under 2 g tension then stimulated with histamine (30 μM before starting the concentration-response curve for melittin (△). Experiments were performed in the absence (△) or in the presence of 1 μM indomethacin (●) added 30 min before the first dose of melittin. The tension induced by histamine was 1583 ± 232 mg. Values are means ± s.e.mean for 6 experiments. *Values significantly different between control preparations stimulated with melittin and preparations stimulated with melittin in the presence of indomethacin: $P < 0.05$.

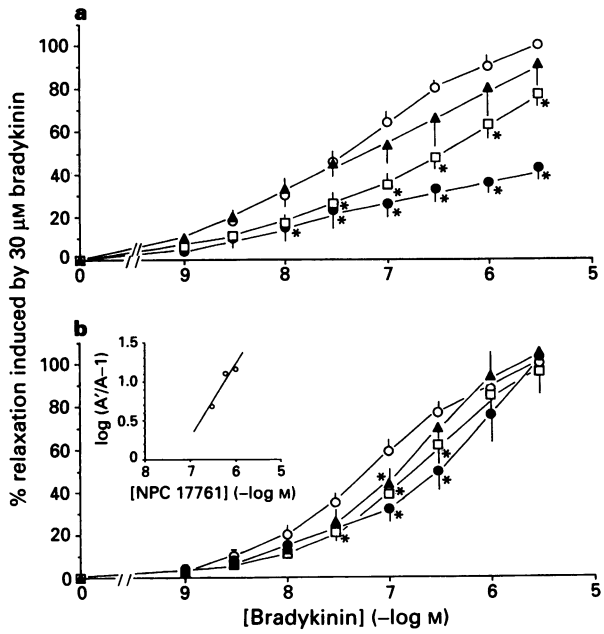


**Figure 3** Effect of bradykinin receptor antagonists on bradykinin-induced relaxation on precontracted guinea-pig tracheal preparations. (a) Tracheal preparations were suspended under 2 g tension then stimulated with histamine (30 μM) before starting the concentration-response curve for bradykinin in the absence (○) or in the presence of Hoe 140 (▲, 0.1 μM; □, 0.3 μM and ●, 0.6 μM on bradykinin-induced relaxation. The tension induced by histamine was 1418 ± 315 mg. Values are means ± s.e.mean for 5 experiments. *Values significantly different between control preparations stimulated with bradykinin and preparations stimulated with bradykinin in the presence of Hoe 140: $P < 0.05$. (b) Tracheal preparations were suspended under 2 g tension then stimulated with histamine (30 μM) before starting the concentration-response curve for bradykinin in the absence (○) or in the presence of NPC 17761 (▲, 0.3 μM; □, 0.6 μM and ●, 1 μM) on bradykinin-induced relaxation. The tension induced by histamine was 1706 ± 173 mg. Insert: Schild plot for NPC 17761-induced relaxation. Values are means ± s.e.mean for 11 experiments. *Values significantly different between control preparations stimulated with bradykinin and preparations stimulated with bradykinin in the presence of NPC 17761: $P < 0.05$. Bradykinin effects are expressed as % of the relaxation induced by bradykinin (30 μM) in the absence of antagonist.

## Discussion

We demonstrate that bradykinin can induce relaxation or contraction of guinea-pig isolated trachea both in the presence or absence of the epithelium. The effects of bradykinin are inhibited by the cyclo-oxygenase inhibitor, indomethacin and by the $B_2$ receptor antagonists, Hoe 140 and NPC 17761. In previous studies it had been suggested that bradykinin-induced relaxation was related to the presence of the epithelium (Bramley *et al.*, 1990; Schlemper & Calixto, 1994). Bramley *et al.* (1990) showed that in the presence of indomethacin the response of the guinea-pig tracheal preparation to bradykinin was changed from a relaxation to a contraction. Therefore, it was suggested that bradykinin-induced relaxation was related to an epithelium-dependent cyclo-oxygenase mechanism (Bramley *et al.*, 1990) or to a cyclo-oxygenase and nitric oxide mechanism (Schlemper & Calixto, 1994). However, in these studies, the experimental conditions were not the same for the tracheal preparations with or without epithelium. Intact tracheal preparations were allowed to develop a spontaneous tone. Treatment with indomethacin reduced the release of prostaglandins (Bramley *et al.*, 1990) and the spontaneous tone (Schlemper & Calixto, 1994). Tracheal preparations without epithelium did not develop a spontaneous tone (Bramley *et al.*, 1990). Moreover, after the induction of tone by acetylcholine, the contractile response elicited by bradykinin was changed to a relaxation (Bramley *et al.*, 1990). The results of the present study show that when tracheal preparations with or without epithelium were equilibrated at a resting tone, bradykinin induced a concentration-related contraction. When tracheal preparations were precontracted, bradykinin induced a concentration-related relaxation. However, in the absence of epithelium, the relaxation induced by bradykinin was smaller (63 ± 13% relaxation in the presence of epithelium vs. 42 ± 6% relaxation in the absence of epithelium). This may be related to a reduced production of prostaglandins in tracheal preparations without epithelium (Bramley *et al.*, 1990). Such a dual behaviour, related to the tone of the guinea-pig isolated trachea and independent of the epithelium, has also been reported for prostaglandin $E_2$ (Braunstein *et al.*, 1988). Melittin, a direct activator of phospholipase $A_2$, can also induce contraction or relaxation in guinea-pig tracheal preparations with or without epithelium. Melittin-induced effects were unaffected by bradykinin-receptor antagonists. Another difference between the effects of bradykinin and melittin is the sensitivity of the contractile response to melittin to 5-lipoxygenase inhibition (Yamamoto *et al.*, 1990; Shikada & Tanaka, 1993). This observation is in agreement with the activation of phospholipase $A_2$ by melittin, since arachidonic acid is the substrate of both cyclo-oxygenase and 5-lipoxygenase.

Another important finding of the present study is the fact that indomethacin can suppress the relaxation induced by bradykinin. These results suggest that, upon blockade of prostaglandin synthesis, bradykinin cannot induce the release of nitric oxide or that nitric oxide alone is unable to induce tracheal relaxation (Schlemper & Calixto, 1994). In the presence of indomethacin, the bradykinin-induced contraction was reduced, although a small contraction persisted for bradykinin concentrations up to 1 μM. These results agree with previous reports that bradykinin-induced effects *in vitro* are largely related to prostaglandin synthesis in guinea-pig tracheal preparations (Farmer *et al.*, 1989; Bramley *et al.*, 1990).

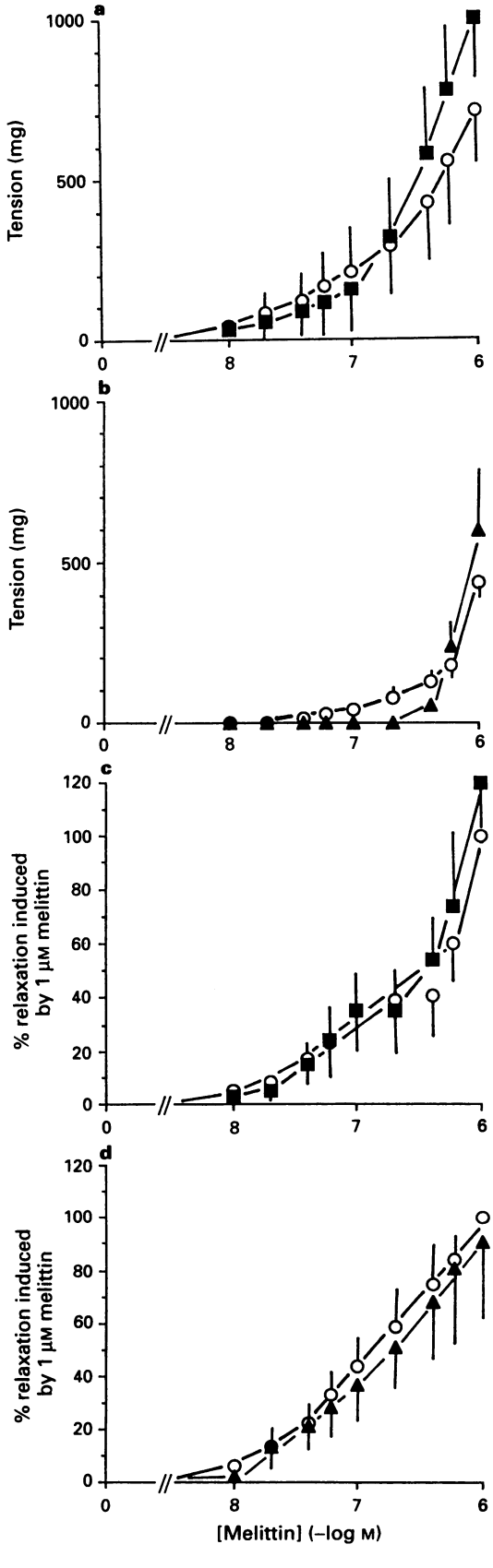


**Figure 4** Effect of bradykinin receptor antagonists on melittin-induced contraction and relaxation on guinea-pig tracheal preparations. Tracheal preparations were suspended under 2 g tension before starting the concentration-response curve for melittin in the absence (○) or (a) in the presence of Hoe 140 (■, 0.6 μM, $n = 6$) or (b) in the presence of NPC 17761 (▲, 1μM, $n = 3$). Alternatively, tracheal preparations were suspended under 2 g tension then stimulated with histamine (30 μM) before starting the concentration-response curve for melittin in the absence (○) or (c) in the presence of Hoe 140 (■, 0.6 μM, $n = 6$) or (d) in the presence of NPC 17761 (▲, 1μM, $n = 5$). The tensions induced by histamine were 1367 ± 195 mg (c) and 1290 ± 447 mg (d).

Under the conditions used in the present study, the responses obtained were fully reproducible. We therefore investigated the possibility of the activation of a bradykinin receptor leading to an induction of relaxation. Kinins receptors have been subdivided into two types (Regoli & Barabé, 1980): $B_1$, activated by [des-Arg$^9$]-bradykinin, with a higher potency than bradykinin, and antagonized by [Leu$^8$, des-Arg$^9$]-bradykinin, and $B_2$ for which these agents have low affinity. The first $B_2$-receptor antagonist, [D-Phe$^7$]-bradykinin, was described by Vavrek & Stewart (1985) and has since been followed by many analogues with improved affinities and selectivities (see Hall, 1992). However, these $B_2$-receptor antagonists maintain low affinity and partial agonist activity especially in the guinea-pig trachea (Stewart & Vavrek, 1990; Regoli *et al.*, 1990). Such compounds led Farmer *et al.* (1989), to suggest the presence of a putative bradykinin $B_3$-receptor in airway smooth muscle. Recently, Farmer & DeSiato (1994) described a novel nonpeptide bradykinin $B_2$ receptor antagonist (WIN 64338) selective for ileal receptors, i.e. without effect on bradykinin-induced contraction of guinea-pig trachea. $B_1$-receptor agonists and $B_2$-receptor antagonists have been described as having no effect on bradykinin-induced relaxation (Rhaleb *et al.*, 1988). Therefore, it was proposed that bradykinin may activate directly one of the enzymes involved in the arachidonic acid cascade, including phospholipase $A_2$. Alternatively, these authors proposed that the guinea-pig trachea may contain a new type of receptor for kinins which is not blocked by the $B_1$- or $B_2$-receptor antagonists. Bramley *et al.* (1989) have reported that bradykinin-induced contraction was inhibited by the $B_2$-receptor antagonist, D-Arg,[Hyp$^3$,Thi$^{5,8}$,D-Phe$_7$]-bradykinin whereas the bradykinin-induced relaxation was potentiated. These observations led them to suggest that this antagonist may act as a partial agonist for other bradykinin receptors. The results of the present study do not agree with a direct activation of phospholipase $A_2$ by bradykinin nor with the involvement of a non-$B_1$, non-$B_2$ bradykinin receptor. We show that bradykinin-induced relaxation is reduced in the presence of $B_2$-receptor antagonists, Hoe 140 or NPC 17761. As for the contraction induced by bradykinin (Trifilieff *et al.*, 1992; 1993a), Hoe 140 behaved as a non-competitive antagonist and NPC 17761 as a competitive antagonist on bradykinin-induced relaxation. The $B_1$-receptor agonist, [des Arg$^9$]-bradykinin was without effect on guinea-pig trachea. The non-competitive properties of Hoe 140 in the guinea-pig trachea remain unexplained. The existence of $B_2$-receptor subtypes cannot be excluded considering the recent results of Farmer & DeSiato (1994). Also, a low-affinity binding site has been detected in tracheal preparations with epithelium but not in preparations without epithelium (Trifilieff *et al.*, 1992). This binding site may be related to the activation of a receptor in epithelial cells which is not involved in bradykinin-induced tone or may belong to non-receptor proteins such as enzymes involved in the degradation of kinins. For example, endopeptidase 24.11 has been shown to be located mainly in the epithelium (Johnson *et al.*, 1985).

In summary, the present study provides consistent evidence supporting the view that both bradykinin-induced contraction or relaxation are mediated by a $B_2$-receptor in guinea-pig trachea. The response, relaxation or contraction, is not related to the presence or absence of the epithelium but to the tone of the isolated trachea.

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# Evidence that nitric oxide from the endothelium attenuates inherent tone in isolated pulmonary arteries from rats with hypoxic pulmonary hypertension

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**1** The inherent contractile tone, and its modulation by the endothelium, have been studied in isolated pulmonary artery preparations taken from rats in which pulmonary hypertension was induced by exposure to a hypoxic environment (10% $O_2$) for 14 days. Control rats were housed in room air.

**2** All preparations in which the endothelium was left intact relaxed in response to acetylcholine (43 ± 4% and 54 ± 9%, reversal of the noradrenaline-induced contraction in control and hypoxic rats, respectively) indicating that the endothelium was functional in both groups of rats.

**3** Exposure of the preparations to $Ca^{2+}$-free physiological salt solution containing 2 mM EGTA for 30–40 min had no effect on preparations from control rats but caused relaxation in preparations from hypoxic rats. The relaxation (taken as a measure of the inherent tone in the preparations) was larger in preparations without endothelium (14.5 ± 1.9 mN $mm^{-2}$; $n = 5$) than in preparations with endothelium (9.1 ± 1.2 mN $mm^{-2}$; $n = 5$).

**4** In preparations from hypoxic rats the magnitudes of the contractions to 80 mM $K^+$ and to noradrenaline (0.1 μM) were less than in preparations from control rats. This may have been because the preparations from hypoxic rats were already partially contracted due to the inherent tone.

**5** The nitric oxide (NO) synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME, 0.1–100 μM) had negligible effect on preparations from control rats or on endothelium-denuded preparations from hypoxic rats, but produced concentration-dependent contractions (maximum contraction 7.4 ± 0.7 mN $mm^{-2}$ ($n = 4$) with 100 μM) in endothelium-intact preparations from hypoxic rats. This effect of L-NAME was prevented by L-arginine (1 mM) but not by D-arginine (1 mM).

**6** Contractions to L-NAME were also seen in endothelium-intact arteries from control rats if the preparations were first partially contracted by exposure to $K^+$, endothelin, U46619 (thromboxane-mimetic) or noradrenaline.

**7** It is concluded that isolated pulmonary artery rings from hypoxic rats, but not those from control rats, have substantial inherent tone. This inherent tone is normally attenuated by the generation of an endothelium-derived factor that is probably NO. A stimulus for the release of NO from the endothelium may be the contraction of the underlying smooth muscle, whether the contraction is inherent in the tissue, as in preparations from hypoxic rats, or is induced by a vasoconstrictor spasmogen.

**Keywords:** Hypoxia; pulmonary hypertension, rat pulmonary artery; endothelium; inherent tone, nitric oxide; $N^G$-nitro-L-arginine methyl ester (L-NAME)

## Introduction

Chronic exposure of rats to a hypoxic environment causes pulmonary hypertension accompanied by characteristic pathophysiological changes such as right ventricular hypertrophy and pulmonary vascular remodelling (Reid, 1979; Wanstall *et al.*, 1992). Pulmonary arteries from these rats, whether studied in perfused lungs or as isolated ring preparations, show not only altered reactivity to various vasoactive agents but also some inherent contractile tone (Emery *et al.*, 1981; Wanstall & O'Donnell, 1992; Wanstall *et al.*, 1992).

Our initial evidence that pulmonary artery ring preparations from hypoxic rats had inherent tone, i.e. were not fully relaxed at the applied resting force, was obtained in a study on relaxant responses to the vasodilator drug, pinacidil, in preparations precontracted with a spasmogen. Pinacidil not only reversed the contraction induced by the spasmogen but further relaxed the preparations to below the resting baseline (Wanstall & O'Donnell, 1992). In corresponding experiments with nitroprusside, the evidence for inherent tone (i.e. relaxation to below the resting baseline) was seen only in preparations from which the endothelium had been removed (Wanstall *et al.*, 1992). This suggested that a substance(s) generated by the endothelium might attenuate this inherent tone. The aim of the current study was to compare quantitatively the inherent tone in pulmonary arteries from hypoxic rats in preparations with and without endothelium, and to investigate whether nitric oxide (NO) generated from endothelial cells might be involved in attenuating the inherent tone.

A preliminary account of some of these data was presented to a joint meeting of the British and Italian Pharmacological Societies in Rome (Wanstall & Hughes, 1994).

## Methods

Male Wistar rats (6 weeks old; 165–235 g) were housed in room air (control rats) or in 10% oxygen (hypoxic rats) for 14 days (Wanstall *et al.*, 1992). On the day of the experiment each rat was anaesthetized with pentobarbitone (90 mg $kg^{-1}$, i.p.), the trachea was cannulated and the rat was artificially ventilated. The thorax was opened and a heparin-filled hypodermic needle (23G, with the sharp point removed),

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connected to a Bentley Trantec pressure transducer (Model 60-800), was inserted into the right ventricle and carefully advanced into the main pulmonary artery. The pulmonary artery pressure (PAP) was recorded on a Ugo Basile Gemini recorder (Wanstall & O'Donnell, 1990). The placement of the needle in the pulmonary artery was confirmed from the waveform of the pressure record, which differed from that obtained when the needle was withdrawn to the right ventricle. Mean PAP was taken as diastolic PAP + 1/3 (systolic – diastolic PAP). A blood sample was removed for measurement of haematocrit. The pulmonary artery, heart and lungs were removed and the weights (corrected for body weight) of the right ventricle (RV) and of the left ventricle plus septum (LV + S), the ratio RV/(LV + S) and the ratio of lung wet to dry weight determined. Increases in mean PAP, haematocrit, RV/(LV + S) or lung wet/dry weight in hypoxic rats, compared with controls, were taken as indicators of pulmonary hypertension, polycythemia, right ventricular hypertrophy and lung oedema respectively.

Single rings (3 mm) of main pulmonary artery were set up in physiological salt solution (PSS) at 37°C in a vertical organ bath. The composition of the PSS was (mM): NaCl 118, KCl 5.9, $CaCl_2$ 1.5, $MgSO_4$ 0.72, $NaHCO_3$ 25, glucose 11.7, ascorbic acid 1.14 (95% $O_2$/5% $CO_2$; pH 7.4). When appropriate, the endothelium was removed by gently rubbing the luminal surface of the preparations with small forceps. Force in the circular muscle was recorded isometrically. The resting forces for the preparations were 10 mN (control rats) or 20 mN (hypoxic rats), unless otherwise stated, to take into account the differing diastolic pulmonary artery pressures to which the vessels from the two different groups of rats are exposed *in vivo*. These forces were based on data from a previous study in which we determined the points of intersection of the isobars (based on the Law of Laplace) for 10 or 20 mmHg with plots of passive wall tension vs. internal radius in preparations from control and pulmonary hypertensive rats, respectively (Wanstall & O'Donnell, 1990). The values of wall tension at these points of intersection were converted to force by multiplying by twice the length (3 mm) of the preparations. This procedure is a modification of the procedure described by Mulvany & Halpern (1977) and Angus *et al.*, (1986). Retrospective analysis of the data showed that, in the preparations used in the present study, these selected resting forces were equivalent to pressures of 10.3 ± 0.1 mmHg (control rats, $n = 25$) and 20.5 ± 0.3 mmHg (hypoxic rats, $n = 25$). The diastolic pulmonary artery pressures in the rats were: control rats 14 ± 2 mmHg; hypoxic rats 24 ± 1 mmHg.

At the end of each experiment, the distance between the wires (with the preparation under the selected resting force, see above) was measured using the micrometer attached to the force transducer. The wet weight of the preparation (after blotting for 30 s between filter papers) was determined. The cross-sectional area of the preparation ($mm^2$), in the plane perpendicular to the direction of the developed force, was then calculated as described by Wanstall & O'Donnell (1992) from the formula:

$$\text{cross sectional area} = w(hd)^{-1}$$

where h = the distance between the wires plus the diameters of the two wires (mm), w = wet weight (mg) and d = density = 1.06 mg $mm^{-3}$ (Murphy, 1980). Since all preparations were the same length (3 mm) an increase in cross-sectional area reflected an increase in vessel wall thickness and hence the presence of pulmonary vascular hypertrophy.

### *Experimental protocols*

All pulmonary artery preparations were allowed to equilibrate in PSS for 1 h. They were then checked for the presence or absence of a functional endothelium by submaximally contracting the preparations with 0.1 μM noradrenaline and, when the contraction had reached equilibrium, adding 1 μM acetylcholine (ACh). A reference contraction to $K^+$ depolarizing PSS (in which 80 mM NaCl was replaced with 80 mM KCl) was then obtained. The preparations were washed with PSS so that baseline force was restored, and then one of three different experimental protocols was carried out, as follows:

#### *Protocol A*

The PSS bathing the preparation was replaced with $Ca^{2+}$-free PSS (PSS with the $Ca^{2+}$ omitted) containing 2 mM EGTA. The resulting decrease in force (relaxation), which occurred over a period of 30–40 min of washing in the $Ca^{2+}$-free solution, was a measure of the inherent tone in the preparation.

#### *Protocol B*

A cumulative concentration-response curve to L-NAME (0.1, 1, 10, 100 μM), an inhibitor of NO synthase, was obtained. Contractile responses were taken as an indication that there was attenuation of tone by endogenous NO. In some experiments, L- or D-arginine (1 mM) was included in the PSS 15 min before the L-NAME. This was to confirm that the effects of L-NAME were due to inhibition of NO synthase.

#### *Protocol C*

Preparations (from control rats only) were submaximally contracted with $K^+$ (20 mM), noradrenaline (0.1 μM), U46619 (10 nM; thromboxane-mimetic) or endothelin-1 (ET-1;5 nM). At equilibrium (approximately 30 min) a cumulative concentration-response curve to L-NAME was obtained as in protocol B above.

### *Analysis of data*

Contractions or relaxations were measured as force (mN) and were normalized for vessel wall thickness by dividing values of force by the cross sectional area of the tissue to give values in mN $mm^{-2}$. The maximum relaxation (from resting baseline force) induced by $Ca^{2+}$-free PSS containing EGTA was used to quantify inherent tone in the preparations. Relaxations to ACh were also expressed as 'percentage reversal' of the noradrenaline-induced contraction.

### *Drugs and solutions*

Acetylcholine chloride (ACh, Sigma); D- and L-arginine (Sigma); endothelin-1 (Auspep, Australia); ethylene glycol-*bis*(β-amino-ethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA; Sigma); (−)-noradrenaline acid tartrate (Sigma); $N^G$-nitro-L-arginine methyl ester (L-NAME; Sigma); U46619 (9,11-dideoxy-11$_\alpha$, 9$_\alpha$-epoxymethano-prostaglandin $F_{2\alpha}$; Upjohn); Solutions of acetylcholine (10 mM), endothelin-1 (10 μM) and L-NAME (10 mM) were prepared in deionised water, of noradrenaline (10 mM) in 10 mM HCl and of U46619 (10 mM) in ethanol. All dilutions were made in PSS and were kept on ice during the experiment.

### *Statistical analyses*

Mean values were calculated from data obtained in preparations from a number ($n$) of different animals and are quoted together with their standard errors (s.e.mean). The statistical significance of differences between mean values has been assessed by Student's $t$ test (for values of neg log $EC_{50}$ and mN $mm^{-2}$) or Mann Whitney U test (for percentage values).

## Results

### *Effects of hypoxia on the rats*

Chronic (14 day) exposure of rats to 10% oxygen (hypoxic rats) resulted in changes consistent with the development of hypoxic pulmonary hypertension, *viz.* retarded growth, polycythemia, elevated pulmonary artery pressure, right ventricular hypertrophy and pulmonary vascular hypertrophy, but no lung oedema (Table 1).

### *Responses to ACh on noradrenaline-contracted pulmonary artery preparations*

The pulmonary artery endothelium was functional in control rats and also in rats exposed to hypoxia for 14 days, in that all preparations with intact endothelium relaxed in response to ACh (Table 2). Preparations from which the endothelium had been removed did not relax to ACh. The ACh responses in preparations with endothelium, when expressed as 'percentage reversal' of the contractile response to 0.1 μM noradrenaline, were not significantly different in arteries from hypoxic or control rats (Table 2). However the actual magnitude (mN mm$^{-2}$) of the relaxant responses to ACh on these preparations, and also the contractile responses to noradrenaline (on preparations with or without endothelium), were both significantly less in preparations from hypoxic rats than in those from control rats (Table 2).

### *Effects of $Ca^{2+}$-free PSS containing EGTA on pulmonary artery preparations*

Replacement of the PSS with $Ca^{2+}$-free PSS containing EGTA had no effect on the resting force of pulmonary artery preparations from control rats, whether the endothelium was present or not. This suggested that these preparations were already fully relaxed, i.e. had no inherent tone (Figure 1). In contrast, in pulmonary artery preparations from hypoxic rats, marked relaxation occurred when the PSS was replaced with $Ca^{2+}$-free PSS plus EGTA, suggesting that these preparations were not fully relaxed when in normal PSS, i.e. they had inherent tone (Figure 1). Furthermore the relaxations produced by $Ca^{2+}$-free PSS plus EGTA (stippled columns in Figure 1) were significantly greater on preparations without endothelium (14.5 ± 1.9 mN mm$^{-2}$, $n = 5$) than on those with endothelium (9.1 ± 1.2 mN mm$^{-2}$, $n = 5$, $P < 0.05$). This suggested that an endothelium-derived substance might attenuate, in part, the inherent tone in pulmonary arteries from hypoxic rats.

On all preparations from hypoxic rats, whether with or without endothelium, (i.e. on those preparations that had inherent tone), the contractile responses to 80 mM $K^+$ were significantly smaller than those in preparations from control rats (Figure 1, open columns). The combined values for the

**Table 1** Effects of exposure of rats to 10% oxygen (hypoxic rats) or room air (control rats) for 14 days

| | *Control rats* ($n = 29$) | *Hypoxic rats* ($n = 25$) |
|---|---|---|
| Weight after exposure (g) | 312 ± 6 | 245 ± 5††† |
| Haematocrit (%) | 42 ± 1 | 59 ± 1*** |
| Mean PAP (mmHg) | 17 ± 1[a] | 28 ± 1*** |
| RV/(LV + S) | 0.27 ± 0.01 | 0.50 ± 0.01*** |
| RV/body weight (mg/g)[b] | 0.54 ± 0.01 | 1.11 ± 0.02*** |
| (LV + S)/body weight (mg/g)[b] | 2.02 ± 0.02 | 2.23 ± 0.03*** |
| Cross-section of pulmonary artery preparations (mm$^2$) | 0.54 ± 0.02 | 0.98 ± 0.02*** |
| Lung wet/dry weight | 4.61 ± 0.05 | 4.68 ± 0.02 |

Values are means ± s.e.mean. $n$ = number of rats.
[a]Pulmonary artery pressure (PAP) was determined in 16 of the 29 control rats. [b]These values indicate that the increase in RV/(LV + S) in hypoxic rats reflects an increase in RV weight (i.e. RV hypertrophy) and not a decrease in the weight of (LV + S).
†††Value significantly less than value in control rats $P < 0.001$, Student's $t$ test. ***Value significantly greater than value in control rats $P < 0.001$, Student's $t$ test.

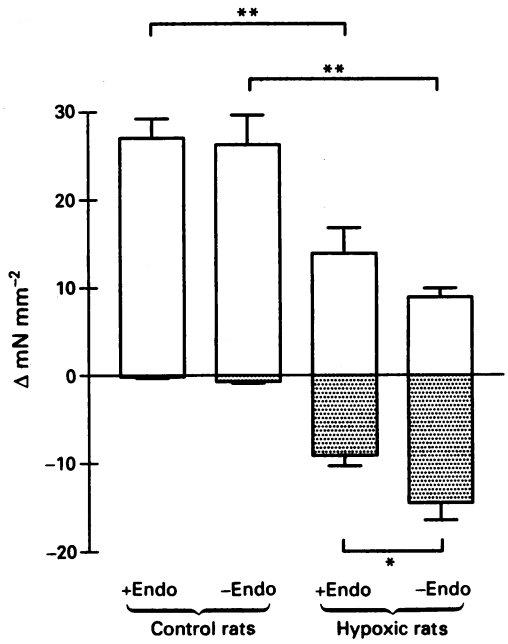


**Figure 1** Relaxation, from resting baseline, induced by $Ca^{2+}$-free PSS containing 2 mM EGTA (representing inherent contractile tone; stippled columns) and contractions, from resting baseline, induced by 80 mM $K^+$ (open columns) in rat pulmonary artery preparations. Data, in mN mm$^{-2}$, obtained in preparations with (+ Endo) and without (− Endo) endothelium, taken from control and hypoxic rats are shown. $n = 5$ rats for each group of data. *Inherent contractile tone in preparations without endothelium significantly greater than that in preparations with endothelium, $P < 0.05$ (Student's $t$ test). **Contraction to 80 mM $K^+$ in preparations from hypoxic rats significantly smaller than that in corresponding preparations from control rats, $P < 0.01$ (Student's $t$ test).

**Table 2** Relaxant responses to acetylcholine (ACh, 1 μM) in noradrenaline (NA, 0.1 μM)-contracted pulmonary artery preparations taken from control and hypoxic rats

| | *With endothelium* | | *Without endothelium* | |
|---|---|---|---|---|
| | *Control rats* ($n = 18$) | *Hypoxic rats* ($n = 16$) | *Control rats* ($n = 11$) | *Hypoxic rats* ($n = 9$) |
| *ACh relaxation* | | | | |
| % reversal of NA | 43 ± 4 | 54 ± 9 | 2 ± 1 | No |
| mN mm$^{-2}$ | 9.6 ± 1.0 | 4.6 ± 0.5*** | 0.7 ± 0.3 | relaxation |
| *NA contraction* | | | | |
| mN mm$^{-2}$ | 23.5 ± 2.0 | 9.2 ± 0.9*** | 29.6 ± 3.2 | 5.5 ± 0.6*** |

Values are means ± s.e.mean, $n$ = number of animals.
***Value significantly smaller than the corresponding value in control rats $P < 0.001$, (Student's $t$ test).

contractile force inherent in the tissue (quantified as the relaxation that occurred in $Ca^{2+}$-free PSS containing EGTA, and referred to as inherent tone) plus the contractile force generated by 80 mM $K^+$ were not significantly different in the four groups of tissues (Figure 1; combined values (mN $mm^{-2}$, $n = 5$): *control*, endothelium-intact 27.2 ± 2.2; endothelium-denuded 27.0 ± 3.2; *hypoxic*, endothelium-intact 23.0 ± 2.9; endothelium-denuded 23.4 ± 1.8). These observations indicated that when there was inherent tone in the preparations there was a corresponding reduction in the size of the $K^+$-induced contraction.

### *Effects of the NO-synthase inhibitor, L-NAME, on pulmonary artery preparations*

To investigate whether NO might be the endothelium-derived substance attenuating the inherent tone in pulmonary arteries from hypoxic rats, experiments were carried out in which the generation of NO was inhibited by L-NAME. Addition of L-NAME to pulmonary arteries from hypoxic rats resulted in concentration-dependent contractions in preparations with the endothelium present, but not in preparations with the endothelium removed (Figure 2a). The contractions to L-NAME were abolished by L-arginine but were unaffected by D-arginine (Figure 2b) confirming that the effects of L-NAME were due to inhibition of NO synthase. These data showed that pulmonary arteries from hypoxic rats could gain tone if the endothelial production of NO was prevented.

In contrast L-NAME had negligible effect on pulmonary arteries (endothelium intact) from control rats (maximum contraction 0.4 ± 0.16 mN $mm^{-2}$, $n = 4$). The lack of effect was not due to the lower passive force used in these preparations (10 mN), since L-NAME also had a negligible effect in control preparations set at the higher passive force of 20 mN that was used in preparations from hypoxic rats (maximum contraction to L-NAME 0.6 ± 0.14 mN $mm^{-2}$, $n = 4$).

### *Effects of L-NAME on $K^+$-contracted pulmonary artery preparations from control rats*

To investigate whether NO might be released from the endothelium when the tone was acutely increased by a spasmogen, preparations from control rats were partially contracted with $K^+$, ET-1, noradrenaline or U46619 and then exposed to L-NAME (0.1–100 μM). The concentrations of the spasmogens (see Methods) were selected so that the contractile responses were submaximal and approximately matched the inherent tone seen in pulmonary arteries from hypoxic rats (9.1 and 14.5 mN $mm^{-2}$, respectively, see above). The contractions to the spasmogens were (mN $mm^{-2}$): *with endothelium*: $K^+$ 16.9 ± 3.5 ($n = 5$); ET-1 10.7 ($n = 1$); noradrenaline 12.0 ($n = 1$); U46619 8.8 ($n = 1$); *without endothelium*: $K^+$ 15.5 ± 3.6 ($n = 4$). Provided the endothelium was intact, L-NAME (1–100 μM) produced contractile responses on preparations when pre-contracted with each of these spasmogens. The data on preparations contracted with 20 mM $K^+$ are shown in Figure 2c. Comparable contractile responses to L-NAME were obtained on the preparations that were precontracted with ET-1, noradrenaline or U44619 (data not shown). In contrast, contractions to L-NAME were not seen on $K^+$-contracted preparations if the endothelium was removed, except for 1/4 preparations where the highest concentration (100 μM) caused a contraction of 7.5 mN $mm^{-2}$ (Figure 2c).

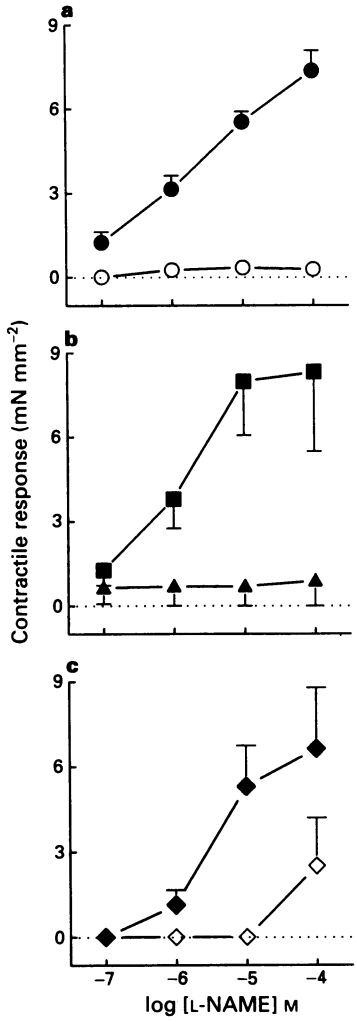


**Figure 2** Concentration-response (contraction) curves to $N^G$-nitro-L-arginine methyl ester (L-NAME) in pulmonary artery preparations. Standard errors of mean responses are shown. (a) Data in preparations taken from hypoxic rats (i.e. preparations with inherent tone), with (● $n = 4$) and without (○ $n = 4$) endothelium. (b) Data in preparations (with endothelium) taken from hypoxic rats, in the presence of 1 mM L-arginine (▲ $n = 4$) or 1 mM D-arginine (■ $n = 3$). (c) Data in $K^+$ (20 mM)-contracted preparations, with (◆ $n = 5$) and without (◇ $n = 4$) endothelium, taken from control rats.

## Discussion

The data obtained in the present study (a) have shown that isolated pulmonary artery preparations from rats exposed to a hypoxic environment for 14 days have inherent tone, (b) have confirmed that these arteries still have a functional endothelium and (c) suggest that the inherent tone is attenuated by NO released from the endothelium.

Inherent tone was quantified in the present study by measuring the relaxation (from resting baseline) induced by bathing the preparations for 30–40 min in $Ca^{2+}$-free PSS containing 2 mM EGTA. Using this criterion, inherent tone was seen in arteries from hypoxic rats but not from control rats, confirming previous observations using a different approach (see Introduction: Wanstall & O'Donnell, 1992; Wanstall *et al.*, 1992). Moreover the inherent tone was significantly larger in endothelium-denuded preparations than in endothelium-intact preparations. This provided evidence that the inherent tone was modulated by a factor from the endothelium. The factor responsible for modulating the inherent tone is probably NO since L-NAME, over the concentration-range known to inhibit NO synthase (Rees *et al.*, 1990), contracted endothelium-intact arteries from hypoxic rats. These contractions were inhibited by L-arginine but not by D-arginine. The absence of responses to L-NAME in endothelium-denuded tissues confirmed that the endothelium was the source of the NO. It is interesting to note that the maximum contraction to L-NAME (approximately 7 mN $mm^{-2}$) was as big as the difference between the

inherent tone in endothelium denuded and intact preparations (5.4 mN mm$^{-2}$). Thus, it is possible that release of NO could account completely for the modulation of the inherent tone by the endothelium.

Preparations without inherent tone, (i.e. fully relaxed preparations obtained from control rats) did not contract in response to L-NAME, suggesting that there was no basal release of NO. Nor did they contract in response to L-NAME if the passive (resting) tension was increased by stretching the tissues. However, L-NAME did produce contractions in arteries (endothelium-intact) from control rats if the preparations were pre-contracted with $K^+$ to a level corresponding to the inherent tone seen in arteries from hypoxic rats. Therefore, we postulate that in rat pulmonary artery, release of NO from the endothelium may occur as a consequence of smooth muscle contraction, whether inherent in the tissue or induced by a spasmogen. This postulate was supported by the finding that L-NAME also contracted preparations that were pre-contracted with endothelin-1, U46619 (thromboxane-mimetic) or noradrenaline, and implies that any procedure which results in contraction of rat pulmonary artery should be accompanied by, and may be modulated by, the release of NO.

Our findings that endothelium-derived NO modulates (a) inherent tone in pulmonary arteries from hypoxic rats and (b) contractions induced by various spasmogens in arteries from control rats are compatible with recent data obtained in perfused lungs from hypoxic rats (Barer *et al.*, 1993; Oka *et al.*, 1993) and in isolated pulmonary arteries from normal sheep (Bansal *et al.*, 1993). In lungs taken from hypoxic rats and perfused via the pulmonary artery, the resting perfusion pressure is higher than in lungs from control rats. The elevated pressure is due partly to inherent muscle tone in the pulmonary vasculature in hypoxic rats (Barer *et al.*, 1993). In lungs from hypoxic rats the resting perfusion pressure was increased by the addition of an NO-synthase inhibitor to the perfusate (Oka *et al.*, 1993; Barer *et al.*, 1993). This suggested that NO was being released and was modulating the vasoconstrictor tone. In contrast, in lungs from control rats NO synthase inhibitors did not increase resting perfusion pressure but they did augment vasoconstrictor responses to alveolar hypoxia (Oka *et al.*, 1993; Barer *et al.*, 1993), endothelin or almitrine (Barer *et al.*, 1993). An increase in intraluminal pressure did not in itself cause the release of NO (Oka *et al.*, 1993; Barer *et al.*, 1993) and it was concluded that the stimulus for the release of NO in perfused lungs was an increase in shear stress resulting from the narrowing (constriction) of the pulmonary vessels (Barer *et al.*, 1993).

In sheep isolated pulmonary arteries, L-NAME caused contractions if the preparations were first partially contracted with phenylephrine but not if the preparations were under resting conditions (Bansal *et al.*, 1993). Thus, our experiments and those of Bansal *et al.* (1993) show that in isolated pulmonary arteries, as in perfused lungs, NO is released only when the smooth muscle is contracted. In isolated artery ring preparations there is neither intraluminal pressure nor shear stress. Thus the contraction of the smooth muscle, *per se*, appears to lead to the release of NO.

It has previously been postulated that in hypoxic rats, there may be an increase in NO release from the pulmonary vascular endothelium *in vivo*, and that this could result in desensitization of soluble guanylate cyclase (Wanstall *et al.*, 1992; Crawley *et al.*, 1992). This hypothesis was proposed in order to explain the decreased sensitivity to nitroprusside that is seen in pulmonary arteries taken from chronically hypoxic rats (Wanstall & O'Donnell, 1992; Wanstall *et al.*, 1992; Crawley *et al.*, 1992). The current *in vitro* finding that the inherent tone in pulmonary arteries from hypoxic rats is associated with NO release is compatible with this hypothesis. It was previously suggested that any augmented release of NO *in vivo* in hypoxic rats might be due to the increases in pulmonary artery pressure and/or pulmonary blood flow (Wanstall *et al.*, 1992; Crawley *et al.*, 1992); the results of the present study indicate that the actual contraction of the pulmonary vascular smooth muscle may also be partly responsible for the NO release.

The mechanism underlying the inherent tone is not known and was not the subject of this study. The procedure used in the present study to relax the inherent tone (i.e. prolonged exposure to $Ca^{2+}$-free PSS containing 2 mM EGTA) not only prevents $Ca^{2+}$ influx but also affects contractions that rely on intracellular sources of $Ca^{2+}$ (Wheeler & Weiss, 1979; Wanstall, unpublished). Thus no conclusions can be drawn from the experiments in this study as to the mechanism responsible for the inherent contractile tone. However, it is possible that the tone could be due, at least in part, to depolarization of the smooth muscle cell membrane, as described by Suzuki & Twarog (1982) and Smirnov *et al.* (1994) in pulmonary arteries from hypoxic rats. It was noted that contractions to both noradrenaline and potassium were smaller in preparations from hypoxic rats. This may be simply because the arteries are already partially contracted due to the presence of the inherent tone. The finding that summation of the inherent tone and the potassium-induced contraction gave values that were not statistically significantly different in all arteries (endothelium-intact and denuded from hypoxic and control rats) supports this view. It also argues against any suggestion that the overall contractile ability of the vascular smooth muscle is impaired in preparations from hypoxic rats, at least after 2 weeks of hypoxic exposure.

In conclusion, we have quantified the inherent tone in isolated pulmonary arteries from hypoxic rats. This tone, not seen in arteries from control rats, was considerable, amounting to about half the overall contractile ability of the artery preparations. It was shown to be modulated by NO released from the endothelium and we postulate that the stimulus for the release of NO may be the actual contraction of the vascular smooth muscle. If the inherent tone contributes to the elevation in pulmonary artery pressure in these rats, it follows that the severity of the pulmonary hypertension may depend on the functional state of the endothelium. The rats in this study were exposed to hypoxia for only 2 weeks, but more prolonged hypoxic exposure may result in impairment of endothelial cell function (Adnot *et al.*, 1991), diminished NO production, unopposed inherent tone and exacerbation of the elevation in pulmonary artery pressure. This principle could also apply to human subjects with secondary chronic hypoxic pulmonary hypertension, since functional impairment of the endothelium has been reported in pulmonary arteries from patients with various chronic end-stage lung diseases that are generally accompanied by pulmonary hypertension (Dinh-Xuan *et al.*, 1991).

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# The kinin $B_1$ receptor antagonist des-Arg$^9$-[Leu$^8$]bradykinin: an antagonist of the angiotensin $AT_1$ receptor which also binds to the $AT_2$ receptor

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**1** Agonists and antagonists of kinin $B_1$ and $B_2$ receptors were evaluated *in vitro* for their effects against angiotensin II (AII)-induced contractile responses in the rabbit aorta and for their binding properties to angiotensin $AT_1$ and $AT_2$ receptors from purified membrane of rat liver and lamb uterus respectively.

**2** In aortic rings, the kinin $B_1$ receptor antagonist, des-Arg$^9$-[Leu$^8$]bradykinin (BK) (3-100 μM) caused a concentration-dependent decrease in sensitivity and a depression of the maximum response to AII. Des-Arg$^{10}$-[Leu$^9$]kallidin (KD), des-Arg$^9$-BK, des-Arg$^{10}$-KD, BK or KD at 3 μM had no effect against AII-induced contractions.

**3** Des-Arg$^9$-[Leu$^8$]BK (3 or 100 μM) did not affect contractions of aortic rings to histamine, potassium chloride, endothelin-1, 5-hydroxytryptamine, noradrenaline and the thromboxane $A_2$-mimetic, U46619.

**4** Des-Arg$^9$-[Leu$^8$]BK displaced [$^{125}$I]-Sar$^1$-AII binding to the $AT_1$ subtype in rat liver membranes with a $K_i$ value of 1.1 ± 0.4 μM. Values of $K_i$ for des-Arg$^9$-BK and KD were 45 ± 13 μM and 25 ± 22 μM, respectively. The other kinin derivatives des-Arg$^{10}$-KD, BK and des-Arg$^{10}$-[Leu$^9$]KD at concentrations up to 100 μM did not bind to the $AT_1$ receptor.

**5** All the kinin derivatives except BK bound to $AT_2$ receptors in lamb uterus membranes. Values of $K_i$ for des-Arg$^9$-[Leu$^8$]BK, des-Arg$^{10}$-[Leu$^9$]KD, des-Arg$^9$-BK, des-Arg$^{10}$-KD and KD were 0.3 ± 0.1, 0.7 ± 0.1, 1.2 ± 0.3, 1.5 ± 0.3 and 7.0 ± 1.6 μM, respectively.

**6** In conclusion, des-Arg$^9$-[Leu$^8$]BK is an insurmountable antagonist of AII-induced contractions in the rabbit aorta and also binds with a relatively high affinity to $AT_1$ and $AT_2$ receptors in isolated membrane fractions. These additional properties of des-Arg$^9$-[Leu$^8$]BK should be considered when it is used as an antagonist to characterize kinin $B_1$ receptors.

**Keywords:** Kinin $B_1$ and $B_2$ receptors; angiotensin $AT_1$ and $AT_2$ receptors; rabbit aorta; vascular reactivity; binding study

## Introduction

Kinin receptors have been divided into $B_2$ and $B_1$ subtypes on a pharmacological basis (Regoli & Barabé, 1980). Kallidin (KD) and bradykinin (BK) which represent the tissular and circulating forms of kinins respectively, stimulate preferentially the $B_2$ receptor subtype (Regoli & Barabé, 1980; Hall, 1992). In contrast, kinin derivatives lacking the C-terminal arginine such as des-Arg$^9$-BK and des-Arg$^{10}$-KD have been described as selective agonists at kinin $B_1$ receptors (Regoli & Barabé, 1980). Kinin $B_1$ receptors are further characterized by the ability of des-Arg$^9$-[Leu$^8$]BK and des-Arg$^{10}$-[Leu$^9$]KD to antagonize solely $B_1$ receptor-mediated responses (Regoli & Barabé, 1980). In this regard, des-Arg$^9$-[Leu$^8$]BK has been described as a specific kinin $B_1$ receptor antagonist (Regoli & Barabé, 1980; Regoli *et al.*, 1990) and is widely used to assess the participation of kinin $B_1$ receptors in experimental pathological situations (Corrêa & Calixto, 1993; Perkins *et al.*, 1993).

In the present study, we show that des-Arg$^9$-[Leu$^8$]BK is an antagonist of angiotensin $AT_1$ receptors and also binds to the $AT_2$ subtype. The results were obtained from functional assays on isolated rabbit aorta contracted with angiotensin II (AII) and from binding experiments conducted in membrane preparations from rat liver and lamb uterus.

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## Methods

### *Rabbit isolated aorta*

Male New Zealand White rabbits weighing 2.5 to 3.5 kg were killed with an overdose of i.v. sodium pentobarbitone. The thoracic aorta was dissected out and immediately placed in a Krebs solution of the following composition (in mM): NaCl 119, KCl 4.7, $KH_2PO_4$ 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, ethylenediaminetetracetic acid (EDTA) 0.026, glucose 5.5, and bubbled with 95% $O_2$ plus 5% $CO_2$. Eight rings of 4 mm in length were prepared from the aortic segment and the endothelium was rubbed off *in vitro* with a pipe cleaner which was introduced into the vessel lumen and gently moved back and forth several times. Rings were then suspended on triangular stainless-steel wires (0.3 mm in diameter) in 20 ml jacketed organ baths maintained at 37°C. One hook was suspended from a Gould-Statham $UC_2$ or $UTC_2$ transducer, and the other was fixed to a plastic support leg. Changes in isometric tension were recorded continuously on two-channel recorders (Gould BS272 or Linseis Type 7025). Rings were left unstretched for 30 min and were then stretched in a stepwise fashion to a passive force of 2 g.

After 1 h of equilibration, arterial rings were contracted with an isotonic high KCl (75 mM)-containing Krebs solution and the baths washed 3 times with normal Krebs solution. This procedure was repeated 30 min later. After return to the baseline, BK (3 μM), KD (3 μM), des-Arg$^9$-BK (3 μM), des-

$Arg^{10}$-KD (3 μM), des-$Arg^9$-[$Leu^8$]BK (3 to 100 μM) or des-$Arg^{10}$-[$Leu^9$]KD (3 μM) was added and 10 min was then allowed before the cumulative concentration-response curve to AII (0.0003 to 1 μM). The effect of the kinin $B_1$ receptor antagonist, des-$Arg^9$-[$Leu^8$]BK (3 or 100 μM) against cumulative concentration-response curves to histamine, KCl, endothelin-1, 5-hydroxytryptamine, noradrenaline or U46619 was also evaluated. Only one concentration-contraction curve was obtained in any one ring of artery.

### *Binding experiments*

Liver membranes were purified from male Wistar rats weighing 200 to 250 g (Iffa Credo, L'Arbresle, France) according to Neville (1968) and stored frozen in liquid nitrogen. Competition binding assays were carried out in 50 mM Tris-HCl buffer at pH 7.4 containing 5 mM $MgCl_2$, 1 mg $ml^{-1}$ bovine serum albumin (BSA) and 1 mg $ml^{-1}$ bacitracin, using [$^{125}$I]-$Sar^1$-AII as ligand, 15 μg membrane protein per assay (final volume 90 μl) and 30 min reaction times at 30°C.

Lamb uteri obtained from animals freshly-slaughtered at a local abattoir were ground in about 8 volumes of ice-cold grinding medium (10 mM Tris-HCl buffer at pH 7.4, 1 mM EDTA, 0.3 M KCl) using a Polytron apparatus, then a Dounce homogenizer. After 15 min of gentle stirring, the homogenate was filtered through two layers of gauze, then centrifuged for 20 min at 600 *g*. The supernatant was centrifuged for 20 min at 20,000 *g*. The resulting crude membrane pellets were washed twice with 10 mM Tris-HCl buffer at pH 7.4 containing 1 mM EDTA. Aliquots of the membrane suspension kept in the same buffer were stored frozen in liquid nitrogen. Before performing $AT_2$ binding experiments, membranes were treated with 20 mM dithiotreitol (DTT) added to the binding medium maintained at pH 7.8, in order to inactivate $AT_1$ receptor sites (Whitebread *et al.*, 1989). Competition binding assays were carried out in a binding medium containing 50 mM Tris-HCl buffer at pH 7.4, 125 mM NaCl, 6.5 mM $MgCl_2$, 1 mM EDTA, 2 mg/$ml^{-1}$ BSA, 1 mg/$ml^{-1}$ bacitracin, with [$^{125}$I]-$Sar^1$-AII used as displaced ligand, 50 μg membrane protein per assay (final volume 90 μl) and 30 min reaction times at 30°C. Kinin derivatives were tested in 0.001–100 μM range of concentrations. Protein measurements were performed according to Lowry *et al.* (1951).

### *Calculations and statistical analysis*

The negative logarithm of the concentration of agonist needed to reach 50% ($EC_{50}$) of the maximal response to this agonist in the rabbit aorta was calculated by least-square regression analysis (Tallarida & Murray, 1981). After testing for linearity and parallelism, $EC_{50}$ values for contraction and maximal responses ($E_{max}$) were compared by Student's *t* test. Data obtained from binding experiments are given as mean ± s.d.mean. In binding experiments, values of $K_i$ were determined as described by Ekins *et al.* (1968). n indicates the number of animals in vascular reactivity studies and the number of experiments in binding studies. The level of significance was $P<0.05$.

### *Drugs*

Bacitracin, bradykinin acetate, des-$Arg^9$-bradykinin acetate, des-$Arg^9$-[$Leu^8$]bradykinin acetate, dithiotreitol, histamine dihydrochloride, noradrenaline hydrochloride, 5-hydroxytryptamine creatinine sulphate complex and U46619 (1,5,5-hydroxy-11,9-(epoxymethano) prosta-5z, 13E-dienoic acid) were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.). Neosystem (Strasbourg, France) provided des-$Arg^{10}$-kallidin acetate and des-$Arg^{10}$-[$Leu^9$]kallidin acetate. Endothelin-1 and $Sar^1$-angiotensin II were from Bachem (Bubendorf, Switzerland). $Sar^1$-angiotensin II was radioiodinated (1800 Ci $mmol^{-1}$) as previously described (Bonnafous *et al.*, 1988). All drugs were made up in distilled water or dimethylsulphoxide and kept on ice and protected from light.

## Results

### *Vascular reactivity*

The effect of des-$Arg^9$-[$Leu^8$]BK on the cumulative concentration-contraction curve to AII is shown in Figure 1. Des-$Arg^9$-[$Leu^8$]BK (3-100 μM) produced a concentration-related, insurmountable antagonism of AII. The $EC_{50}$ value was 8.7 ± 0.1 in the absence and 7.8 ± 0.2, 7.3 ± 0.4, 6.8 ± 0.3, 6.3 ± 0.4 in the presence of 3, 10, 30 and 100 μM des-$Arg^9$-[$Leu^8$]BK, respectively. The maximum response was reduced to 65.4 ± 7.6%, 52.1 ± 7.2%, 47.1 ± 7.5% and 53.6 ± 10.4% in the presence of 3, 10, 30 and 100 μM des-$Arg^9$-[$Leu^8$]BK, respectively. Des-$Arg^9$-BK, BK, des-$Arg^{10}$-[$Leu^9$]KD, des-$Arg^{10}$-KD and KD at 3 μM had no effect against the cumulative response-curve to AII ($n = 4$/group; data not shown). Des-$Arg^9$-[$Leu^8$]BK at 3 μM had no effect against concentration-contraction curves to histamine, potassium chloride, endothelin-1 and the thromboxane $A_2$ mimetic, U46619 (Figure 2). Furthermore, des-$Arg^9$-[$Leu^8$]BK at 100 μM did not affect contractile responses to noradrenaline and 5-hydroxytryptamine, two agents that similar to AII produce contractions *via* $IP_3$-mediated increases in intracellular $Ca^{2+}$ (Figure 2).

### *Binding experiments*

Des-$Arg^9$-[$Leu^8$]BK bound to the rat liver $AT_1$ receptor (Figure 3 and Table 1) with a $K_i$ value of 1.1 ± 0.4 μM ($n = 4$). KD and des-$Arg^9$-BK displayed lower affinities towards $AT_1$ receptors with $K_i$ values of 25 ± 22 μM ($n = 3$) and 45 ± 13 μM ($n = 3$) respectively whilst BK, des-$Arg^{10}$-KD and des-$Arg^{10}$-[$Leu^9$]KD had no affinity for the $AT_1$ receptor ($K_i > 100$ μM, Table 1).

Kinin derivatives were also tested for their ability to reverse [$^{125}$I]-$Sar^1$-AII binding to the $AT_2$ receptor in lamb uterus membranes. As for the rat liver $AT_1$ receptor, des-$Arg^9$-[$Leu^8$]BK showed the highest affinity with a $K_i$ value of 0.3 ± 0.1 μM ($n = 4$, Figure 3 and Table 1). BK had no binding affinity up to 100 μM whilst KD and the other des-Arg compounds displayed $K_i$ values in the micromolar range (Table 1).

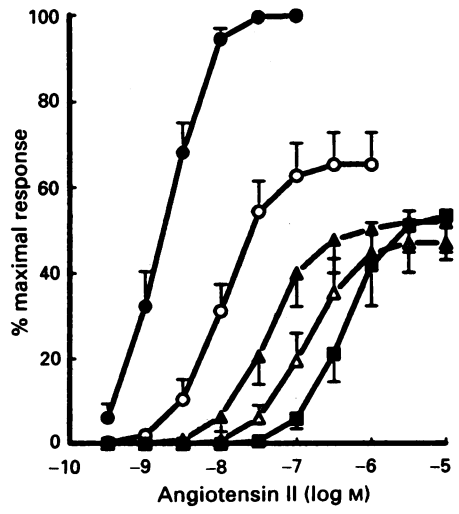


**Figure 1** Cumulative concentration-contraction curves for angiotensin II in the rabbit aorta in the absence (●) and presence of 3 (○), 10 (▲), 30 (△) and 100 μM (■) des-$Arg^9$-[$Leu^8$]BK. Values represent means ± s.e.mean of 6 experiments.

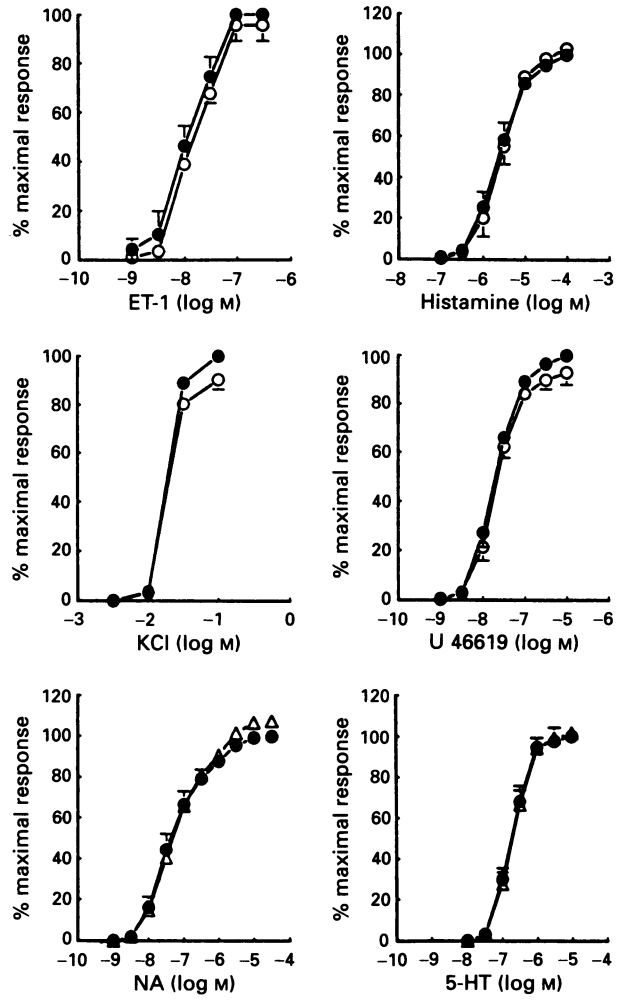


**Figure 2** Concentration-response curves to endothelin-1 (ET-1), histamine, potassium chloride (KCl), U46619, noradrenaline (NA), 5-hydroxytryptamine (5-HT) obtained in the absence (●) or presence of 3 μM (○) or 100 μM (△) des-Arg$^9$-[Leu$^8$]BK. Values represent means ± 1 s.e.mean of 4 experiments.

**Table 1** Binding of kinin derivatives to angiotensin II $AT_1$ and $AT_2$ receptor subtypes

| *Kinin derivatives* | $K_i$ (μM) $AT_1$ (rat liver) | $K_i$ (μM) $AT_2$ (lamb uterus) |
|---|---|---|
| BK | >100 | >100 |
| des-Arg$^9$-BK | 45 ± 13 | 1.2 ± 0.3 |
| des-Arg$^9$-[Leu$^8$]BK | 1.1 ± 0.4 | 0.3 ± 0.1 |
| KD | 25 ± 22 | 7.0 ± 1.6 |
| des-Arg$^{10}$-KD | >100 | 1.5 ± 0.3 |
| des-Arg$^{10}$-[Leu$^9$]KD | >100 | 0.7 ± 0.1 |

Competition experiments were carried out as described in the Methods section, using [$^{125}$I]-Sar$^1$-AII (0.2 nM) as radioactive ligand and 0.001–100 μM concentrations of the various kinin derivatives.
Values are means ± s.e. of 3–4 independent experiments. The assays within each experiment were carried out in triplicate.
Values of $K_i$ were calculated as described by Ekins *et al.* (1968).

## Discussion

The kinin $B_1$ receptor antagonist, des-Arg$^9$-[Leu$^8$]BK has been reported to have no antagonist effect against a number of other contractile agonists including AII (Regoli & Barabé, 1980; Regoli *et al.*, 1990). In the present study however, we have shown that des-Arg$^9$-[Leu$^8$]BK not only binds to $AT_1$ and $AT_2$ receptors but is also an insurmountable antagonist of AII-induced contractions in the rabbit aorta. The reason for the insurmountable antagonism by des-Arg$^9$-[Leu$^8$]BK which has been described for other peptide (Scanlon *et al.*, 1984) and nonpeptide (Timmermans *et al.*, 1991) $AT_1$ receptor antagonists, is unknown.

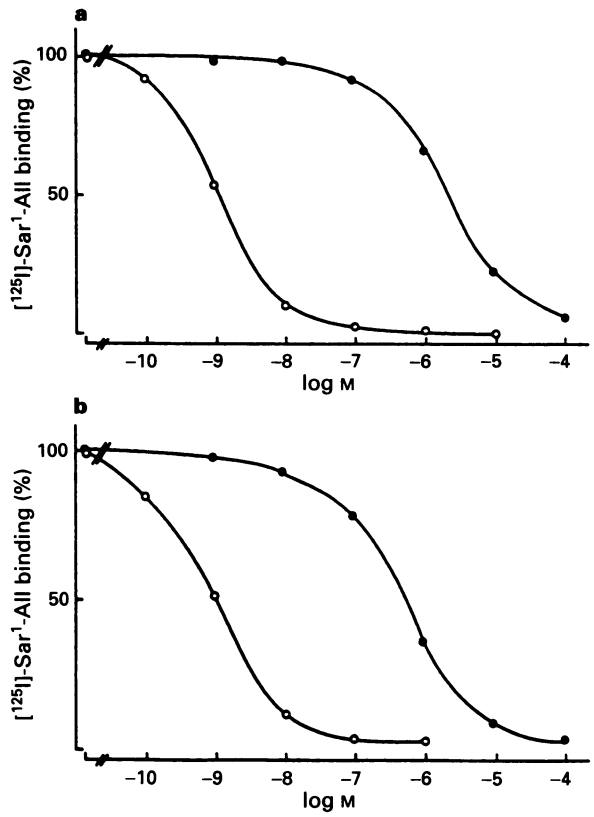


**Figure 3** Binding of des-Arg$^9$-[Leu$^8$]BK to the angiotensin $AT_1$ and $AT_2$ receptors. Representative typical competition experiments carried out as described in the Methods section, using [$^{125}$I]-Sar$^1$-AII (0.2 nM) as radioactive ligand in the presence of Sar$^1$-AII (○, 0.0001 to 10 μM) or des-Arg$^9$-[Leu$^8$]BK (●, 0.001 to 100 μM). Each point was obtained from a triplicate run. (a) Competition binding to $AT_1$ receptor from rat liver. The value of $K_i$ for des-Arg$^9$-[Leu$^8$]BK in this experiment was 0.8 μM. (b) Competition binding to $AT_2$ receptor from lamb uterus. The value of $K_i$ for des-Arg$^9$-[Leu$^8$]BK in this experiment was 0.16 μM.

Angiotensin receptors have been divided into $AT_1$ and $AT_2$ types both of which have been cloned (Murphy *et al.*, 1991; Sasaki *et al.*, 1991; Kambayashi *et al.*, 1993; Mukoyama *et al.*, 1993). $AT_1$ receptors mediate most of the biological responses hitherto attributed to AII whilst the physiological role of $AT_2$ receptors remains uncertain (Timmermans *et al.*, 1993). Binding studies performed in rat liver membrane preparations indicate that des-Arg$^9$-[Leu$^8$]BK bound to the $AT_1$ receptor whilst BK, KD, des-Arg$^{10}$-[Leu$^9$]KD and des-Arg$^{10}$-KD did not bind to the $AT_1$ receptor at concentrations up to 100 μM. In addition, des-Arg$^9$-BK, BK, des-Arg$^{10}$-[Leu$^9$]KD, des-Arg$^{10}$-KD and KD at 3 μM had no effect on AII-induced contractions. Thus, des-Arg$^9$-[Leu$^8$]BK appears to be unique amongst kinin derivatives in its additional antagonist effects at the $AT_1$ receptor. Furthermore, in preliminary studies des-Arg$^9$-[Leu$^8$]BK reversed the binding of [$^{125}$I]-Sar$^1$-AII or tritiated nonpeptide $AT_1$ antagonists to intact CHO cells transfected with cDNA encoding the $AT_1$ receptor (J.C. Bonnafous & A. Duvoid, personnal communication).

Membranes from lamb uterus contain a mixed population

of $AT_1$ and $AT_2$ receptors. Therefore, in order to assess the binding property of des-Arg$^9$-[Leu$^8$]BK and other kinins to the $AT_2$ receptor in this tissue, membranes were first treated with DTT to selectively abolish the binding of AII to the $AT_1$ subtype (Whitebread *et al.*, 1989). Under these conditions, various kinin derivatives bound to the $AT_2$ receptor with the following potency ($K_i$, μM): des-Arg$^9$-[Leu$^8$]BK (0.3), des-Arg$^{10}$-[Leu$^9$]KD (0.7), des-Arg$^9$-BK (1.2), des-Arg$^{10}$-KD (1.5) and KD (7.0). BK was inactive at 100 μM. Although the number of kinin related compounds tested was limited, some simple structure-activity relationships appear. Compounds lacking the C-terminal arginine had a better affinity than KD and BK and a leucine residue in position 8 or 9 of des-Arg$^9$-BK and des-Arg$^{10}$-KD respectively, also enhanced the binding affinity.

In conclusion, we have demonstrated that the previously accepted selective $B_1$ receptor antagonist des-Arg$^9$-[Leu$^8$]BK is an insurmountable antagonist of AII-induced contraction in rabbit aorta and binds to both $AT_1$ and $AT_2$ receptors. Thus, these additional properties of des-Arg$^9$-[Leu$^8$]BK should be considered in studies in which des-Arg$^9$-[Leu$^8$]BK is used to assess the participation of kinin $B_1$ receptors. Furthermore, these findings raise the question of how kinin derivatives recognize $AT_1$ and $AT_2$ receptors. Future cloning of the kinin $B_1$ receptor might allow delineation of the structural basis for such recognition through the comparison of binding epitopes of angiotensin II and kinin receptors.

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# G-protein involvement in muscarinic receptor-stimulation of inositol phosphates in longitudinal smooth muscle from the small intestine of the guinea-pig

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**1** Aluminium fluoride (AlF), pertussis toxin (PTX) and cholera toxin (ChTX) have been used to examine the involvement of G-proteins during muscarinic acetylcholine receptor (AChR) stimulation of inositol phospholipid hydrolysis in fragments of longitudinal smooth muscle from the small intestine of the guinea-pig.

**2** Carbachol (CCh) induced time- and concentration-dependent increases in [$^3$H]-inositol monophosphates, [$^3$H]-inositol (1,4) bisphosphate, [$^3$H]-inositol (1,3,4) trisphosphate, [$^3$H]-inositol (1,4,5) trisphosphate ([$^3$H]-Ins (1,4,5)$P_3$) and [$^3$H]-inositol tetrakisphosphates measured by h.p.l.c. These increases were inhibited $>95\%$ in the presence of the muscarinic AChR antagonist atropine (0.5 $\mu$M).

**3** AlF transiently increased the basal levels of [$^3$H]-Ins (1,4,5)$P_3$ but increases in the levels of the other [$^3$H]-inositol phosphates occurred more slowly. CCh-induced increases in the levels of all the [$^3$H]-inositol phosphates were strongly inhibited in the presence of AlF.

**4** PTX had no effect on basal levels of any of the [$^3$H]-inositol phosphates but reduced the effects of CCh on these; ChTX had no effects on either basal or CCh-stimulated levels.

**5** It was concluded that muscarinic AChR-stimulated increases in the levels of [$^3$H]-inositol phosphates occur via both a PTX-sensitive G-protein and a PTX-insensitive mechanism. The actions of AlF may suggest the involvement of an inhibitory G-protein in the regulation of muscarinic AChR-stimulated inositol phospholipid turnover.

**Keywords:** Smooth muscle; G-proteins; aluminium fluoride; inositol phosphates; pertussis toxin; cholera toxin; muscarinic acetylcholine receptor

## Introduction

In smooth muscle, muscarinic acetylcholine receptors (AChRs) are linked via G-proteins to stimulation of inositol phospholipid hydrolysis and inhibition of adenylyl cyclase (Kamm & Stull, 1989; Noronha-Blob *et al.*, 1989). These effects are thought to be mediated by different subtypes of receptor and in the longitudinal muscle of guinea-pig ileum, radioligand binding and contraction studies using selective antagonists have suggested that 70% of the muscarinic AChRs are $M_2$ and the other 30% are $M_3$. The $M_3$ subtype of the receptor mediate contraction (Giraldo *et al.*, 1988; Michel & Whiting, 1990; Barocelli *et al.*, 1993).

The G-protein $\alpha$ subunits involved in phospholipase C (PLC) activation and adenylyl cyclase inhibition have been divided into two classes based on their sensitivity to PTX (Casey & Gilman, 1988). Studies using transfected mammalian cell lines have suggested that individual muscarinic AChR subtypes evoke distinct cellular responses through differential interaction with these two classes of G-proteins (Peralta *et al.*, 1988; Ashkenazi *et al.*, 1989; Lechleiter *et al.*, 1990). It has been suggested that the $m_1$, $m_3$ and $m_5$ receptor subtypes primarily activate a PTX-insensitive G-protein pathway leading to a potent stimulation of PLC-mediated phosphoinositide hydrolysis (Peralta *et al.*, 1988; Ashkenazi *et al.*, 1989) whereas the $m_2$ and $m_4$ receptor subtypes primarily mediate both the activation of PLC and inhibition of adenylyl cyclase through PTX-sensitive pathways (Ashkenazi *et al.*, 1989). Stimulation of phospholipase $A_2$, phospholipase D, adenylyl cyclase, Ins(1,4,5)$P_3$-independent receptor-operated $Ca^{2+}$ channels and $Ca^{2+}$-dependent tyrosine kinases by $m_1$, $m_3$ and $m_5$ have also been reported (Nathanson, 1987; Baumgold & Fishman, 1988; Conklin *et al.*, 1988; Stratton *et al.*, 1989; Sandmann *et al.*, 1991; Felder *et al.*, 1993). Despite these results there is no conclusive evidence that an individual muscarinic AChR subtype cannot link to several different G-protein or other signal transducing systems.

G-proteins have been shown to be directly activated by AlF. The AlF mixture gives $AlF_4^-$ (AlF) (Sternweis & Gilman, 1982) and Bigay *et al.* (1987) showed that the AlF complex, which has a chemical structure similar to that of $PO_4^-$, binds to the nucleotide binding site of the $\alpha$-subunit of the G-protein, close to the $\beta$-phosphate of GDP, and consequently activates the G-protein by dissociating the $\alpha$ from the $\beta\gamma$ subunits. Thus, when the nucleotide site contains GDP (as in the inactive state) AlF becomes associated with the GDP and mimics the $\gamma$-phosphate of GTP. In many cell types AlF has been shown to induce $Ca^{2+}$ mobilization and phosphoinositide hydrolysis by direct activation of a G-protein coupled with PLC (Blackmore *et al.*, 1985; Guillon *et al.*, 1986; Paris & Pouysségur, 1987; Toyjo *et al.*, 1991) and to cause contraction in various smooth muscles (Marc *et al.*, 1988; Zeng *et al.*, 1989; Cushing *et al.*, 1990; Hall *et al.*, 1990; Ratz & Blackmore, 1990; Kawase & Van Breemen, 1992). AlF also affects cyclic AMP levels through $G_i$ and $G_s$ (Cook *et al.*, 1985).

PTX and ChTX have both been used extensively in aiding the identification of the G-proteins linked to adenylyl cyclase (Stryer & Bourne, 1986) and have more recently been used to determine the involvement of G-proteins in PLC activity (Hepler & Gilman, 1992). In several cell types PTX blocks agonist-stimulated PLC activation (Brandt *et al.*, 1985; Nakamura & Ui, 1985). In smooth muscle, variable effects of PTX have been found on agonist-stimulated phospholipase C activation e.g. responses have been shown to be markedly inhibited in the rabbit pulmonary artery (Hohlfeld *et al.*, 1990) and in cultured cells from rat aorta (Bruns & Marmé,

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1987) partially inhibited in rat and human myometrium (Anwer *et al.*, 1989; Phaneuf *et al.*, 1993) and not affected in pig coronary arteries (Sasaguri *et al.*, 1986) a vascular smooth muscle cell line (Muldoon *et al.*, 1989) or rat portal vein myocytes (Loirand *et al.*, 1990). In guinea-pig myometrial smooth muscle, treatment with ChTX was shown to inhibit the muscarinic inositol phosphate response (Marc *et al.*, 1988) and inhibit agonist-induced contractions in rat portal vein (Zhang *et al.*, 1993). The fact that in some cells agonist-induced phosphoinositide breakdown is inhibited by PTX whereas in others it is not led to the conclusion that more than one type of G-protein was associated with PLC (Simon *et al.*, 1991).

In this study, both AlF, which by mimicking the γ phosphate of GTP activates all G-proteins in a non-selective way, and treatment with PTX and ChTX have been used to identify G-protein modulation of muscarinic AChR-induced increases in [$^3$H]-inositol phosphate levels at early times.

## Methods

### *Preparation and assay of labelled fragments*

The preparation of the longitudinal layer from the small intestine of the guinea-pig is a modification of the method by Paton & Zar (1968). Female guinea-pigs (250–350 g) were killed by cervical dislocation and exsanguination. The small intestine was exposed and rinsed in pregassed Krebs Ringer buffer (KRB) and the longitudinal layer from the whole length of the small intestine except the terminal 5 cm, was carefully pulled away from the underlying tissue using fingers. Tissue which had been in contact with the fingers was discarded and the rest of the strips immersed in warm KRB solution. These strips were rinsed several times in fresh KRB. Each strip was examined under the microscope and any circular muscle peeled off. Chopped fragments of 350 μM × 350 μM were prepared with a McIlwain tissue chopper. Rinsed fragments were transferred to one or more wells of a 24-well multidish and incubated in 1 ml KRB containing 50 μCi [$^3$H]-inositol and kept in a humidified atmosphere containing 95% $O_2$/5% $CO_2$ at 30°C for 20 h. When required, smooth muscle fragments were incubated with 6 μg ml$^{-1}$ PTX or 5 μg ml$^{-1}$ ChTX for 20 h at 30°C (the activity of ChTX is impaired at temperatures above 30°C (Gill & Meren, 1983)).

Labelled and washed fragments were divided into aliquots to give approximately 1 mg protein/tube. Experiments were performed at 37°C with or without the appropriate concentration of CCh or AlF (to give 10 μM aluminium chloride and 10 mM sodium fluoride final concentrations). When the effects of AlF were studied the concentration of $CaCl_2$ in the KRB solution was reduced to 0.8 mM; this was to prevent the formation of insoluble $CaF_2$ detected at 2.4 mM $Ca^{2+}$ (Blackmore *et al.*, 1985; Paris & Pouysségur, 1987). This decrease in $Ca^{2+}$ did not affect agonist-induced stimulation of [$^3$H]-inositol phosphates. At the end of the test period the samples were stopped, processed, assayed by anion-exchange h.p.l.c. within 7 days and the elution profiles of the labelled inositol phosphate samples determined as described in Prestwich & Bolton (1991). The protein concentration of the samples was determined using the method of Lowry *et al.* (1951).

KRB solution was gassed throughout with 95% $O_2$, 5% $CO_2$ and had the following composition (final, mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, $NaH_2PO_4$ 1.2, glucose 11.5, $MgCl_2$ 1.2, $CaCl_2$ 2.5 and the pH was 7.25 when equilibrated.

### *Materials*

PTX and ChTX were obtained from Boehringer. [$^3$H]-inositol was obtained from Amersham International Ltd. [$^3$H]-inositol contained a PT6 tablet which absorbed any radiolysis products, which meant no purification of the label was necessary before use. All the other chemicals and drugs were obtained from either Sigma Chemical Co. Ltd or BDH.

### *Data analysis*

Results are expressed as d.p.m. mg$^{-1}$ protein. The average protein concentration was 1.10 ± 0.02 mg/tube ($n = 20$). Agonist stimulated responses were compared with control values and the results expressed as % change compared to control (except Figure 2).

Data are expressed as the mean ± s.e.mean of at least three experiments performed on different occasions. The data presented are paired, i.e. each control was performed on the same day as the test, using tissue from the same animal unless otherwise stated. The statistical significance was assessed by use of a Student's $t$ test of pair differences. $P$ values that were less than 0.05 were considered to be significant. Calculations were performed using the computer programme INSTAT (GraphPAD software, U.S.A.). Time in the figures is plotted on a logarithmic axis because of the wide range of incubation times employed in the experiments.

The identity of the various [$^3$H]-inositol phosphate isomers except for one of the [$^3$H]-inositol bisphosphates, was determined by comparing their elution profiles from the h.p.l.c. with those of labelled standards (see Prestwich & Bolton, 1991). From the elution profiles of other workers, the other [$^3$H]-inositol bisphosphate is likely to be inositol (3,4) bisphosphate.

## Results

### *Muscarinic AChR activation*

Addition of the stable muscarinic agonist carbachol (CCh) to fragments labelled with [$^3$H]-inositol resulted in time- and concentration-dependent increases in the levels of all the [$^3$H]-inositol phosphates measured (Figures 1 and 2) with an $EC_{50}$ of about 10 μM (Figure 3). In the presence of CCh (100 μM) a significant increase in the level of [$^3$H]-Ins(1,4,5)$P_3$ was seen at the earliest time point measured, 2s (223 ± 27% above control, $P < 0.05$) this peaked at 10 s (437 ± 54% above control, $P < 0.05$) and was followed by a decline towards basal values (Figure 1). The [$^3$H]-Ins(1,4)$P_2$ isomer rapidly increased upon stimulation with CCh and the increase was greatest at 30 s (651 ± 49% above control, $P < 0.01$) and was less at later times. The levels of the other isomers measured rose more slowly than [$^3$H]-Ins(1,4,5)$P_3$, and were still increasing at the end of the 5 min incubation period. The response of total [$^3$H]-inositol phosphates to 100 μM CCh was reduced in the presence of 100 nM atropine by 45.8 ± 9.6% ($n = 6$) and by 95 ± 1.32% ($n = 6$) in the presence of 500 nM atropine.

### *Effect of aluminium fluoride*

Incubation of smooth muscle fragments with AlF (10 mM sodium fluoride plus 10 μM aluminium chloride) from 20 s up to 30 min resulted in increases in the levels of the [$^3$H]-inositol phosphates (Figure 1) but the increases in the isomers were less than seen with 100 μM carbachol except in the case of [$^3$H]-Ins$P_1$ and [$^3$H]-sIns$P_2$. AlF caused a significant increase in [$^3$H]-Ins(1,4,5)$P_3$ levels within 20 s (44 ± 7%, $P < 0.05$) and also at 60 s (69 ± 9% above control, $P < 0.01$). By 5 min the levels were returning towards basal (25 ± 7% above control) but still significantly increased ($P < 0.05$); by 10 min the levels of [$^3$H]-Ins(1,4,5)$P_3$ had almost returned to basal values (10 ± 3% above control) and by 30 min incubation with AlF, the levels were less than basal values (Figure 1). A similar profile was seen for the [$^3$H]-inositol tetrakisphosphate isomers (55 ± 6% above control at 60 s, $P < 0.01$) and by 5 min the levels were returning towards basal

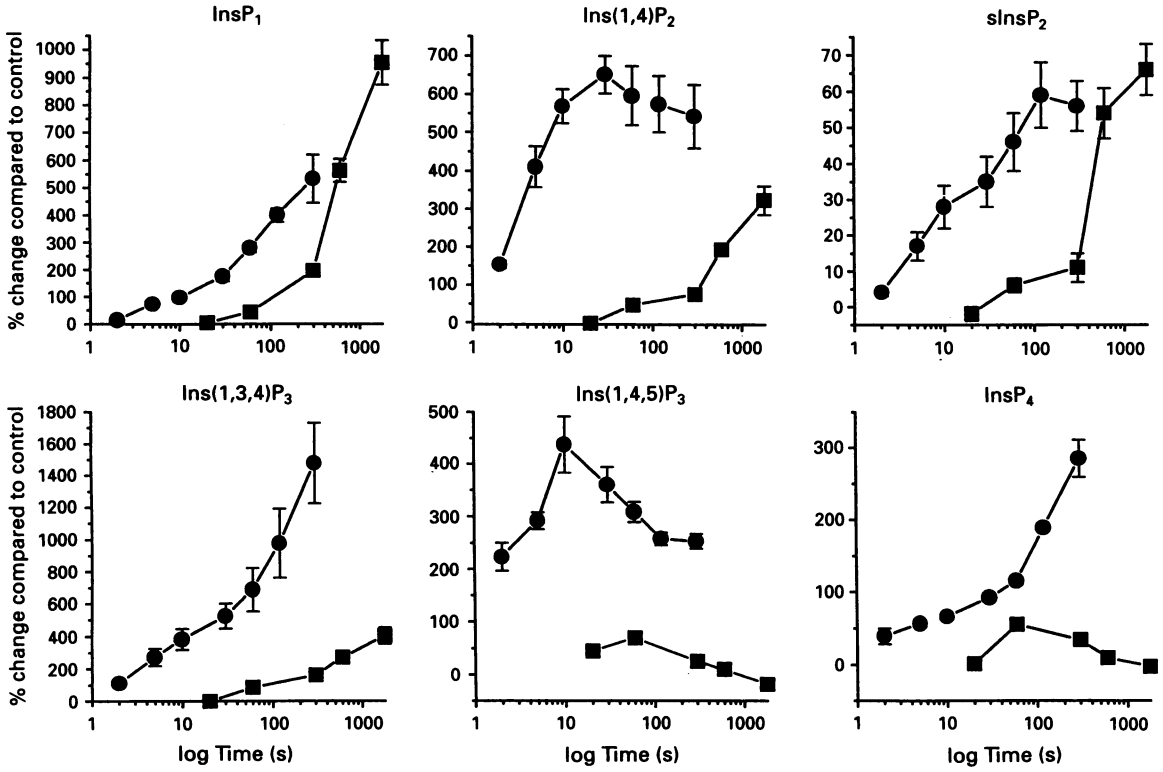


**Figure 1** Comparison of [$^3$H]-inositol phosphate levels in the presence of AlF or carbachol (CCh). [$^3$H]-inositol-labelled smooth muscle fragments were incubated for 20 s, 1 min, 5 min, 10 min or 30 min in the presence of AlF (10 mM NaF plus 10 μM $AlCl_3$) (■) or 2 s, 5 s, 10 s, 30 s, 1 min, 2 min or 5 min in the presence of 100 μM CCh (●). Results represent the mean ± s.e.mean (where it exceeds symbol width) of three experiments performed on separate occasions and are expressed as the % change in [$^3$H]-inositol phosphates compared to control where the control was in the absence of AlF or CCh. These experiments were not paired. Increases in the levels of [$^3$H]-inositol monophosphates, ($InsP_1$); [$^3$H]-inositol (1,4)bisphosphate ($Ins(1,4)P_2$); [$^3$H]-inositol (3,4) bisphosphate ($sInsP_2$); [$^3$H]inositol (1,3,4) trisphosphate, ($Ins(1,3,4)P_3$); [$^3$H]-inositol (1,4,5) trisphosphate, ($Ins(1,4,5)P_3$) and [$^3$H]-inositol tetrakisphosphates ($InsP_4$) are shown.

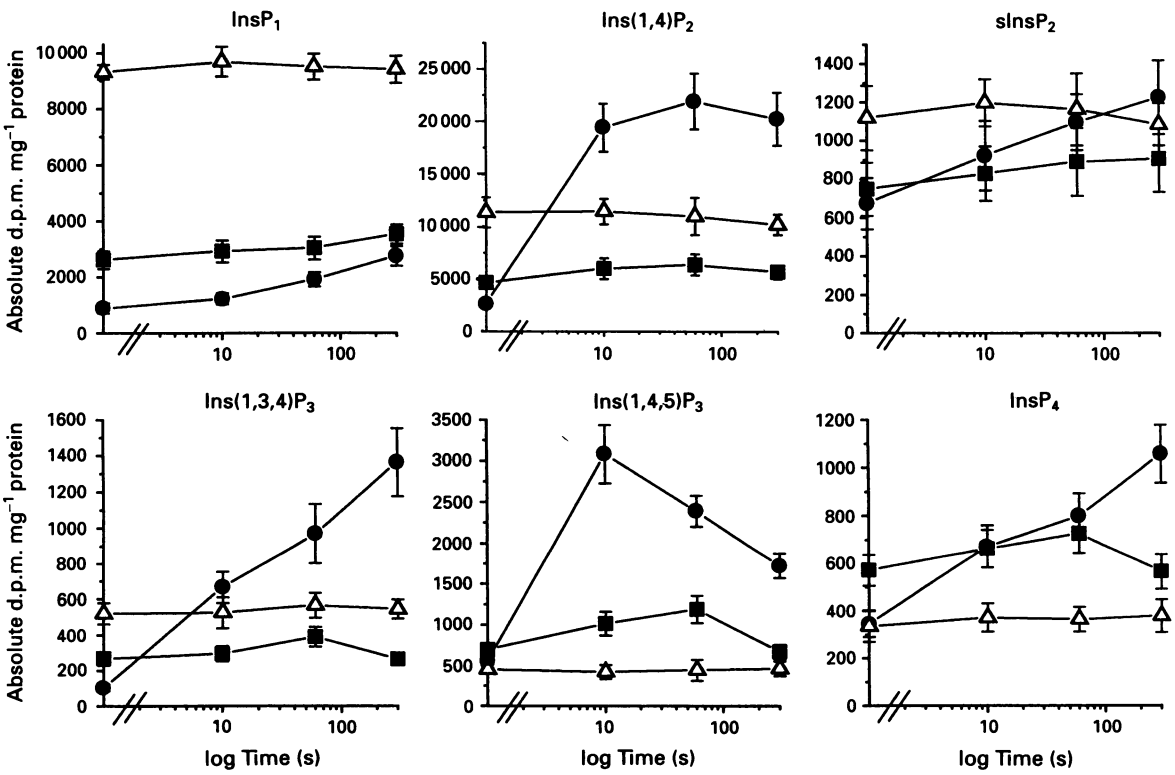


**Figure 2** The effect of AlF (10 mM sodium fluoride and 10 μM aluminium chloride) on carbachol (CCh)-induced increases in [$^3$H]-inositol phosphate levels. [$^3$H]-inositol-labelled smooth muscle fragments were stimulated with 100 μM CCh for 10 s, 60 s or 5 min in the absence (●) or in the presence of AlF added 5 min (■) or 30 min. (△) before CCh. Results represent the mean ± s.e.mean (where it exceeds symbol width) of three experiments performed on separate occasions and are expressed as absolute d.p.m. $mg^{-1}$ protein. See also legend to Figure 1.

(34 ± 3% above control, $P<0.05$) and by 30 min the levels had returned to basal. However, AlF caused a gradual increase in the other [$^3$H]-inositol phosphates. These increased very slowly over the first 5 min incubation, then increased more rapidly and were still increasing at the end of the 30 min incubated period. [$^3$H]-inositol monophosphates increased 43 ± 4% above control by 60 s ($P<0.01$) and 953 ± 111% above control by 30 min ($P<0.01$). The [$^3$H]-inositol (1,4) bisphosphate increased 45 ± 7% above control at 60 s ($P<0.05$) and 323 ± 38% above control by 30 min ($P<0.01$). The increase seen in the [$^3$H]-inositol (1,3,4) trisphosphate isomer was 84 ± 7% above control by 60 s ($P<0.01$) and 403 ± 58% above control by 30 min, ($P<0.01$). A comparison between the time courses of increases of [$^3$H]-inositol phosphates in the presence of CCh (100 μM) and AlF (Figure 1) shows, however, that the increases in [$^3$H]-inositol phosphates in response to AlF were much slower than for CCh.

The effect of several concentrations of NaF on this muscle have been studied (Watson *et al.*, 1988) so we chose to study the effects of a maximally effective concentration at numerous time points to follow progressive activation of G-protein effects on inositol phosphates levels in these experiments. Incubation with AlF for 1 min prior to addition 10 or 100 μM CCh for 10 s reduced the CCh-induced increases in the levels of [$^3$H]-Ins(1,4,5)$P_3$ by 8.4 ± 2.3% (not significant) but significantly reduced all the other [$^3$H]-inositol phosphates by an average of 56 ± 4.4 ($P<0.05$). However, when fragments were incubated for 5 min or 30 min in the presence of AlF and 100 μM CCh added either for 10 s, 60 s or 5 min, the CCh-induced increases in the levels of all the [$^3$H]-inositol phosphates including [$^3$H]-Ins(1,4,5)$P_3$ were almost completely inhibited at each time point by an average of 86 ± 2.5% for a 5 min incubation with AlF and 97 ± 1.2% for a 30 min incubation with AlF ($P<0.01$) (Figure 2). It will be seen that the effects of AlF on all inositol phosphate isomers levels were less when 5 min incubation was used than the effects of CCh (100 μM) after 5 min incubation (Figure 1).

Application of AlF for 5 min increased the measured levels of labelled inositol phosphates and 30 min incubation produced further increases in all inositol phosphates measured except [$^3$H]-Ins(1,4,5)$P_3$ and [$^3$H]-Ins$P_4$ (Figure 1). Although there was no increase in basal levels of these two isomers upon 30 min incubation with AlF, the effects of CCh on these were completely inhibited; 5 min incubation with AlF produced substantial reduction in the effect of CCh on [$^3$H]-Ins(1,4,5)$P_3$ (Figure 2). In the case of the levels of [$^3$H]-Ins(1,4)$P_2$ and [$^3$H]-Ins(1,3,4)$P_3$, modest rises were produced by AlF, less than the effects of CCh alone, but nevertheless the action of CCh was still abolished. In the case of [$^3$H]-Ins$P_1$ the effect of incubation with AlF for 5 min was comparable to, and the effect of 30 min much greater than, the effect of CCh alone and incubation with AlF completely prevented (30 min AlF) or substantially reduced (5 min incubation) the effects of CCh.

### *Pertussis and cholera toxin treatment*

The increases in the levels of all the [$^3$H]-inositol phosphates observed following a 10 s stimulation with increasing concentrations of CCh were significantly reduced after PTX treat-

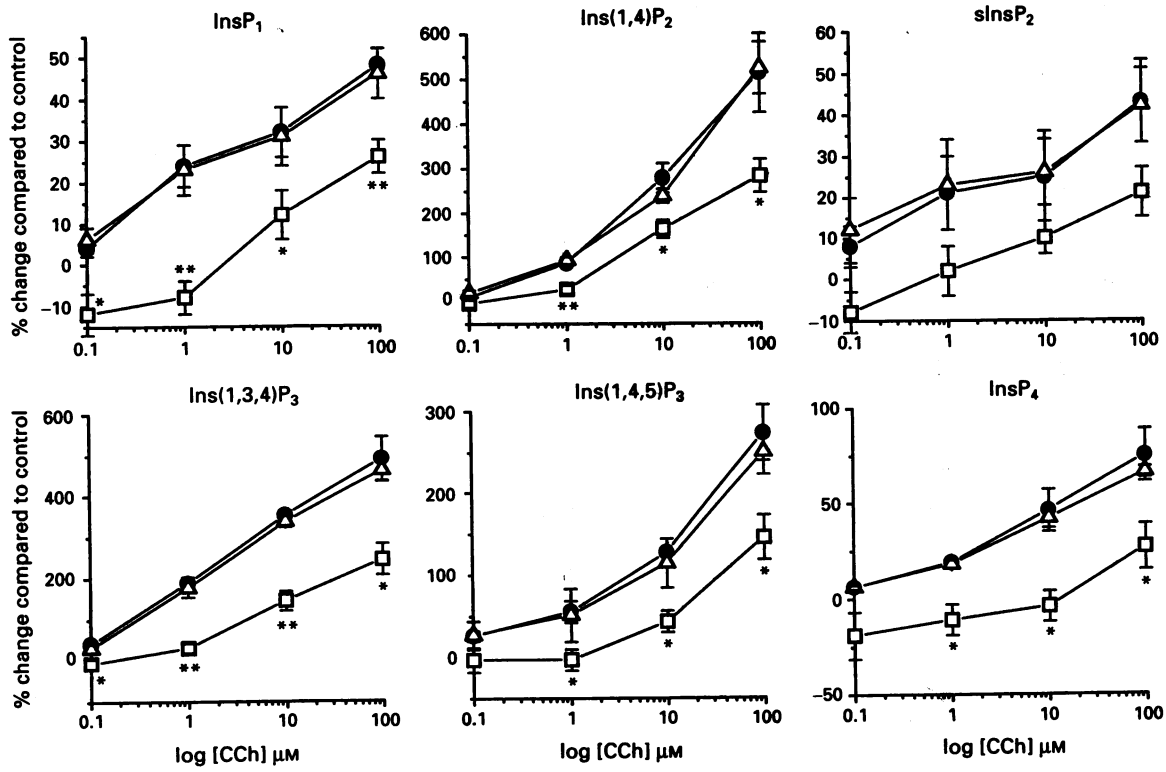


**Figure 3** The effects of pertussis toxin (PTX) and cholera toxin (ChTX) treatments on carbachol (CCh)-induced increases in [$^3$H]-inositol phosphate levels. [$^3$H]-inositol-labelled smooth muscle fragments were incubated in either normal KRB or KRB with 6 μg ml$^{-1}$ PTX or 5 μg ml$^{-1}$ ChTX for 20 h at 30°C. At the end of the incubation period each set of fragments was washed and divided and the increases in the levels of [$^3$H]-inositol phosphates in the presence of increasing concentrations of CCh for 10 s was determined. The symbols represent, the effect of CCh with no treatment (●) after treatment with PTX (□) and after treatment with ChTX (△). Results represent the mean ± s.e.mean (where it exceeds symbol width) of three experiments performed on separate occasions and are expressed as the % change compared to control, where the control is taken as the value in the absence of CCh. PTX and ChTX had no significant effect on basal [$^3$H]-inositol phosphate production. Statistical analysis comparing the increases in labelled inositol phosphates in the presence of CCh alone and after treatment with PTX are shown in the figure and represent the following: $*P<0.05$; $**P<0.01$.

ment (Figure 3). The time-dependent increases in the levels of [$^3$H]-inositol phosphates in the presence of 100 μM CCh were also significantly reduced although the pattern of increase for each of the isomers was similar (Figure 4). PTX had no significant effect on the basal release of [$^3$H]-inositol phosphates (basal d.p.m. mg$^{-1}$ protein for [$^3$H]-Ins(1,4,5)$P_3$ in control fragments was 976 ± 8 and in PTX treatment fragments was 884 ± 140, and for total [$^3$H]-inositol phosphates this was 26592 ± 3758 and 290004 ± 4202 without and with PTX treatment respectively). The peak response to [$^3$H]-Ins(1,4,5) $P_3$ still occurred at 10 s but the % stimulation after treatment with PTX was significantly less (258 ± 82%) compared to the response in fragments which had not been treated (516 ± 49%, $P<0.01$) (Figure 4). Despite the fact that cells were incubated with a high concentration of PTX (6 μg ml$^{-1}$) and for a long period (20 h) part of the CCh-induced increases in [$^3$H]-inositol phosphate levels was PTX-insensitive. This indicates that a PTX-insensitive G-protein or a G-protein-independent mechanism is involved in mediating part of the CCh-induced increases in the levels of [$^3$H]-inositol phosphates. In contrast to PTX, treatment with ChTX had no effect on either the basal levels of [$^3$H]-inositol phosphates or on CCh-induced increases in [$^3$H] inositol phosphates levels (Figure 3).

## Discussion

Application of μM concentrations of CCh results in a rapid and transient increase in Ins(1,4,5)$P_3$ levels in this smooth muscle. The Ins(1,4,5)$P_3$ causes $Ca^{2+}$ release from the stores (Komori & Bolton, 1991) in the sarcoplasmic reticulum and this contributes to the initial transient phase of contraction. The actual concentration in the muscle of Ins(1,4,5)$P_3$ is determined by the balance between synthesis and degradation. Thus the rapid decay of the Ins(1,4,5)$P_3$ signal could be due either to a slowing of the initially elevated rate of synthesis of Ins(1,4,5)$P_3$ or to an accelerated removal by phosphorylation and dephosphorylation. One feature of the muscarinic AChR-induced Ins(1,4,5)$P_3$ response is that, even when the agonist is continuously present, the intracellular Ins(1,4,5)$P_3$ levels peak and then start to return towards basal levels within a short period of time. Although phosphorylation by one or more specific kinases may play a major role in this desensitization, feedback mechanisms involving G-protein subunits may also contribute to the termination or slowing down of the signalling response.

### *Aluminium fluoride*

AlF has been shown to increase $[Ca^{2+}]_i$, increase $Ca^{2+}$ influx, deplete phosphatidylinositol (4,5) bisphosphate and increase Ins(1,4,5)$P_3$ levels (Blackmore *et al.*, 1985). This suggests that AlF can mimic the effects of $Ca^{2+}$-mobilizing agents by activating a G-protein involved in the coupling of receptors to PLC-mediated hydrolysis of phosphatidylinositol (4,5) bisphosphate.

The concentration of AlF used in this study (10 mM sodium fluoride plus 10 μM aluminium chloride) has previously been shown on single cells from longitudinal smooth muscle of the small intestine to abolish the transient outward currents probably caused by spontaneous release of calcium from stores within 2 min of application in a similar way to that of GTPγS and CCh (Bolton & Lim, 1989). This suggests it causes a depletion of calcium from the internal stores via Ins(1,4,5)$P_3$ release in these cells. Using longitudinal smooth muscle from the small intestine of the guinea-pig, NaF was found to cause a concentration-depen-

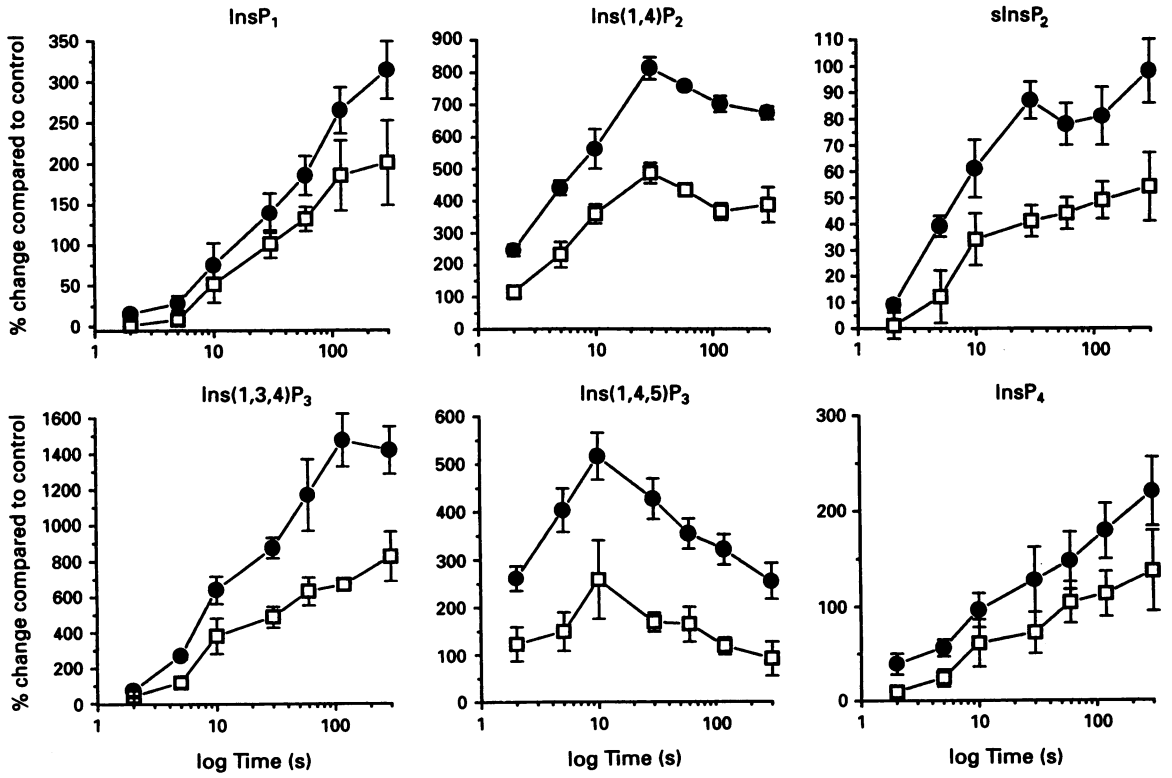


**Figure 4** The effect of pertussis toxin (PTX) on carbachol (CCh)-induced increases in [$^3$H]-inositol phosphate levels with time. [$^3$H]-inositol-labelled smooth muscle fragments were incubated in either normal KRB or KRB with 6 μg ml$^{-1}$ PTX for 20 h at 30°C. At the end of the incubation period each set of fragments was washed and divided and the production of [$^3$H]-inositol phosphates in the presence of 100 μM CCh for 2 s, 5 s, 10 s, 30 s, 60 s, 120 s and 300 s was determined with (□) or without (●) PTX treatment. Results represent the mean ± s.e.mean of three experiments performed on separate occasions and are expressed as the % change compared to control, where the control is taken as the value in the absence of CCh.

dent increase in the levels of total inositol phosphates (although lithium was present and only total inositol phosphates were measured at one time point of 20 min) and this was maximal at 10 mM NaF. No further increase was produced by the addition of $AlCl_3$ suggesting that contamination with $Al^{3+}$ ions was sufficient to activate inositol phosphate formation maximally when NaF was added alone (Watson *et al.*, 1988). $AlCl_3$ with 10 mM NaF has also been shown to result in maximal increases in inositol phosphates in other smooth muscle cells (Marc *et al.*, 1988).

Although the muscarinic AChR-stimulation of [$^3$H]-inositol phosphate levels occurs within seconds, the AlF response is much slower, taking several minutes before increases could be seen in most of the isomers (Figure 1). Such a slow or gradual development of the response has been observed by others in several cell types (Matozaki *et al.*, 1988; Baukal *et al.*, 1988) and in smooth muscle cells (Marc *et al.*, 1988). The increases in the levels of the [$^3$H]-inositol phosphates were generally smaller with AlF than with CCh and after 10 min incubation only the [$^3$H]-inositol monophosphate isomer levels and [$^3$H]-sInsP$_2$ reached a value close to that of CCh at 5 min. The peak increase in the levels of [$^3$H]-Ins(1,4,5)P$_3$ at 60 s in the presence of AlF are only 69 ± 9% above control compared to 437 ± 54% at 10 s and 308 ± 19% at 60 s in the presence of CCh (Figure 1).

Although initially AlF may act by stimulating a G-protein linked to [$^3$H]-phosphatidylinositol(4,5)bisphosphate hydrolysis, it also may have other effects which inhibit phosphatidylinositol(4,5)bisphosphate hydrolysis with time such as inhibition of the kinases that convert phosphatidylinositol(4) phosphate to phosphatidylinositol(4,5) bisphosphate (Claro *et al.*, 1990) resulting in depletion of the (labelled) phosphatidylinositol(4,5) bisphosphate pool or inhibition of ATP hydrolysis (Biffen & Martin, 1989). The response of Ins(1,4,5)P$_3$ to AlF may then decrease whereas increases in lower inositol phosphates may still occur.

AlF inhibited the CCh-induced increase in [$^3$H]-inositol phosphate levels (Figure 2). This inhibition was evident when the absolute increases in d.p.m. mg$^{-1}$ protein in the presence of CCh alone were compared with the absolute increases with CCh in the presence of AlF. AlF alone had modest and slowly developing effects on [$^3$H]-Ins(1,4,5)P$_3$ and [$^3$H]-InsP$_4$ (Figure 1) but severely inhibited the effects of CCh (100 μM) on these; it also inhibited the effects of CCh on [$^3$H]-Ins(1,4)P$_2$ and [$^3$H]-Ins(1,3,4)P$_3$ after 5 min incubation (Figure 2) despite having a much smaller stimulatory effect on these isomers than CCh (Figure 1). Only in the case of [$^3$H]-InsP$_1$ did AlF alone have a comparable effect to CCh alone; again AlF blocked the effect of CCh. It may be that depletion of phosphatidylinositol(4,5) bisphosphate (Biffen & Martin, 1989; Claro *et al.*, 1990) in AlF prevents much action of PLC to form higher inositol phosphate isomers either when AlF is present alone or when CCh is added in addition. However, persistent action of PLC on phosphatidylinositol may allow the formation of [$^3$H]-InsP$_1$ in increasing amount throughout 30 min incubation with AlF although CCh may under these conditions be unable to cause further increases in PLC activity. However, if AlF stimulates PLC maximally in this way, it does not explain why it does not increase [$^3$H]-Ins(1,4,5)P$_3$ at early times although CCh does so.

Inhibition is perhaps better explained if AlF stimulated an inhibitory G-protein linking muscarinic AChRs to PLC as suggested by other workers (Godfrey & Watson, 1988; Geet *et al.*, 1990). A 1 min incubation with AlF (which causes a small, but its maximal, stimulation of [$^3$H]-Ins(1,4,5)P$_3$) had no significant effect on the CCh-induced increases in the levels of [$^3$H]-Ins(1,4,5)P$_3$ but inhibited the CCh-induced increases in all other [$^3$H]-inositol phosphates. This may indicate the existence of both stimulatory and inhibitory G-proteins, such that, with longer incubation in AlF, inhibitory G-protein effects become apparent which block the action of CCh. Released βγ subunits combining with α subunits due to the non selectivity of AlF or a direct effect of the βγ subunit on the PLC enzyme itself may also occur and both inhibition of agonist-stimulated inositol phosphate production (Moriarty *et al.*, 1989; Boyer *et al.*, 1989) and stimulation of PLC by βγ subunits has recently been identified (Clapham & Neer, 1993). Other mechanisms can be envisaged. With time the levels of the [$^3$H]-inositol phosphates other than [$^3$H]-Ins(1,4,5)P$_3$ continue to increase and the levels of 1,2-diacylglycerol will continue to rise resulting in the activation of protein kinase Cs, which may act as negative feedback regulators (Katada *et al.*, 1985; Olianas & Onali, 1993).

### *Toxin treatment*

The results using ChTX (Figure 3) suggest that G$_s$α, the βγ subunits produced from activation of Gs, or the increases in cyclic AMP that would be expected to occur following ChTX treatment are not involved in muscarinic AChR activated inositol phospholipid hydrolysis. However, PTX did partially inhibit the CCh-induced [$^3$H]-inositol phosphate production by 53% at 2 s and by 50% at 10 s for [$^3$H]-Ins(1,4,5)P$_3$. Komori *et al.* (1992) showed that although incubation with PTX (6 μg ml$^{-1}$ for 6 h) inhibited the agonist-induced cationic current, CCh-induced release of $Ca^{2+}$ from stores was not affected (which has been shown in these cells to be due to an increase in Ins(1,4,5)P$_3$). This suggests that sufficient [$^3$H]-Ins(1,4,5)P$_3$ is still produced following PTX treatment to cause $Ca^{2+}$ store release with high concentrations of CCh. Similar results have been found in myometrial cells (Phaneuf *et al.*, 1993). Long incubations with PTX (20 h) may result in the ADP-ribosylation of a slowly ribosylated PTX sensitive G-protein (Neylon *et al.*, 1992) or down regulation of the β subunit of G-proteins, resulting in a non-specific inhibition of all G-proteins present (Watkins *et al.*, 1989). Hence, although the PTX effects in the present study may be due to the presence of a G-protein, the altered component of the G-protein is not necessarily an identified PTX-sensitive α subunit.

In many of the studies where a PTX-sensitive G-protein has been shown to be involved in agonist-induced inositol phospholipid hydrolysis, the presence of only one G-protein has been assumed to link the receptor with the PLC. Partial inhibition of muscarinic AChR agonist-induced production of inositol phosphates has been observed in reconstitution studies (Ashkenazi *et al.*, 1989; Lazareno *et al.*, 1993). A PTX-sensitive G-protein coupled transfected m$_2$ receptors to phosphatidylinositol hydrolysis and at least one PTX-sensitive and one PTX-insensitive G-protein coupled transfected m$_1$ and m$_3$ receptors to phosphatidylinositol hydrolysis. Lazareno *et al.* (1993) found that m$_2$ and m$_4$ receptors were associated with PTX-sensitive G-proteins whereas m$_1$ and m$_3$ were associated with both PTX-sensitive and -insensitive G-proteins. This suggests that in the present study both the PTX-sensitive and PTX-insensitive G proteins may be linked to the M$_3$ receptors which are thought to be the predominant receptors involved with contraction in this smooth muscle. However M$_2$ receptors associated with PTX-sensitive G proteins may also have a role in the agonist induced increases in the levels of inositol phosphates (Thomas *et al.*, 1993). The identification of the muscarinic receptor subtype linked to the inositol phosphates response is not a trivial problem and although both M$_2$ and M$_3$ subtypes have been suggested to exist in this tissue, their individual roles in contraction via increases in inositol phosphates have been determined mainly by the use of antagonists which show only modest selectivity for any one subtype (Caulfield, 1993; Doods *et al.*, 1994; Eglen *et al.*, 1994).

The identification of the G-protein α subunit linking the PTX-sensitive activation of PLC mediated by muscarinic AChRs, and several other types of G-protein-coupled receptor has proved elusive although recently Gα$_i$2 and Gα$_i$3 have been shown to be coupled to transfected muscarinic AChR m$_2$ and the stimulation of PLC (Dell'Acqua *et al.*, 1993).

Recent reports describing βγ subunit activation of PLCβ2 raises the possibility that the PTX sensitive PLC activation in these experiments may be mediated by βγ subunits (Clapham & Neer, 1993). Muscarinic AChRs expressed in COS cells activate PLCβ2 only in the presence of Gβγ, indicating that mAChR can initiate signalling cascades working through Gβγ (Katz *et al.*, 1992). However, different regions of PLCβ2 are activated by α and βγ subunits and so it is possible that α and βγ subunits may independently modulate a single PLCβ2 molecule at the same time. (Bong Lee *et al.*, 1993). If some βγ-subunit complexes have abilities to stimulate or inhibit a particular effector, then the forms of βγ associated with different G-proteins that interact with individual receptors will be an important determinant of agonist regulation.

## Conclusion

The inhibitory effect of AlF on CCh-induced increases in labelled inositol phosphates may indicate the activation by AlF of an inhibitory G-protein although other possibilities cannot as yet be excluded. Muscarinic AChR responses are coupled to PLC via PTX-sensitive G-protein(s) and by a PTX-insensitive mechanism.

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# Effect of the $Ca^{2+}$-ATPase inhibitor, cyclopiazonic acid, on electromechanical coupling in the guinea-pig ureter

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**1** We have investigated the effect of the sarcoplasmic reticulum (SR) $Ca^{2+}$-ATPase inhibitor, cyclopiazonic acid (CPA), on electromechanical coupling in the guinea-pig ureter. All experiments were performed in capsaicin-pretreated (10 μM for 15 min) ureters to prevent the release of sensory neuropeptides from afferent nerves.

**2** In organ bath experiments, electrical field stimulation (EFS, 10 Hz for 1 s, 5 ms pulse width, 60 V) produced tetrodotoxin- (1 μM) resistant phasic contractions which were enhanced by Bay K 8644 (1 μM) and abolished by nifedipine (10–30 μM).

**3** CPA (10 μM) enhanced the EFS-evoked contractions both in the absence and presence of Bay K 8644. The effect of CPA was concentration-dependent between 1 and 30 μM. The response to 10 μM CPA was biphasic: the maximal enhancement (58 ± 3% increase) was observed within 10–20 min from CPA administration, followed by a decline to a new steady state (25 ± 5% increase over baseline) at 50–60 min. The effect of CPA was reversed by washout.

**4** Ryanodine (100 μM) produced a prompt enhancement of the EFS-evoked contractions of the guinea-pig ureter, which peaked at 42 ± 3% increase over baseline; the co-administration of CPA (10 μM) and ryanodine (100 μM) produced a peak effect (60 ± 8% enhancement) which was not different from that produced by CPA alone. With either ryanodine alone *or* ryanodine plus CPA, the enhancement of the EFS-induced contractions was biphasic, showing a time-course similar to that observed with CPA alone. Tetraethylammonium (10 mM) produced a significantly larger effect (93 ± 13% increase over baseline) and its effect was sustained throughout the 60 min observation period.

**5** In the presence of Bay K 8644, superfusion for 30 min with a low $Na^+$ medium (60% of extracellular $Na^+$ replaced by $Li^+$ or choline) reduced the amplitude of EFS-evoked contractions by 20–35%. In both $Li^+$- and choline-substituted media, spontaneous activity developed during superfusion with low $Na^+$ Krebs solution which was suppressed by 10 μM nifedipine. CPA (10 μM) produced a marked enhancement of the EFS-evoked contractions in low-$Na^+$ medium (both $Li^+$- and choline-substituted) and this effect was sustained throughout the 60 min observation period.

**6** In the absence of Bay K 8644, the response of the ureter smooth muscle to EFS is characterized by a refractory period: an interval of about 30 s was required between two applied stimuli to produce a second response comparable in size to that elicited by the first stimulus. CPA (10 μM, 10–20 min before) markedly reduced the refractory period of the guinea-pig ureter to EFS.

**7** CPA (10 μM, 30–60 min before) increased the phasic component of contraction produced by 80 mM KCl. The tonic component of the response to KCl was slightly but not significantly reduced by CPA, and a 'hump' in the tonic contraction was observed at 1–2 min from addition of KCl.

**8** In sucrose gap experiments, 10 μM CPA produced a sustained depolarization of the membrane and reduced the latency between application of electrical stimuli and onset of the action potential; these effects were maintained throughout the 60 min superfusion with CPA. CPA also transiently prolonged the plateau phase of the action potential and increased the peak amplitude of contraction: these effects peaked at about 10–20 min from start of superfusion with CPA and then declined. At the peak of its enhancing effect on contraction amplitude, CPA prolonged the contractile phase of the contraction-relaxation cycle.

**9** Superfusion with a low-Na, choline-substituted Krebs solution produced a reversible membrane depolarization. In the presence of Bay K 8644 (1 μM), action potentials and phasic contractions were superimposed on this depolarization which were abolished by nifedipine (10 μM).

**10** These findings indicate that CPA augments the excitability and affects the contraction-relaxation cycle of the smooth muscle of the guinea-pig ureter, implying a role for sarcoplasmic reticulum $Ca^{2+}$-ATPase in the regulation of electromechanical coupling. The effects of CPA resemble those produced by ryanodine and the effect of the two agents on the amplitude of contractions is non-additive. It appears that following blockade of the CPA-sensitive SR $Ca^{2+}$ pump, other mechanism(s) may come into action to reduce intracellular $Ca^{2+}$. The $Na^+/Ca^{2+}$ exchanger could be involved in the compensatory changes responsible for the fading of the response to CPA.

**Keywords:** Guinea-pig ureter; electromechanical coupling; cyclopiazonic acid; sarcoplasmic reticulum $Ca^{2+}$ pump; ryanodine; smooth muscle

## Introduction

The mechanisms determining contraction of the ureter in response to direct electrical stimulation represent an excellent example of electromechanical coupling in smooth muscle. From both the electrical and mechanical point of view, the ureter smooth muscle is quiescent but when subjected to depolarization, it produces an action potential and accompanying phasic contraction: L-type voltage-sensitive calcium ($Ca^{2+}$) channels play a fundamental role in excitation-

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contraction coupling at this level, since the action potential and contraction are both powerfully altered by administration of drugs with antagonist or agonist action on these $Ca^{2+}$ channels (Shuba, 1977; Brading *et al.*, 1983; Maggi *et al.*, 1994a). As expected from the pivotal role of voltage-sensitive $Ca^{2+}$ channels, the manipulation of potassium ($K^+$) permeability also exerts remarkable effects on the action potential and contractility of the guinea-pig ureter (Shuba, 1977; Imaizumi *et al.*, 1989b; Lang, 1989; Maggi *et al.*, 1994b). The contribution of intracellular $Ca^{2+}$ handling mechanisms to electromechanical coupling in the ureter smooth muscle has been little investigated.

In a previous study (Maggi *et al.*, 1994a), we found that ryanodine, a plant alkaloid which blocks $Ca^{2+}$ handling by the sarcoplasmic reticulum (SR) by acting on the calcium-induced calcium release channel (CICR), increases the excitability of the guinea-pig ureter by producing membrane depolarization and reducing the refractory period. Interestingly, ryanodine does not reduce the amplitude of depolarization-induced phasic contraction but, especially after addition of the L-type $Ca^{2+}$ channel agonist, Bay K 8644, it prolongs the contractile cycle of the ureter; these observations suggest that $Ca^{2+}$ handling by the SR regulates excitability and the contractile cycle of the ureter smooth muscle especially to enable removal of cytoplasmic $Ca^{2+}$ while the SR may not be essential as a source of activator $Ca^{2+}$ for contraction.

In this study, we have determined the effect of cyclopiazonic acid (CPA) a mycotoxin from *Aspergillus* and *Penicillum*, on the electrical and mechanical responses of the guinea-pig ureter to depolarizing stimuli. CPA is a specific inhibitor of the SR $Ca^{2+}$-ATPase and is increasingly used as a tool for studying excitation-contraction coupling in smooth muscles (Deng & Kwan, 1991; Low *et al.*, 1992; Uyama *et al.*, 1992; 1993; Bourreau *et al.*, 1992). CPA acts by preventing the binding of $Ca^{2+}$ to the high affinity sites on SR, thereby impeding the $Ca^{2+}$-dependent phosphorylation of SR proteins (Goeger & Riley, 1989). A comparison of the effect of CPA and ryanodine on electromechanical coupling of the ureter appears of interest since the two drugs would affect the same structure (SR) by acting on different targets. Since the response to CPA was found to have a biphasic time course, the possible involvement of $Na^+/Ca^{2+}$ exchanger (Aickin *et al.*, 1984; 1987; Aickin, 1987; Aaronson & Benham, 1989) in this phenomenon was assessed by studying the effect of CPA following reduction of extracellular $Na^+$ which was replaced with lithium ($Li^+$) or choline chloride.

## Methods

### *General*

Male albino guinea-pigs weighing 250–300 g were stunned and bled. The ureters (from the inferior renal pole to their entry into the bladder) were excised, cleaned of adhering fat and connective tissue and placed in oxygenated and warmed (95% $O_2$ and 5% $CO_2$, pH 7.4 at 37°C) Krebs solution of the following composition (mmol/l): NaCl 119, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, $MgSO_4$ 1.5, KCl 47, $CaCl_2$ 2.5 and glucose 11. In some experiments (see below) a low-$Na^+$ Krebs solution was prepared by replacing 86.4 mM NaCl with an equimolar amount of LiCl or choline Cl. Considering the overall $Na^+$ content of the normal Krebs solution (144 mM) this replacement corresponds to 60% reduction of extracellular $Na^+$. Because of the existence of a capsaicin-sensitive inhibitory innervation in the guinea-pig ureter, all experiments were performed in capsaicin-pretreated (10 μM for 15 min) ureters to eliminate this neural inhibitory influence on ureteral excitability (Maggi & Giuliani, 1991).

### *Organ bath experiments*

The ureter was placed in a Petri dish, a segment about 2 cm long was excised from its middle portion and the specimen mounted in a 5 ml organ bath for isotonic recording (load 2 mN) of mechanical activity along the longitudinal axis, as described previously (Maggi & Giuliani, 1991; Maggi *et al.*, 1994b).

In a first series of experiments, we investigated the effect of CPA on the phasic contractions produced by electrical depolarization of the ureter. With this aim, trains of pulses (10 Hz for 1 s, 60 V) of long width (5 ms) were automatically delivered at 1 min intervals by means of platinum wire electrodes placed at the top and bottom of the organ bath (electrical field stimulation, EFS) by means of a Grass S88 stimulator. In most experiments, the effects of CPA on EFS-evoked contractions were investigated in the presence of Bay K 8644 (1 μM) which provides reproducible responses to EFS over prolonged periods of time by reducing the refractory period of the guinea-pig ureter (Maggi *et al.*, 1994a; and see Results, Figure 1).

In some experiments we studied the effect of Low-$Na^+$ Krebs solution ($Li^+$- or choline-substituted, see above) on the response to EFS and on the effect produced by CPA: two ureters from the same animal were studied in parallel, one of which was exposed to the low-$Na^+$ Krebs solution and the other served as control. Both ureters were initially exposed to capsaicin in Krebs solution and then 1 μM Bay K 8644 was added, which was present throughout the experiments. After having recorded a steady response to EFS, the ureters were perfused with low-$Na^+$ Krebs solution or normal Krebs solution for 30 min at a rate of 5 ml $min^{-1}$. At this stage, the superfusion was stopped and the response to EFS was allowed to reach a new steady state: CPA (10 μM) was added to the bath and its effects observed for 60 min.

In a second series of experiments, we determined the effect of CPA on the refractory period of the capsaicin-pretreated guinea-pig ureter to EFS, as described previously (Maggi *et al.*, 1994a): a conditioning stimulus (10 Hz for 1 s, 5 ms pulse width, 60 V) was applied either in the absence or the presence of 10 μM CPA and a test stimulus of the same intensity was delivered at 3, 5, 10 or 30 s interval from the conditioning stimulus. The amplitude of the phasic contraction to the test stimulus was expressed as a % of that evoked by the conditioning stimulus. These experiments were performed at 10–20 min from administration of CPA, corresponding to the period of maximal enhancement of the evoked responses by the drug.

In a third series of experiments we studied the effect of CPA on the biphasic contractile response produced by application of 80 mM KCl to the capsaicin-pretreated guinea-pig ureter; both the phasic and tonic components of this response are produced through the activation of L-type, nifedipine-sensitive, calcium channels (Maggi & Giuliani, unpublished; Maggi *et al.*, 1994c). Two reproducible control responses to KCl were obtained at 30 or 60 min intervals and the effect of CPA (10 μM) determined after 30 or 60 min contact time.

### *Sucrose gap experiments*

A single sucrose-gap, modified as described in details by Artemenko *et al.* (1982) and Hoyle (1987) was used to investigate changes in membrane potential and mechanical activity in response to electrical stimulation. The ureters were superfused with oxygenated Krebs solution at a rate of 1 ml $min^{-1}$. The temperature of the solution was kept constant at 35 ± 0.5°C. The experiments were performed in ureters pre-exposed to capsaicin (10 μM for 15 min) and in the presence of 1 μM Bay K 8644 (Maggi *et al.*, 1994a,b,c).

EFS with single pulses using parameters sufficient to produce direct excitation of smooth muscle (20–40 V, 0.5–2 ms pulse width) evoked an action potential and accompanying contraction of the guinea-pig ureter (Shuba *et al.*, 1977;

Brading *et al.*, 1983). To study the effects of CPA, action potentials and contractions of the ureters were produced at 2.5 min intervals: when steady state responses had been obtained, superfusion with CPA (10 μM) were started and maintained for 60 min. In other experiments the ureters were superfused with the low-$Na^+$ choline-substituted medium for 3 min at 15–20 intervals. The effect of Bay K 8644 (1 μM) and nifedipine (10 μM) on changes in membrane potential produced by the low-$Na^+$ choline-substituted medium were assessed after 15 min contact time.

### *Statistical analysis*

Each value is mean ± s.e. mean. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data or by means of analysis of variance, when appropriate. Linear regression was performed by the least squares method: $EC_{50}$ and 95% confidence limits were calculated accordingly.

### *Drugs*

Drugs used were: cyclopiazonic acid (CPA), capsaicin, nifedipine and tetrodotoxin (Sigma), ryanodine and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) (Calbiochem), tetraethylammonium (TEA, Serva).

## Results

### *Organ bath experiments*

*Effect of CPA on the response to EFS* EFS (10 Hz for 1 s, 5 ms pulse width, 60 V) induced phasic contractions of the guinea-pig ureter ranging from 20–40% of the maximal response to 80 mM KCl (Figure 1a, b and c): all ureters developed phasic contractions upon EFS application but the responses became irregular during repetitive cycles of

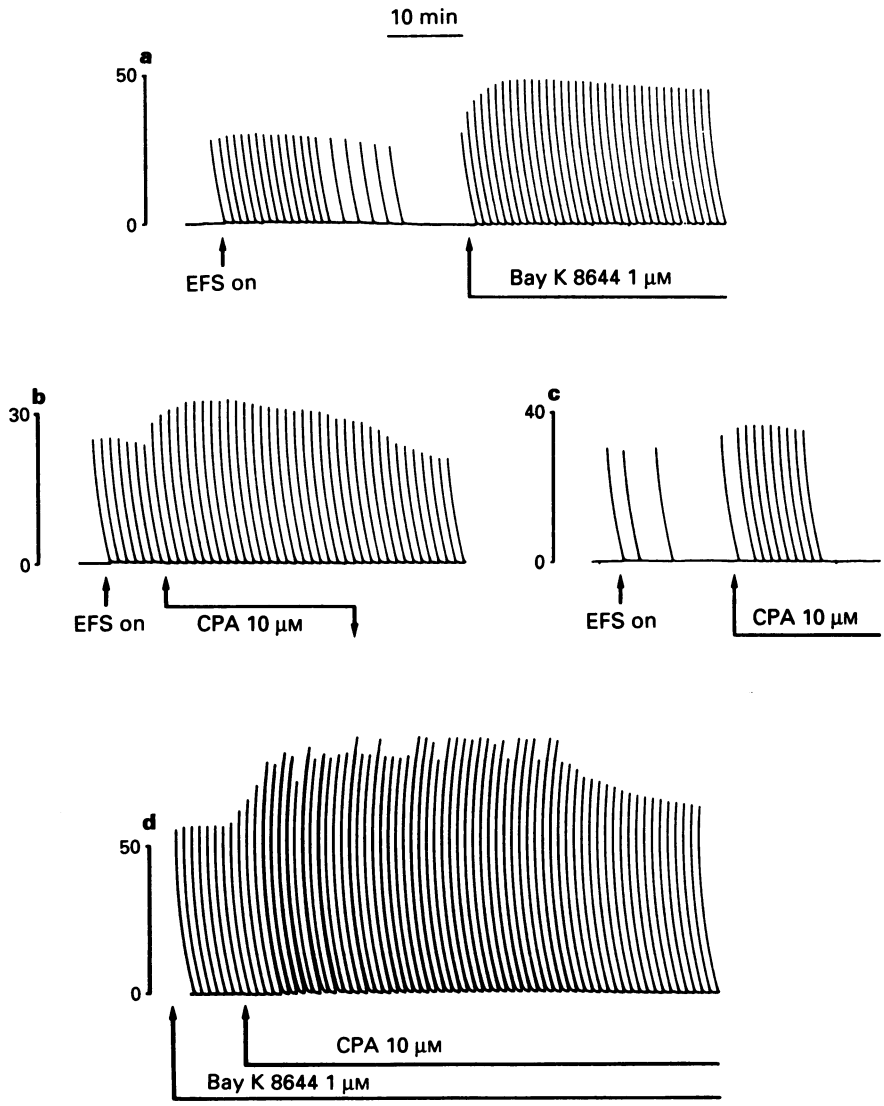


**Figure 1** Typical tracings showing the effect of cyclopiazonic acid (CPA) on electrical field stimulation-(EFS) induced contractions of the guinea-pig isolated ureter in the absence and presence of Bay K 8644. Phasic contractions of the ureter were evoked by EFS (train of pulses at a frequency of 10 Hz for 1 s, 60 V, 5 ms pulse width, applied every 60 s). In the absence of Bay K 8644, most ureters responded to EFS for a limited period of time after which the response waned (a and c). Either Bay K 8644 (a) or CPA (c) caused resumption of the response to EFS. CPA added before spontaneous fading of the response enhanced the EFS-induced contractions (b; tracings shown in panels a and b were obtained in paired ureters from the same animal). In the presence of Bay K 8644, CPA produced a prompt enhancement of the response to EFS which showed a spontaneous decline at 40–50 min from CPA administration (d). Vertical bars are percentage response to KCl.

stimulation; the most frequently observed pattern was the failure of some applied stimuli to produce a response (Figure 1a and c). In 5 experiments CPA (10 μM) was applied to one ureter while the other served as control (Figure 1a and b): CPA enhanced the amplitude of the evoked contractions by 36 ± 6% and prevented the decline observed in untreated ureters (cf. Figure 1a and b). If added when the response had become irregular or had disappeared, CPA (10 μM) produced a transient excitatory effect on the responsiveness of the ureter (Figure 1c). As noted in previous studies (e.g. Uyama *et al.*, 1992), the effect of CPA was reversed by washout.

As shown in Figure 1a, the addition of the dihydropyridine calcium channel agonist, Bay K 8644 (1 μM) restored the ability of the ureter to respond to each applied stimulus and enhanced the amplitude of the EFS-evoked contractions. In the presence of Bay K 8644, the response to EFS ranged from 40–65% of the maximal response to KCl (80 mM): these responses were unaffected by tetrodotoxin (1 μM) and were suppressed by 10–30 μM nifedipine. In the presence of Bay K 8644, CPA (1–30 μM, $n = 6$) produced a concentration-dependent enhancement of the amplitude of electrically evoked contractions of the ureter (Figure 1d and 2a). The effect of each concentration developed in 10–20 min to a steady state. When the effect of 10 μM CPA, administered as a single concentration to the bath, was compared to that produced by 10 μM CPA during a cumulative concentration-response curve, the two effects averaged 37 ± 6 and 55 ± 8% increase over baseline, respectively ($n = 6$ each, Figure 2a). The enhancement of the evoked contractions produced by CPA was reversed by washing at any time after its application. In the presence of 10–30 μM CPA, an increased excitability of the preparation was also evident because multiphasic responses to EFS and/or spontaneous contractions were observed in some preparations.

*Time course of the effect CPA and comparison with ryanodine* In these experiments ($n = 11$), the response to 10 μM CPA was studied over a time course of 60 min. As shown in Figure 1d and 2b, the maximal enhancement was produced at 5–20 min from CPA application (Table 1). At 30–40 min from CPA application, a relatively sudden fading of the enhancement of the evoked contractions was observed and their amplitude equilibrated at a new level, significantly lower than the peak effect of CPA (Figure 1d and 2b, Table 1). When multiphasic responses to EFS had been produced at the peak of the enhancing effect of CPA, these usually disappeared at the new steady state.

For comparative purposes, we investigated the effect of ryanodine, on the response to EFS. The concentration of ryanodine (100 μM) used in this study produces maximal effects on excitation-contraction coupling of the guinea-pig ureter (Maggi *et al.*, 1994a); the effect of combined administration of CPA (10 μM) and ryanodine (100 μM) was also determined (Figure 2b).

Either ryanodine (100 μM, $n = 15$) or ryanodine plus CPA (100 and 10 μM, respectively, $n = 7$) produced an enhancement of the EFS-induced contractions of the guinea-pig ureter, qualitatively and quantitatively similar to that described above for CPA alone (Figure 2b, Table 1). In the presence of ryanodine alone *or* CPA plus ryanodine, a fading of the potentiating effect was observed at 30–40 min from drug administration (Figure 2b): at 60 min, the degree of enhancement produced by ryanodine or ryanodine plus CPA

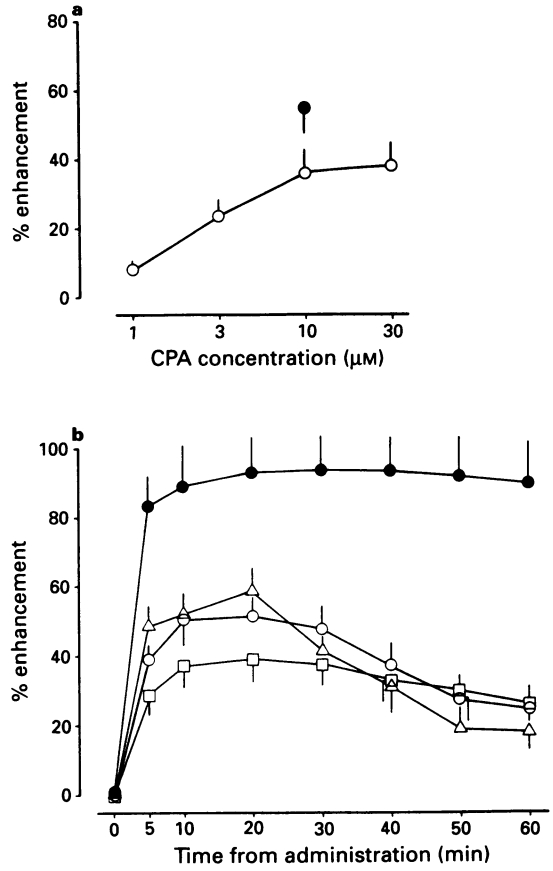


**Figure 2** (a) Concentration-dependent enhancement of the response to EFS by cyclopiazonic acid (CPA ○) produced during a cumulative response curve vs. the response produced by 10 μM CPA (●) administered as a single concentration to the bath. All values are mean ± s.e.mean of 6 experiments performed in the presence of 1 μM Bay K 8644. (b) Time course of the potentiation of EFS-induced contractions of the guinea-pig isolated ureter by CPA (10 μM, ○), ryanodine (100 μM, □), CPA plus ryanodine (△) or tetraethylammonium (10 mM) (●). Each value is mean ± s.e.mean of 6–15 experiments performed in the presence of 1 μM Bay K 8644.

**Table 1** Enhancement by cyclopiazonic acid (CPA), ryanodine and tetraethylammonium (TEA) of EFS-induced contractions in the guinea-pig ureter.

| | n | *Peak effect* (% increase) | *Time to peak* (min) | *Effect at 60 min* (% increase) |
|---|---|---|---|---|
| CPA 10 μM | 11 | 58 ± 3 | 15 ± 2 | 25 ± 5** |
| Ryanodine 100 μM | 15 | 42 ± 3 | 18 ± 3 | 25 ± 3** |
| CPA 10 μM plus ryanodine 100 μM | 7 | 60 ± 8 | 20 ± 4 | 18 ± 5** |
| TEA 10 mM | 6 | 93 ± 12* | 22 ± 3 | 90 ± 12 |

All experiments were performed in the presence of Bay K 8644, 1 μM. For each group, the maximal enhancement of EFS-induced contractions and the time required for development of maximal effect have been calculated as well as the enhancement observed at 60 min from drug application.
Each value is mean ± s.e.mean from $n$ experiments.
*Significantly different from CPA or ryanodine, alone or in combination, $P < 0.05$.
**Significantly different from the peak response to the same drug, $P < 0.05$.

was significantly less than the corresponding peak effect (Table 1). Overall, the results indicate that the effect of CPA and ryanodine are non-additive.

The non-additivity of the enhancement produced by CPA and ryanodine is not linked to the attainment of a maximal contractile effect with each drug alone: tetraethylammonium (TEA, 10 mM) produced a rapid enhancement of EFS-evoked contractions which was sustained throughout the 60 min observation period ($n = 6$, Figure 2b, Table 1). Furthermore, when TEA (10 mM) was added 20–30 min after CPA (10 μM) administration (i.e. at the peak of CPA action), it produced a further prompt enhancement of the EFS-evoked contractions by 35 ± 6% ($n = 4$). The amplitude of EFS evoked contractions in the presence of Bay K 8644 and TEA equalled the response produced by a maximally effective concentration of KCl (80 mM, $n = 4$).

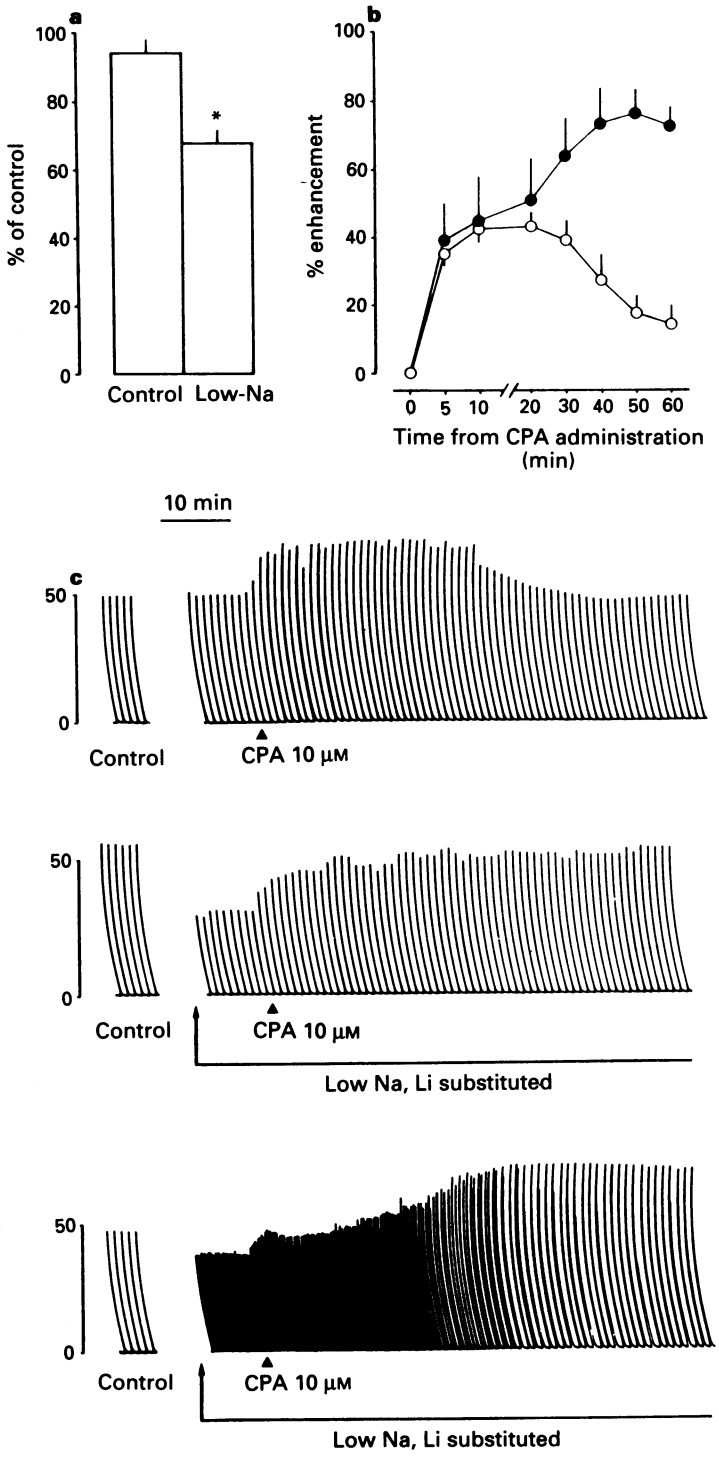


**Figure 3** (a) Amplitude of EFS-evoked contractions after superfusion with normal Krebs solution (control) or low $Na^+$, $Li^+$-substituted Krebs solution in the presence of 1 μM Bay K 8644. Each value is mean ± s.e.mean of 7 experiments. $^*P<0.05$ vs control. (b) Effect of cyclopiazonic acid (CPA, 10 μM) on EFS-induced contractions in normal Krebs solution (○) or in low $Na^+$, $Li^+$-substituted Krebs solution (●) in the presence of 1 μM Bay K 8644. Each value is mean ± s.e.mean of 7 experiments. (c) Typical tracings comparing the effect of CPA on EFS-evoked contractions of the guinea-pig ureter in normal Krebs (upper panel) or in low $Na^+$, $Li^+$-substituted Krebs solution (middle and low tracings). All experiments were performed in the presence of 1 μM Bay K 8644. In panel (c), vertical bars are percentage of the response to KCl.

*Effect of CPA in a low-$Na^+$ Krebs solution* We postulated that the fading of the response to CPA may involve an increased efficiency of a CPA-insensitive mechanism(s) which removes free $Ca^{2+}$ from the cytoplasm following SR $Ca^{2+}$ ATPase blockade. An involvement of the $Na^+/Ca^{2+}$ exchanger is an interesting possibility, since physiological evidence for the existence of this mechanism has been well documented in the guinea-pig ureter (Aickin *et al.*, 1984; 1987; Aickin, 1987; Aaronson & Benham, 1989). In the absence of selective inhibitors, we studied the effect of reducing extracellular $Na^+$ on the response to EFS and on the intensity and time course of the effect of CPA.

Superfusion with low-$Na^+$, $Li^+$-substituted, Krebs solution (60% reduction of extracellular $Na^+$ by $Li^+$) for 30 min reduced the amplitude of the EFS-evoked contractions (in the presence of Bay K 8644) ($n = 7$, Figure 3a). In 5 out of 7 cases tested the low-$Na^+$, $Li^+$-substituted medium also induced the appearance of spontaneous phasic contractions and/or biphasic responses to EFS (Figure 3c). The spontaneous contractions had a similar amplitude of those evoked by EFS.

CPA (10 μM) enhanced the amplitude of the EFS-induced contractions in low-$Na^+$, $Li^+$-substituted medium as well as the amplitude of spontaneous contractions, when present (Figure 3b and c, Table 2). In the low-$Na^+$, $Li^+$-substituted medium, the effect of CPA ensued and developed as observed in controls during the first 20–30 min from CPA administration (Figure 3b): however, while the expected decline in the enhancement of EFS-induced contractions was observed in control ureters, the effect of CPA in low-$Na^+$, $Li^+$-

**Table 2** Enhancement by cyclopiazonic acid (CPA, 10 μM) of the response to EFS in guinea-pig isolated ureter in normal and low-$Na^+$Krebs solution

| | n | *Peak effect* (% increase) | *Time to peak* (min) | *Effect at 60 min* (% increase) |
|---|---|---|---|---|
| Control | 7 | 44 ± 4 | 12 ± 2 | 15 ± 5* |
| Low-$Na^+$, $Li^+$-substituted | 7 | 73 ± 10 | 31 ± 5 | 72 ± 6 |
| Control | 6 | 57 ± 9 | 17 ± 4 | 23 ± 4* |
| Low-$Na^+$, choline-substituted | 6 | 51 ± 12 | 22 ± 7 | 47 ± 8 |

All experiments were performed in the presence of Bay K 8644, 1 μM. For each group, the maximal enhancement of EFS-induced contractions and the time required for development of maximal effect have been calculated as well as the enhancement observed at 60 min from drug application. Two ureters from the same animal were studied in parallel one of which was superfused with low-Na medium (60% extracellular $Na^+$ replaced with either $Li^+$ or choline) and the other served as control. After superfusion, when the amplitude of the response to EFS had reached a steady state, CPA 10 μM was added to the bath and its effect recorded over 60 min Each value is mean ± s.e.mean from $n$ experiments.
*Significantly different from the peak response to CPA, $P<0.05$.

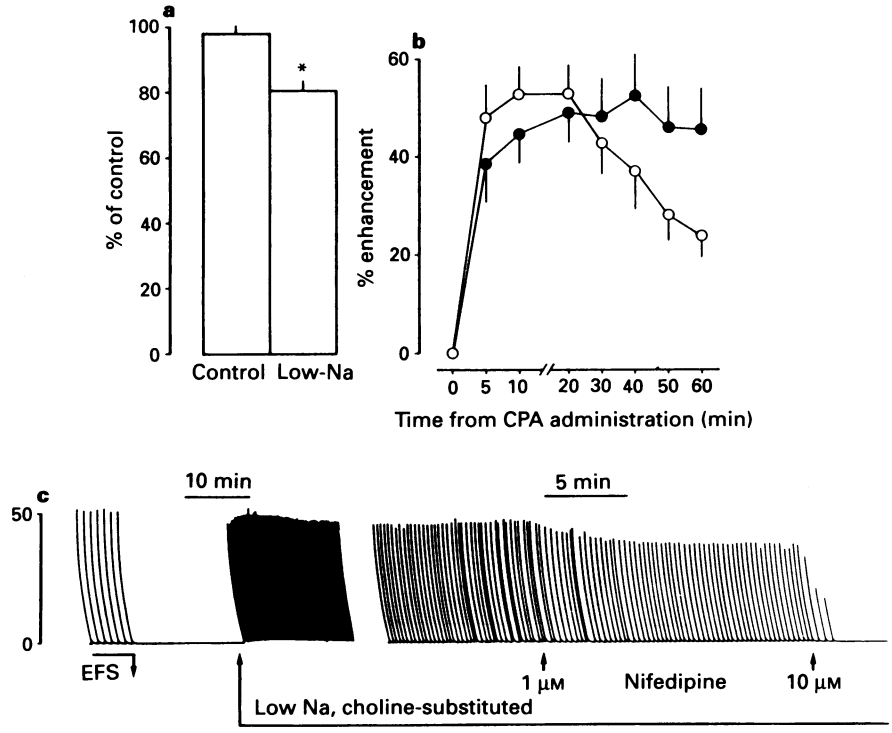


**Figure 4** (a) Amplitude of EFS-evoked contractions after superfusion with normal Krebs solution (Control) or low $Na^+$, choline-substituted Krebs solution in the presence of 1 μM Bay K 8644. Each value is mean ± s.e.mean of 12 experiments. *$P<0.05$ vs control. (b) Effect of cyclopiazonic acid CPA, (10 μM) on EFS-induced contractions in normal Krebs solution (○) or in low $Na^+$, choline-substituted Krebs solution (●) in the presence of 1 μM Bay K 8644. Each value is mean ± s.e.mean of 6 experiments. (c) Typical tracing showing the appearance of spontaneous contractions after superfusion with low $Na^+$, choline-substituted Krebs solution and their inhibition/suppression by nifedipine. All experiments were performed in the presence of 1 μM Bay K 8644. In panel (c), the vertical bar is the percentage response to KCl.

substituted medium was sustained throughout the 60 min observation period and in 3 cases the maximal enhancement was produced at 40–50 min (Figure 3b and c).

In the presence of Bay K 8644, superfusion with a low-$Na^+$, choline-substituted Krebs solution invariably induced the occurrence of a high frequency (see below) spontaneous activity ($n = 12$, Figure 4c). The amplitude of phasic contractions equilibrated, at steady state, at a lower value than that of contractions producted by EFS before reduction of extracellular $Na^+$ (Figure 4a, $n = 6$). In low-$Na^+$, choline-substituted medium, CPA (10 μM) produced a sustained increase in the amplitude of contractions which did not show a significant fading during the 60 min observation period (Figure 4b, Table 2).

The characteristics of spontaneous contractions produced by the low-$Na^+$ choline-substituted medium were investigated in further details (Figure 4c): after having recorded steady responses to EFS, the stimulation was stopped and the ureters were superfused for 30 min with a low-$Na^+$, choline-substituted medium. Spontaneous contractions appeared in all cases tested ($n = 6$) within 5 min from start of superfusion with the low-$Na^+$ medium: at a steady state their frequency averaged $4.3 \pm 0.6$ cycles $min^{-1}$ and their amplitude $86 \pm 1\%$ of that of EFS-evoked contractions. Nifedipine (1 μM) produced $23 \pm 3\%$ inhibition of the amplitude of contractions and abolished the spontaneous activity at 10 μM ($n = 4$, Figure 4c). Washout with normal Krebs solution likewise stopped the spontaneous activity observed during superfusion with low $Na^+$, choline-substituted Krebs solution and the re-application of low-$Na^+$, choline substituted medium induced their re-appearance.

*Effect of CPA on the refractory period of the guinea-pig ureter* The ability of CPA to restore the EFS-induced contractions or prevent their fading in the absence of Bay K 8644 (Figure 1b and c) suggests that CPA increases the excitability of the ureter to depolarizing stimuli. To check this hypothesis and quantitate this effect, we investigated the effect of 10 μM CPA on the refractory period of the guinea-pig ureter, as described previously (Maggi *et al.*, 1994a). In the absence of CPA, only 4 out of 11 preparations developed a response at 3 or 5 s interval between the two stimuli and all ureters responded after a 10 or 30 s interval (Figure 5). In the presence of CPA, 9 out of 11 ureters responded at an interval of 3 s and all ureters responded at 5 s interval between the first and second stimulus. As shown in Figure 5, the amplitude of the response to the test stimulus, expressed as % of the response to the conditioning stimulus was significantly increased by CPA at 3, 5 and 10 s interstimulus intervals.

*Effect of CPA on the response to KCl* The application of 80 mM KCl produced distinct phasic and tonic components of contraction of the ureter (Figure 6). The amplitude of the phasic contraction ranged between 40 and 90% of that of the tonic contraction (determined at 10 min from application of KCl).

CPA (10 μM, 30–60 min before) enhanced the amplitude of the phasic response to 80 mM KCl and prolonged the duration of phasic contractions (Figure 6); the amplitude of the tonic response, determined at 10 min from addition of KCl, was slightly but not significantly reduced. The influence of the two combined effects was best evident when calculating the ratio between the amplitude of the phasic and tonic components of the response to KCl which was significantly enhanced at both 30 and 60 min after CPA administration. After 30 min incubation with CPA the ratio between the phasic and the tonic response to KCl averaged $100 \pm 7\%$ vs. $63 \pm 4\%$ obtained in the same ureters before addition of CPA ($P < 0.05$, $n = 7$). Likewise, after 60 min incubation with CPA, the ratio averaged $89 \pm 8\%$ vs. $59 \pm 4\%$ of the control response ($n = 8$, $P < 0.05$).

At both incubation times (4 out of 7 cases at 30 min, 8 out of 8 cases at 60 min) CPA also modified the shape of the response to KCl: in the presence of CPA, a 'hump' (indicated by arrows in Figure 6) appeared between the initial phasic and the sustained tonic response, peaking at about 2 min from addition of KCl. This hump could be due to to a faster development of the tonic response to KCl in the presence of CPA since: (a) the hump peaked at about the same tension values measured at 10 min from addition of KCl in the absence of CPA and (b) upon superimposition of tracings, the rate of rise of the sustained response appeared much faster in the presence than in the absence of CPA.

*Sucrose gap experiments*

*Effect of CPA on the action potential of the ureter* CPA (10 μM, $n = 5$; Figures 7 and 8) produced a number of changes in the action potential and accompanying phasic contraction of the guinea-pig ureter produced by direct electrical stimulation of smooth muscle (single pulses 30–40 V, 1–2 ms pulse width). All effects ensued within 10–20 min from start of superfusion: some of them were maintained for the whole observation period while others faded within 20–30 min.

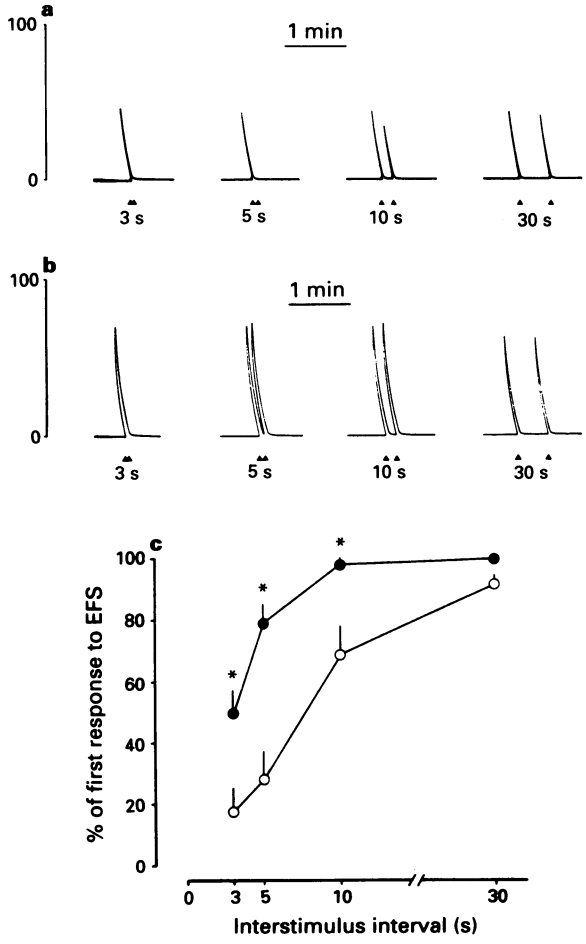


**Figure 5** (a and b) Effect of cyclopiazonic acid (CPA) on the refractory period of the guinea-pig ureter. Two consecutive trains of electrical pulses (at arrowheads) were applied at 3–30 s intervals in the absence (a) or presence (b) of 10 μM CPA. Note that CPA markedly enhanced the ability of the ureter to develop a phasic contraction in response to the second stimulus of each pair, thereby reducing the refractory period. (c) Effect of CPA on the refractory period of the guinea-pig ureter. Each value is mean ± s.e.mean of 11 experiments: (●) CPA 10 μM; (○) control. *$P < 0.05$ vs. controls. In panels (a) and (b) the vertical bars are the percentage of the response to KCl.

The amplitude of the first spike of the action potential was not significantly affected by CPA, being 10.4 ± 1.0, 10.7 ± 1.1 and 10.4 ± 1.1 mV before and at 10 or 60 min of CPA action, respectively ($n = 5$).

The time course of CPA action on membrane potential, latency between stimulus application and onset of action potential, action potential duration (at 90% of repolarization) and contraction is shown in Figure 8. Two effects of CPA ensued within 10–20 min and were maintained or developed further during the 60 min observation period: these were membrane depolarization and reduction in the latency between stimulus application and onset of the action potential. Two effects of CPA peaked within 10–20 min and then subsided: these were a prolongation of action potential duration (59 ± 12% increase) and an increase in amplitude of contraction (34 ± 11% increase) (Figure 8).

When expressed as maximal % change from control values observed in each preparation at any time from CPA administration, the latency between stimulus application and onset of action potential was decreased by 39 ± 11%, while action potential duration (at 90% of repolarization) and the amplitude of contraction were increased by 59 ± 12 and 34 ± 11%, respectively ($n = 5$).

CPA also transiently affected the contraction-relaxation cycle of the guinea-pig ureter: these changes peaked at 10–30 min from start of superfusion with CPA. At the peak of its action, CPA increased the duration of the cycle from 1.97 ± 0.1 to 3.1 ± 0.6 s (57% increase). The increase was relatively larger for the contraction time (CT) than for the relaxation time (RT): CT was increased from 0.87 ± 0.06 to 1.67 ± 0.4 s (92% increase), while RT was increased from 1.1 ± 0.03 to 1.57 ± 0.3 s (42% increase). Accordingly the CT/RT ratio was increased from 80 ± 5 to 111 ± 16% ($n = 5$ for each value).

*Effect of low-Na$^+$, choline-substituted Krebs solution on membrane potential* A brief superfusion (3 min) with low-Na$^+$, choline-substituted Krebs solution produced membrane depolarization by 1.96 ± 0.2 mV ($n = 3$, Figure 9) which was reversible upon returning to normal Krebs solution. The repolarization was quite slow and it took > 10 min after returning to normal Krebs solution for recovery of resting membrane potential. In 2 cases, the depolarization produced by low-Na$^+$, choline-substituted Krebs solution was accompanied by 1–2 action potentials and phasic contractions upon the first challenge, but this effect disappeared upon repeated applications of low-Na$^+$, choline-substituted Krebs solution to the same preparation.

Addition of Bay K 8644 (1 μM) did not change the resting membrane potential but, in its presence, action potentials and phasic contractions were superimposed on the depolarization brought about by the low-Na$^+$, choline-substituted Krebs

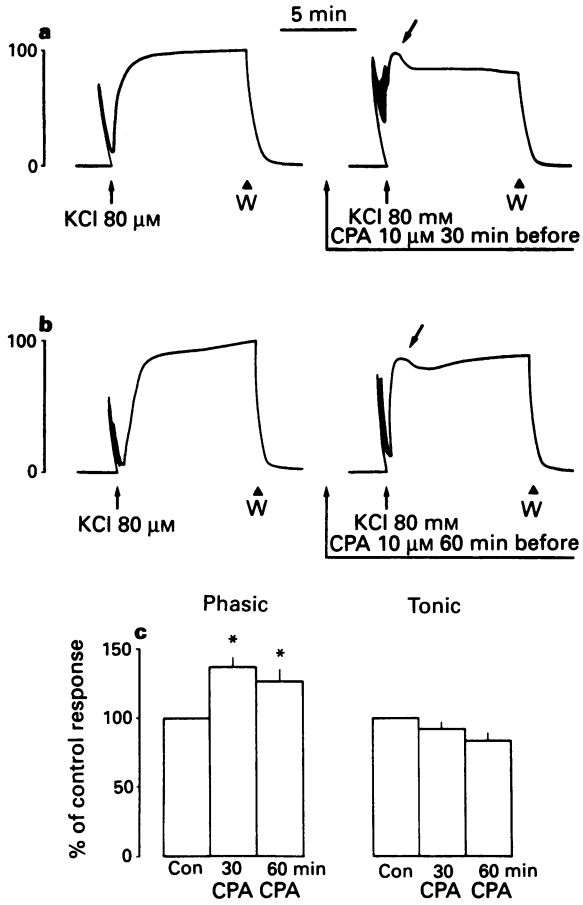


**Figure 6** Effect of cyclopiazonic acid (CPA, 10μM) on the biphasic contractile response produced by 80 mM KCl in the guinea-pig isolated ureter: a and b show the effect of CPA at 30 or 60 min from its addition to the bath. Note that CPA enhanced and prolonged the phasic response to KCl and that a 'hump' (indicated by arrows) appears between the phasic and tonic response to KCl. (c) Effect of CPA on phasic and tonic responses to 80 mM KCl. Each value is mean ± s.e.mean of 7–8 experiments. *$P < 0.05$ vs control. In panels (a) and (b) the vertical bars are the percentage of the response to KCl.

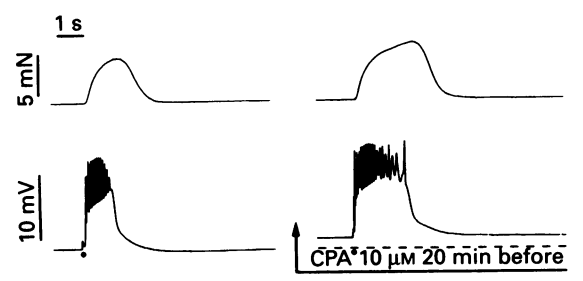


**Figure 7** Effect of 10μM cyclopiazonic acid (CPA) on the action potential and contraction of the guinea-pig isolated ureter induced by EFS (applied at dots) recorded by sucrose gap in the presence of 1 μM Bay K 8644. Lower tracing shows changes in membrane potential, upper tracing shows tension. Note that CPA depolarized the membrane, reduced the latency between stimulus application and onset of the action potential, prolonged the duration of the action potential and enhanced the amplitude and duration of the accompanying phasic contraction.

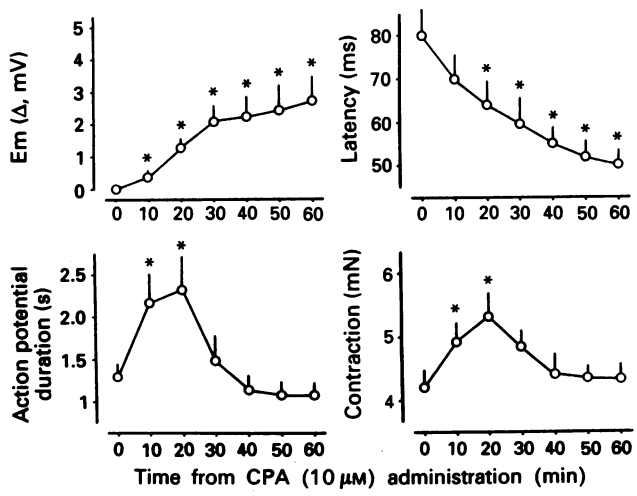


**Figure 8** Time course of the effect of cyclopiazonic acid (CPA) on membrane potential (Em), latency between stimulus application and onset of the action potential, duration of the action potential and amplitude of contractions of the guinea-pig ureter in the presence of 1 μM Bay K 8644. Each value is mean ± s.e.mean of 5 experiments. *$P < 0.05$ vs. controls.

solution. Nifedipine (10 μM) did not affect the resting membrane potential and eliminated the effect of Bay K 8644. In the presence of nifedipine the depolarization observed in response to superfusion with the low-$Na^+$, choline-substituted Krebs solution averaged $1.48 \pm 0.05$ mV ($n = 3$).

Both in the absence and presence of nifedipine a further small depolarization was observed when replacing the low-$Na^+$, choline-substituted Krebs solution with normal Krebs solution (Figure 9).

## Discussion

It is widely accepted that SR $Ca^{2+}$-ATPase plays a role in regulating intracellular $Ca^{2+}$ homeostasis (Grover & Khan, 1992, for review), but the definition of its role in excitation-contraction coupling has been hampered by the lack of selective inhibitors. CPA has been characterized as a highly selective inhibitor of $Ca^{2+}$-ATPase in skeletal and smooth muscle

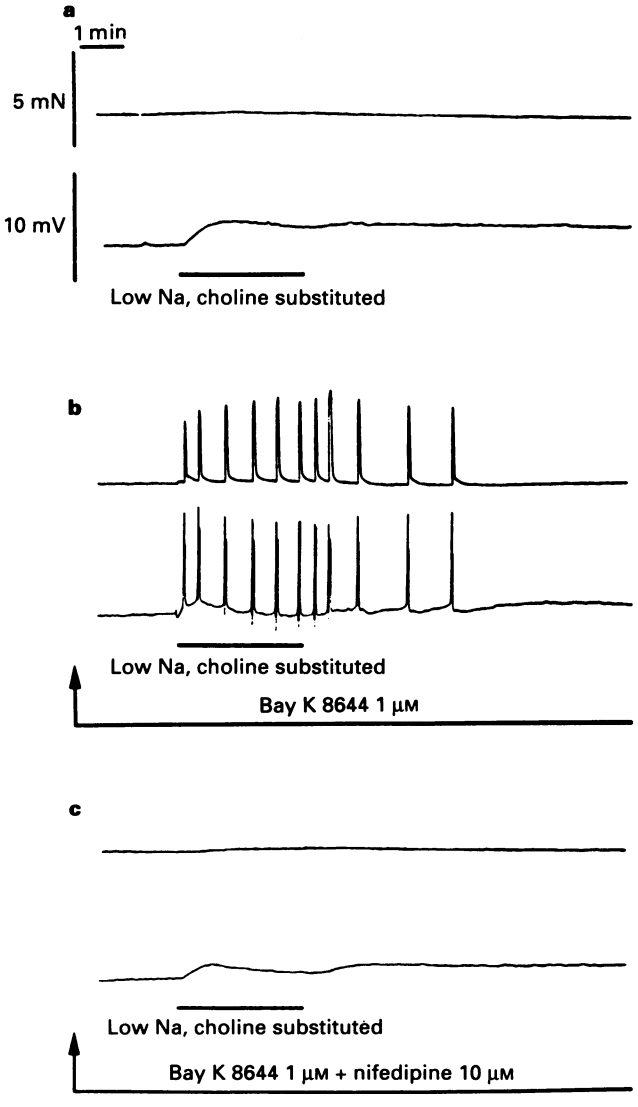


**Figure 9** (a) Effect of a brief superfusion with a low-$Na^+$, choline-substituted Krebs solution on membrane potential (lower tracing in each panel) and tension (upper tracing in each panel) of the guinea-pig ureter recorded by sucrose gap. In the presence of Bay K 8644 (b), action potentials and phasic contraction superimposed on the depolarization induced by superfusion with low $Na^+$ medium; the effect of Bay K 8644 was abolished by nifedipine (c). Note that, in the absence and presence of nifedipine a small depolarization was evident when changing the bathing solution from low $Na^+$ to normal Krebs.

SR (Goeger & Riley, 1989; Seidler *et al.*, 1989; Deng & Kwan, 1991). CPA prevents the binding of $Ca^{2+}$ to its high affinity site on SR and inhibits the $Ca^{2+}$-dependent phosphorylation of the SR proteins (Goeger & Riley, 1989). CPA selectively inhibits the ATP-dependent $Ca^{2+}$ uptake by intracellular storage sites in skinned ileal smooth muscle (Uyama *et al.*, 1992) and blocks contractile responses related to $Ca^{2+}$ mobilization from internal stores by preventing the refilling of the releasable $Ca^{2+}$ pool (Low *et al.*, 1992; Uyama *et al.*, 1992; Bourreau *et al.*, 1993). Furthermore, CPA increases the excitability of smooth muscles and affects $Ca^{2+}$ and $K^+$ currents (Suzuki *et al.*, 1992; Gagov *et al.*, 1993; Uyama *et al.*, 1993). The latter effects have been interpreted as secondary events to SR $Ca^{2+}$-pump inhibition by CPA.

The concentrations of CPA producing effects on electromechanical coupling of the guinea-pig ureter (1–30 μM) are comparable to those used in previous studies on other smooth muscles. Since $Ca^{2+}$ movements into and from the internal store(s) were not directly monitored in this study, we cannot totally exclude the possibility that the effects of CPA may involve some as yet undiscovered action of this compound. The discussion of present findings is based on the assumption that CPA selectively affects SR $Ca^{2+}$ ATPase in the guinea-pig ureter smooth muscle. This assumption is indirectly supported by the observation that many effects produced by CPA resemble those produced by ryanodine (Maggi *et al.*, 1994a). CPA and ryanodine affect SR function(s) by acting at different targets: hence, the effects produced by *both* drugs are likely to be consequent to the impairment of SR $Ca^{2+}$ handling, while qualitative/quantitative differences may either reflect their different mode of action on SR $Ca^{2+}$ handling or may involve nonspecific effects. The exact functional relationship(s) between the ryanodine receptor (CICR channel) and the CPA-sensitive $Ca^{2+}$-ATPase on SR are largely unsettled: replenishment of the SR $Ca^{2+}$ store by $Ca^{2+}$-ATPase is obviously required for CICR. It may be speculated that the activity of the SR $Ca^{2+}$-ATPase is indirectly affected following blockade of the CICR ryanodine receptor. This may account for the observed nonadditivity of the effects of CPA and ryanodine (Figure 2, Table 1).

### *Effect of CPA on electromechanical coupling of the guinea-pig ureter*

SR in smooth muscles can influence intracellular $Ca^{2+}$ in two ways: (a) it can increase cytoplasmic $Ca^{2+}$ by releasing $Ca^{2+}$, resulting in an initiation or augmentation of contraction; (b) it can decrease cytoplasmic $Ca^{2+}$ by uptake system(s) which use ATP to pump $Ca^{2+}$ into the store against a chemical gradient; the second effect can mediate or amplify the relaxation of smooth muscles. Agents which modify intracellular $Ca^{2+}$ handling by the SR can thus be expected to produce either a reduction or an enhancement (or both effects) of the amplitude of evoked contractions, depending on whether the release or storage function of SR predominate. Furthermore, a number of membrane currents can be affected by altering SR $Ca^{2+}$ handling, thereby influencing membrane potential and excitability (see below).

In previous studies, CPA has been used to block the refilling of the internal $Ca^{2+}$ store: this produces a depletion upon consecutive challenges by agents which mobilize $Ca^{2+}$ from the store, thereby eventually abolishing the response under investigation (e.g. Low *et al.*, 1992; Bourreau *et al.*, 1993). In the ureter, CPA did not reduce the amplitude of EFS-evoked contractions produced during repetitive cycles of stimulation, suggesting that release of $Ca^{2+}$ from the internal store does not primarily contribute to the elevation of activator $Ca^{2+}$ which determines electromechanical coupling. This observation is in keeping with our previous results with ryanodine (Maggi *et al.*, 1994a).

On the other hand CPA (and ryanodine) increased the amplitude of EFS-evoked phasic contractions of the ureter:

this effect was more pronounced when tested in the presence of Bay K 8644 but, for CPA, it was also observed without prior addition of the $Ca^{2+}$ channel agonist. This effect was also evident on the phasic response of the ureter to KCl, which is accompanied by the firing of action potentials (Washizu, 1967; Johnishi & Sunano, 1978), and may be explained by assuming that SR $Ca^{2+}$ ATPase plays a role in removing free intracellular $Ca^{2+}$ entering via L-type $Ca^{2+}$ channels activated by depolarization. Blockade of the $Ca^{2+}$ pump would then lead to a more intense/prolonged elevation of free intracellular $Ca^{2+}$ and account for the observed effects on contraction amplitude and duration of the contractile cycle. Clearly, other mechanisms, which can be operationally defined as CPA-insensitive, act to terminate the contractile cycle of the ureter in the presence of CPA; the plasma membrane $Ca^{2+}$ ATPase and the $Na^+/Ca^{2+}$ exchanger are likely candidates for this role (see below).

Both ryanodine (Maggi *et al.*, 1994a) and CPA produced a sustained depolarization of the membrane of the guinea-pig ureter: this effect might be consequent to the blockade of a small spontaneous release of $Ca^{2+}$ from the SR, contributing to the resting membrane potential by activating $K^+$ channels in the vicinity of the SR. Suzuki *et al.* (1992) showed that 10 μM CPA strongly suppresses $Ca^{2+}$-dependent $K^+$-currents in guinea-pig smooth muscles. The prolongation of action potential duration observed with either CPA or ryanodine (Maggi *et al.*, 1994a) in the presence of Bay K 8644 remains unexplained at present. This effect is not consequent to membrane depolarization *per se*, since it is not reproduced by a mild elevation of extracellular $K^+$ (Maggi *et al.*, 1994a). Assuming that CPA elevates intracellular free $Ca^{2+}$ during the action potential, as suggested by the increased developed tension and prolongation of the contractile cycle, it may be speculated that activation of some $Ca^{2+}$-dependent conductance(s) is involved in this effect.

An interesting feature of the effect of CPA on the response to KCl was the appearance of a 'hump' between the phasic and the sustained component of the contractile response: triphasic responses of the guinea-pig ureter to elevated extracellular $K^+$ concentration have been described in previous studies when using higher KCl concentrations than that used here (for e.g. Johnishi & Sunano, 1978). This effect may involve a faster development of KCl depolarization in the presence of CPA which depolarizes the membrane of its own.

### *Time course and fading of the action of CPA*

Previous studies have outlined several aspects in the pharmacology of CPA on smooth muscles which have been confirmed here, including the reversibility of the effect of CPA by washing. In most of the previous studies, the effect of μM concentrations of CPA on smooth muscle excitation-contraction coupling have been studied following a relatively short contact time (e.g. 5–15 min, Suzuki *et al.*, 1992; Janssen & Sims, 1993). We found that some effects of CPA (e.g. depolarization of the membrane) are sustained or anyway develop in full over the 60 min observation period. Other effects, such as the enhancement of the amplitude of contractions showed a peak within 30 min from CPA administration and then faded. We exclude the possibility that this fading involves a degradation of added CPA since: (a) a second addition of CPA (10 μM) at 60 min from the first application in organ bath experiments did not enhance the amplitude of the EFS-evoked contraction (data not shown); (b) a similar biphasic time-course of action of CPA was observed during a prolonged superfusion in sucrose gap experiments. The reason why certain effects of CPA undergo fade is intriguing and may involve different mechanisms, discussed below.

Since CPA has been reported to induce nitric oxide (NO) release from endothelial cells, thereby inducing smooth muscle relaxation (Moritoki *et al.*, 1994), it may be hypothesized that a similar effect is involved in the time-dependent decay of the enhancement of the EFS-evoked contractions. This explanation seems unlikely, however, since we have observed a similar time-course of action of CPA when the ureters had been pre-exposed to the NO-synthase inhibitor, $N^G$-nitro-L-arginine (L-NOARG, 30 μM for 15 min) (data not shown). L-NOARG by itself does not affect the amplitude of the EFS-evoked contractions (Maggi *et al.*, 1994c) indicating that NO production does not play a major role in regulating the response of the guinea-pig ureter to EFS.

The decay of the action of CPA may theoretically involve a reduction of the $Ca^{2+}$ current by elevated intracellular $Ca^{2+}$ following blockade of the SR $Ca^{2+}$ pump, i.e. a kind of $Ca^{2+}$-induced inactivation of the $Ca^{2+}$ current: a similar effect has been described to be produced by CPA after the concomitant administration of ryanodine (Gagov *et al.*, 1993). However, neither the amplitude nor the rate of rise (data not shown) of the first spike of the action potential are modified by CPA. Since the present experiments were performed in the presence of Bay K 8644, which counteracts the inactivation of $Ca^{2+}$ channels (Imaizumi *et al.*, 1989a) it is possible that inactivation of $Ca^{2+}$ channels was partially prevented under our experimental conditions.

Third, other mechanisms for $Ca^{2+}$ extrusion may come into action to compensate for the decreased or blocked $Ca^{2+}$ ATPase function by CPA. These may involve the plasma membrane $Ca^{2+}$ pump (Grover & Khan, 1992 for review) and/or the $Na^+/Ca^{2+}$ exchanger, which may be important for $Ca^{2+}$ extrusion and relaxation in guinea-pig ureter smooth muscle under certain circumstances (Aickin *et al.*, 1984; Aaronson & Benham, 1989). CPA does not affect the plasmalemmal $Ca^{2+}$ ATPase (Seidler *et al.*, 1989), while no data are available about its possible effects on the $Na^+/Ca^{2+}$ exchanger.

To address the possible involvement of a compensatory increase in activity of the $Na^+/Ca^{2+}$ exchanger in the fading of the response to CPA, we studied the effect of a low-$Na^+$ Krebs solution in which extracellular $Na^+$ had been reduced by 60%. This approach has the limitation that direct effects of the substitute used to replace extracellular $Na^+$ also occur in parallel. For instance, $Li^+$ can abolish the response of the ureter to KCl, especially in its tonic component (Washizu *et al.*, 1967). To partially overcome this drawback, we studied two different substitutes for extracellular $Na^+$, $Li^+$ and choline: some of the observed effects were, at least qualitatively, observed with both media and may therefore represent a true consequence of $Na^+$ removal rather than nonspecific effects of the $Na^+$ substitutes.

We speculated that, if the $Na^+/Ca^{2+}$ exchanger becomes more active after blockade of the CPA-sensitive $Ca^{2+}$ pump, producing the fading of the effect of CPA on the amplitude of EFS-evoked contractions, a reduction of the transmembrane gradient to $Na^+$ would counteract the fading of the effect of CPA. The results of these experiments showed that the effect of CPA is maintained when tested in low-$Na^+$ medium. With low-$Na^+$, $Li^+$-substituted solution the peak effect of CPA was actually increased; however, the concomitant reduction of baseline amplitude of EFS-evoked contractions prevents a straight quantitative comparison of the data.

Another effect observed with both types of low-$Na^+$ media was the appearance of spontaneous contractions: this effect was more pronounced when using choline than $Li^+$ as $Na^+$-substitute. The spontaneous activity developing under these conditions was suppressed by nifedipine, indicating that L-type $Ca^{2+}$ channels become activated in these conditions. Electrophysiological recordings showed that the depolarization produced by the low-$Na^+$, choline-substituted medium is the *primum movens* of this response which, in the presence of Bay K 8644, led to development of action potentials and contractions. A depolarization of the ureter smooth muscle upon reduction or total removal of extracellular $Na^+$ has been noted in previous studies in the guinea-pig ureter (e.g. Kuriyama & Tomita, 1970; Aickin *et al.*, 1984; Aickin, 1987).

Again, the results of these experiments are complicated by the possible direct membrane effects of the $Na^+$ substitute; furthermore, since endogenous calcitonin gene-related peptide (CGRP) produces hyperpolarization of the ureter smooth muscle (Santicioli & Maggi, 1994), the results of previous studies on this matter may have been influenced by the release of endogenous CGRP from afferent nerves. The nature of the nifedipine-resistant depolarization produced by the low-$Na^+$, choline-substituted Krebs solution in capsaicin-pretreated ureters needs further investigation.

The present results are in keeping with the hypothesis that the $Na^+/Ca^{2+}$ exchanger becomes more active in removing intracellular free $Ca^{2+}$ after blockade of the CPA-sensitive SR $Ca^{2+}$ pump. Since the $Na^+/Ca^{2+}$ exchanger operates with a 3/1 stoichiometry, this mechanism would be electrogenic and, accordingly, may also participate in the overall membrane depolarization produced by CPA. Clearly, an inhibitor of the $Na^+/Ca^{2+}$ exchanger would be needed for the direct testing of this hypothesis. An abundance of mechanisms operating to lower free intracellular $Ca^{2+}$ and terminate contraction may be expected to be present in the ureter smooth muscle, since any tonic type of contraction would oppose the physiological pattern of motility in this viscus, the phasic pumping of urine to the bladder.

In conclusion, the present findings demonstrate that CPA profoundly affects excitation-contraction coupling in the smooth muscle of the guinea-pig ureter. The striking similarities and the nonadditivity of the effects of CPA and ryanodine (Maggi *et al.*, 1994a) strongly suggest that the effects of CPA are ascribable to an interference of $Ca^{2+}$ handling by the SR $Ca^{2+}$ATPase. Our present and previous (Maggi *et al.*, 1994a) findings strongly support the idea that SR $Ca^{2+}$ handling is not involved in excitation-contraction coupling of the guinea-pig ureter, but plays an essential role in keeping intracellular $Ca^{2+}$ low. The present findings also suggest that the $Na^+/Ca^{2+}$ exchanger may be involved in regulating excitation-contraction coupling at this level, especially after blockade of the SR $Ca^{2+}$ pump.

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# Increase in tone and intracellular $Ca^{2+}$ in rabbit isolated ear artery by platelet-derived growth factor

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**1** The effect of platelet-derived growth factor (PDGF-AB) on tone and intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) was examined in rabbit isolated ear arteries. Arteries were mounted in a myograph and loaded with the $Ca^{2+}$-sensitive fluorescent indicator, fura-2, for concurrent measurements of isometric force and $[Ca^{2+}]_i$.

**2** PDGF-AB contracted rabbit ear artery in a concentration-dependent manner. PDGF-AB induced tone was associated with a rise in $[Ca^{2+}]_i$. In the presence of noradrenaline, PDGF-AB induced a similar rise in $[Ca^{2+}]_i$ but contraction in response to PDGF-AB in the presence of noradrenaline was increased compared with PDGF-AB alone.

**3** PDGF-AB-induced rise in $[Ca^{2+}]_i$ and tone were abolished by removal of extracellular $Ca^{2+}$ (with addition of BAPTA, a $Ca^{2+}$ chelator), and by preincubation with a dihydropyridine calcium channel blocker, (−)-202 791. Bistyrphostin, a selective inhibitor of tyrosine kinases, also inhibited PDGF-AB-induced tone, but had no effect on noradrenaline- or potassium-induced tone.

**4** PDGF-AB contracts rabbit ear artery by increasing $Ca^{2+}$ entry through voltage-operated calcium channels. This effect involves activation of a tyrosine kinase.

**Keywords:** Platelet-derived growth factor; vascular smooth muscle; calcium; tyrosine kinase

## Introduction

Platelet-derived growth factor (PDGF) is a 28–32 kDa peptide originally described in platelets (Kohler & Lipton, 1974), which may have important physiological and pathological roles in the vasculature (Ross *et al.*, 1990). PDGF is now known to be produced by many cell types including endothelial cells, vascular smooth muscle cells and macrophages/monocytes (reviewed in Bobik & Campbell, 1993). PDGF exists in multiple molecular weight isoforms which are composed of two chains (A and B) which share considerable homology. All possible dimeric forms of PDGF have been described (PDGF-AA, PDGF-BB, PDGF-AB), although the dominant isoform produced varies, depending on cell type and experimental conditions (Bobik & Campbell, 1993).

There is also heterogeneity of PDGF receptors. PDGF receptors are 170–180 kDa peptides formed from two subunits (α and β). PDGF receptors may exist in three dimeric forms; αα, αβ and ββ (Hart *et al.*, 1988). These receptor dimers differ in their affinity for the PDGF isoforms; PDGF-AA binds to αα, PDGF-AB binds to αα and αβ, while PDGF-BB binds to αα, αβ and ββ (Seifert *et al.*, 1989). Consequently the different isoforms of PDGF may have different effects depending on which receptor isoforms are expressed by a particular cell type.

All three isoforms of PDGF are reported to be potent mitogens for vascular smooth muscle cells (Ross *et al.*, 1990; Bobik & Campbell, 1993). PDGF-AB is the dominant isoform in human platelets and may account for 50% of the mitogenic effect of platelet activation (Heldin *et al.*, 1981). PDGF also contracts vascular smooth muscle in some (Berk *et al.*, 1986; Berk & Alexander, 1989), but not all sites (Basset *et al.*, 1988). PDGF-induced vasoconstriction is reported to be unaffected by antagonists of α-adrenoceptors, $5\text{-}HT_2$ receptors or cyclo-oxygenase (Berk *et al.*, 1986) and presumably involves a direct action on the PDGF receptor, although the mechanism of this effect is unclear. In the case of all three isoforms the consequence of the interaction of PDGF with the membrane associated receptor is to induce receptor dimerization and autophosphorylation on multiple tyrosine sites of the intracellular domain of the receptor (Pazin & Williams, 1992). These phosphotyrosine residues can then activate a range of intracellular signalling pathways by a process involving binding to peptide domains in the effector proteins bearing homology with src protein (SH2 domains). Phospholipase C-γ, phosphatidyl-inositol-3 kinase, GTPase activating protein for $p21^{ras}$ (GAP) and $p21^{ras}$ all appear to be activated by PDGF in this way (Pazin & Williams, 1992). The role of one or any of these systems in PDGF-induced contraction of intact vascular smooth muscle is unclear, however, changes in intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) play a key role in regulating vascular tone (Somlyo & Himpens, 1989). This study examined the possible role of changes in $[Ca^{2+}]_i$ in the contractile action of PDGF in rabbit isolated ear artery by use of the fluorescent $Ca^{2+}$ indicator fura-2.

## Methods

Branches of rabbit ear artery were mounted in a myograph (Mulvany & Halpern, 1977) containing physiological saline at 37°C and bubbled with 95% $O_2$, 5% $CO_2$ for simultaneous measurement of force and $[Ca^{2+}]_i$ as previously described (Jensen *et al.*, 1992; 1993). The composition of the physiological saline was (in mM): NaCl 118, KCl 4.7, $CaCl_2.6H_2O$ 2.5, $MgSO_4.7H_2O$ 1.17, $NaHCO_3$ 25.0, $NaH_2PO_4.2H_2O$ 1.0, $Na_2EDTA$ 0.03 and glucose 5.5. Arteries were loaded with the $Ca^{2+}$-sensitive fluorescent dye, fura-2 (Tsien, 1981; 1989), by incubation with physiological saline containing fura-2AM (5 μM) and 1 mg $ml^{-1}$ bovine serum albumin for 2 h at 37°C. After thorough washing the myograph was mounted on the stage of an Axiovert 35 microscope (Carl Zeiss, Germany) coupled to a Deltascan D101 high intensity dual wavelength excitation source (Photon Technology International Inc., N.J., U.S.A.) which provided dual excitation fluorescent illumination at 340 and 380 nm. The output beam from the Deltascan was conducted to the microscope by quartz fibre optics. Excitation wavelengths were then reflected up by a dichroic mirror (FT 395 nm, Carl Zeiss, Germany) and focused onto the tissue with a quartz objective

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(Ultrafluor × 10 magnification, 0.20 numerical aperture, Carl Zeiss). Epifluorescence was collected via the objective and passed through the dichroic mirror. The emitted light was then filtered by a band pass filter (BP 500–530 nm, Carl Zeiss, Germany) *en route* to the photomultiplier tube (PMT). A camera output port with an adjustable aperture allowed the field of view to be adjusted so that only the vessel was included. Isometric force and fluorescence signal were captured at 4–8 Hz using a A/D interface (Photon Technology International Inc., N.J., U.S.A.) and an IBM AT microcomputer using commercially available software (Delta, Photon Technology International Inc., N.J., U.S.A.). During the period of the experiments (<90 min), there was no significant leakage or photobleaching of dye.

Measurements of $[Ca^{2+}]_i$ were not calibrated to absolute values using the technique outlined by Grynkiewicz and colleagues (1985) and previously used by us (Jensen *et al.*, 1993) since the effect of the calcium ionophore, ionomycin, was found to be inconsistent in these vessels and was often associated with leakage of dye leading to spurious values for $R_{min}$ and $R_{max}$. Consequently data have been presented as the ratio of the signal at 340 and 380 nm excitation wavelengths with background fluorescence subtracted. Changes in $[Ca^{2+}]_i$ in response to a stimulant were normalised by expressing them as percentage change in the ratio of 340/380 nm signal induced by depolarization with a high potassium solution similar in composition to physiological saline except for equimolar substitution of KCl for NaCl (KPSS).

### *Drugs and chemicals*

Bistyrphostin (Calbiochem, Nottingham, UK), bovine serum albumin (essentially fatty acid free) (Sigma, Poole, UK), cremaphor-EL (Sigma, Poole, UK), dimethylsulphoxide (DMSO) (Sigma, Poole, UK), fura-2AM (1-[2-(5-carbxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid acetoxymethyl (AM) ester (Molecular Probes Ltd., Eugene, U.S.A.), platelet-derived growth factor (PDGF-AB) (Calbiochem, Nottingham, UK), pluronic F-127 (Calbiochem, Nottingham, UK), (−) 202 791 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1, 4-dihydro-2,6-dimethyl-5-nitro-3-pyridene-carboxylate) (a gift from Sandoz AG, Basel). 1 mM stock fura-2AM was made up in 25 µl of DMSO/cremaphor-EL/pluronic F-127. The final concentration of DMSO (0.5%), cremophor-EL (0.1%) and pluronic F-127 (0.02%) in physiological saline did not affect basal tone or the contractile response of vessels. Other chemicals and buffers were obtained from Sigma (Poole, UK).

### *Statistics and data analysis*

All data are presented as means ± s.e.means of $n$ observations. Statistical comparison of data were made by Student's paired $t$ test. $P<0.05$ was considered significant.

Concentration-response data were fitted to a logistic function:

$$y = \frac{A + (B - A)}{1 + \left\{\frac{10^c}{10^x}\right\}^D}$$

Where y = effect at a given concentration of drug; A = minimum effect; B = maximum effect; c = log $EC_{50}$; x = log [drug]; D = 'Hill slope', by non-linear regression using Excel 5.0 (Microsoft, U.S.A.) and a macro written by the author on an IBM compatible PC.

## Results

Cumulative addition of PDGF-AB (1 pM–1 nM) caused a concentration-dependent increase in tone in rabbit isolated ear artery (Figure 1). The $pD_2$ for PDGF-AB-induced contraction was calculated to be 10.50 ± 0.08 ($n = 4$) which corresponds to an $EC_{50}$ value of around 30 pM. Addition of a supramaximal concentration of PDGF-AB (1 nM) caused a

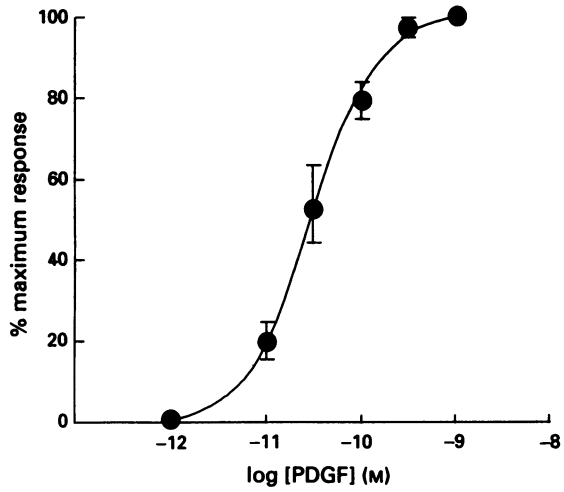


**Figure 1** Concentration-response relationship for the contractile effects of platelet-derived growth factor (PDGF-AB). PDGF-AB was added cumulatively to arteries in physiological saline. Contraction was calculated as % maximum response to PDGF. Points represent means ± s.e.means of 4 separate experiments. The solid line represents the line of best fit obtained by non-linear regression to a logistic function as described in Methods.

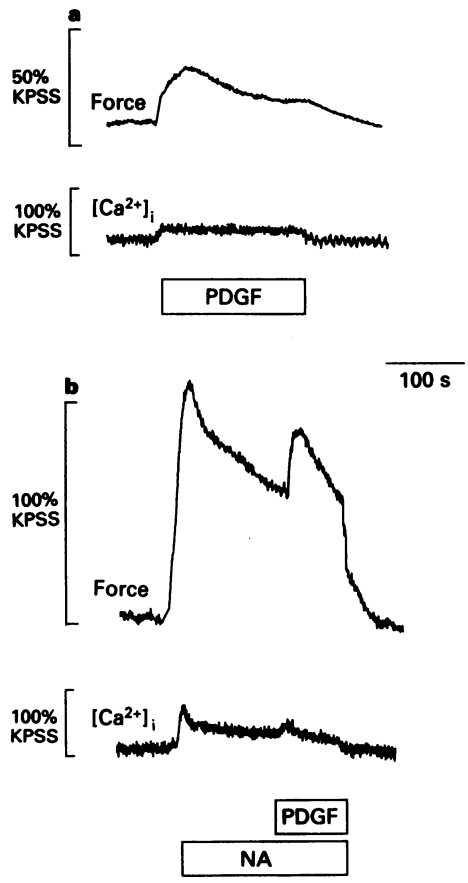


**Figure 2** The effect of platelet-derived growth factor (PDGF-AB) on isometric force and $[Ca^{2+}]_i$ in rabbit isolated ear artery. Figure shows traces from individual experiments showing the effect of PDGF-AB (PDGF; 1 mM) on force (upper trace) and $[Ca^{2+}]_i$ (lower trace) (a) in physiological saline and (b) in physiological saline after precontraction with noradrenaline (NA; 10 µM). Period of exposure to the drugs is indicated by the bars, calibration bars are also shown. Traces are representative of 4 similar experiments.

contraction associated with a rise in $[Ca^{2+}]_i$ which was usually sustained (Figure 2). In general, force and $[Ca^{2+}]_i$ changes in response to supramaximal concentrations of PDGF-AB (1 nM) were smaller than those induced by 10 μM noradrenaline (NA) or high potassium solution (KPSS) (Figure 3). Following precontraction with NA, PDGF-AB induced increases in $[Ca^{2+}]_i$ which were similar to those seen in the absence of NA. However in the presence of NA, PDGF-AB induced significantly greater contractions than in the absence of NA (Figure 3). Preincubation of arteries for 15 min with 5 μM bistyrphostin, a selective inhibitor of receptor-linked tyrosine kinases (Levitzki & Golin, 1991), inhibited PDGF-AB-induced contraction but had no effect on contraction in response to NA or KPSS (Figure 4). In addition both increases in tone and $[Ca^{2+}]_i$ in response to PDGF-AB (1 nM) were abolished by removal of extracellular $Ca^{2+}$ ($n = 3$) or by preincubation for 15 min with 5 μM (−)-202 791, a dihydropyridine calcium antagonist ($n = 3$); in contrast responses to NA were only partially attenuated by these procedures (Figure 5).

## Discussion

PDGF-AB contracts rabbit ear artery in a concentration-dependent manner. The $EC_{50}$ value observed in these studies is similar to that previously reported in rat aortic strips (Berk & Alexander, 1989). PDGF-AB also increases $[Ca^{2+}]_i$ and it is likely that this rise in $[Ca^{2+}]_i$ is the major signal responsible for PDGF-AB-induced contraction in this tissue. The rise in $[Ca^{2+}]_i$ brought about the PDGF-AB in rabbit isolated ear artery appears to depend entirely on influx of extracellular $Ca^{2+}$ and does not appear to involve release of intracellular $Ca^{2+}$ stores. In contrast, noradrenaline does transiently increase tone and $[Ca^{2+}]_i$ under the same conditions indicating that intracellular stores are not completely depleted by re-

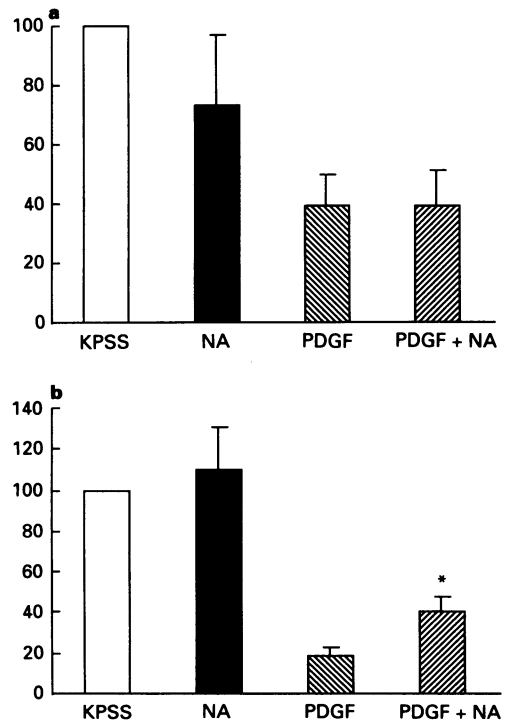


**Figure 3** The effect of contractile stimulants on (a) $[Ca^{2+}]_i$ and (b) isometric force in rabbit ear artery. KPSS = potassium solution, NA = noradrenaline (10 μM), PDGF = platelet derived growth factor-AB (1 nM), PDGF + NA = PDGF-AB (1 nM) added after induction of stable increase in tone and $[Ca^{2+}]_i$ by noradrenaline (10 μM). Data are means ± s.e.means of 4 observations. $^*P < 0.05$ compared with PDGF alone by Student's paired $t$ test.

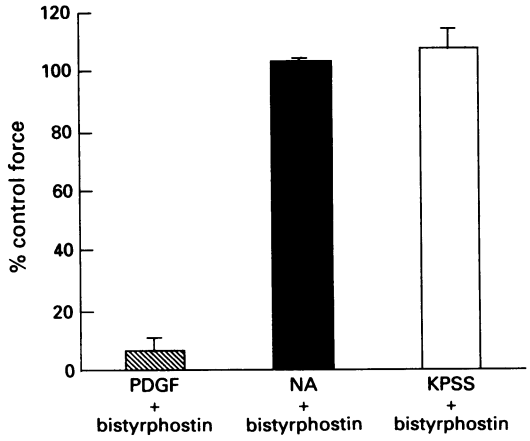


**Figure 4** The effect of bistyrphostin, a selective inhibitor of tyrosine kinase, on contractile responses of rabbit ear artery to platelet-derived growth factor (PDGF; 1 nM), noradrenaline (NA; 10 μM) and potassium solution (KPSS). Tissues were preincubated with bistyrphostin (5 μM) for 15 min prior to application of contractile stimulant. Response were calculated as % of response to the same agonist prior to addition of bistyrphostin. Data are means ± s.e.means of 4 separate experiments.

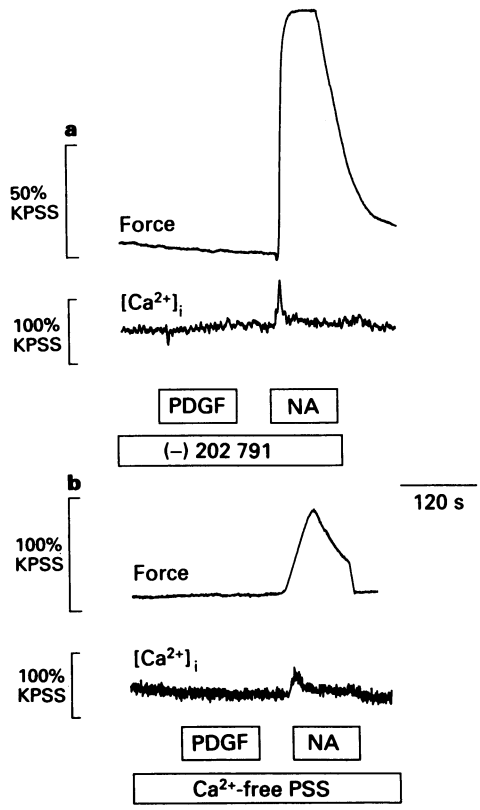


**Figure 5** The role of extracellular $Ca^{2+}$ in the action of platelet-derived growth factor (PDGF). Traces from individual experiments show (a) the effect of preincubation with dihydropyridine calcium channel blocker, (−)-202 791, on the effect of PDGF on force (upper trace) and $[Ca^{2+}]_i$ (lower trace). Period of exposure to drugs is indicated by the bars, PDGF = PDGF-AB (1 nM), NA = noradrenaline (10 μM), (−)-202 791 = (−)-202 791 (5 μM). Calibration bars are shown. (b) The effect of removal of extracellular $Ca^{2+}$ on the effect of PDGF on force (upper trace) and $[Ca^{2+}]_i$ (lower trace). The artery was incubated in a $Ca^{2+}$-free physiological saline containing 1 mM BAPTA ($Ca^{2+}$-free PSS) for 3 min prior to addition of PDGF-AB and maintained in $Ca^{2+}$-free conditions until after washout of noradrenaline (NA). Period of exposure to drugs is indicated by the bars, PDGF = PDGF-AB (1 nM), NA = noradrenaline (10 μM). Calibration bars are shown. Traces are representative of 3 similar experiments.

moval of extracellular $Ca^{2+}$ or a calcium channel blocker. The failure of PDGF-AB to abolish store release by noradrenaline (see Figure 5) and the similarity in the rise in $[Ca^{2+}]_i$ following PDGF-AB application in the presence of noradrenaline (when noradrenaline has already mobilized intracellular $Ca^{2+}$ stores) is also consistent with this interpretation. This finding in intact isolated vascular smooth muscle differs from previous studies of cultured vascular smooth muscle (Berk & Alexander, 1989; Cirillo *et al.*, 1993; Kobayashi *et al.*, 1994), and is somewhat surprising in view of previous reports showing that PDGF induces inositol 1,4,5 trisphosphate ($IP_3$) production by activation of phospholipase C-γ in cultured vascular smooth muscle (Rosengurt, 1986; Kawahara *et al.*, 1988). However, a previous study has also reported that contractile responses to PDGF in rat isolated aorta were abolished by removal of extracellular $Ca^{2+}$ (Berk *et al.*, 1986), so this difference may reflect difference between signal transduction mechanisms linked to PDGF receptors in intact and cultured vascular smooth muscle. The effect of PDGF-AB was blocked by bistyrphostin, a selective inhibitor of receptor-linked tyrosine kinases (Levitzki & Gilon, 1991). The tyrphostins are a group of low molecular weight compounds (Gazit *et al.*, 1989) which have been shown to inhibit the effects of tyrosine kinases in a number of systems (Gazit *et al.*, 1989), including vascular smooth muscle (Bilder *et al.*, 1991), and to have little or no significant effect on a number of serine-threonine kinases (Gazit *et al.*, 1989; Levitzki & Gilon, 1991). Recently Sauro & Thomas (1993) reported that a tyrphostin inhibited both the contractile action of PDGF and PDGF-induced tyrosine phosphorylation at similar concentrations in rat aorta. Our finding is consistent with this previous report and suggests that the action of PDGF is mediated through tyrosine phosphorylation. Di Salvo and colleagues (1993) have previously reported that tyrosine kinase inhibitors suppress noradrenaline-induced contraction, however we, like Sauro & Thomas (1993), saw no effect of a tyrphostin on α-adrenoceptor-induced tone at concentrations that blocked responses to PDGF. The reasons for this discrepancy are unclear but could reflect differences between the tyrphostins used in these studies.

Our findings with the dihydropyridine calcium antagonist, (−)-202 791, suggest that PDGF-AB-induced $Ca^{2+}$ influx takes place largely or wholly as a result of activation of voltage-operated calcium channels. Recently we have also found that PDGF-AB increases voltage-dependent calcium channel currents in rabbit isolated single ear artery cells studied by whole cell voltage clamp techniques (Wijetunge & Hughes, 1994). Since both the contractile effect of PDGF-AB in isolated ear artery and the increase in calcium channel currents seen following PDGF-AB in rabbit single ear artery cells are blocked by inhibitors of tyrosine kinases this suggests that the action of PDGF-AB depends on tyrosine phosphorylation. However, whether tyrosine phosphorylation may directly modulate voltage-operated calcium channels in vascular smooth muscle as has been previously suggested (Wijetunge *et al.*, 1992), or whether some secondary signal transduction mechanism linked to the PDGF receptor is involved remains to be established. As mentioned above precontraction with noradrenaline did not affect the PDGF-AB-induced rise in $[Ca^{2+}]_i$ but the force generated in response to PDGF-AB was increased under these conditions. A number of contractile agents which act through receptors coupled to G proteins, including noradrenaline have been reported to increase the sensitivity of the contractile machinery of vascular smooth muscle to $Ca^{2+}$ (Nishimura *et al.*, 1988; Somlyo & Himpens, 1989; Jensen *et al.*, 1992). Whilst the mechanism of this effect is still debated, it seems that such an effect is the most likely explanation of the increased force production by PDGF-AB in the presence of noradrenaline. Such interactions may have important physiological significance *in vivo*, when a number of contractile influences may be expected to act together on a blood vessel. Signalling systems linked to tyrosine kinases, such as $p21^{rho}$ and $p21^{ras}$ have also been proposed to modulate $Ca^{2+}$-sensitivity in vascular smooth muscle (Hirata *et al.*, 1992; Satoh *et al.*, 1993). Our data suggests that if PDGF-AB increases $Ca^{2+}$-sensitivity in vascular smooth muscle, then noradrenaline can induce an additional enhancement of $Ca^{2+}$-sensitivity, which may indicate that they act by distinct mechanisms. Alternatively PDGF-AB may have little effect on $Ca^{2+}$-sensitivity. Further studies will be required to resolve this issue.

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# Further analysis of the mechanisms underlying the tracheal relaxant action of SCA40

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**1** SCA40 (1nM–10 μM), isoprenaline (1–300 nM) and levcromakalim (100 nM–10 μM) each produced concentration-dependent suppression of the spontaneous tone of guinea-pig isolated trachea. Propranolol (1 μM) markedly (approximately 150 fold) antagonized isoprenaline but did not antagonize SCA40. The tracheal relaxant action of SCA40 was unaffected by suramin (100 μM) or 8-(*p*)-sulphophenyltheophylline (8-SPT; 140 μM).

**2** An isosmolar, $K^+$-rich (80 mM) Krebs solution increased tracheal tone, antagonized SCA40 (approximately 60 fold), antagonized isoprenaline (approximately 20 fold) and very profoundly depressed the log concentration-effect curve for levcromakalim. Nifedipine (1 μM) did not itself modify the relaxant actions of SCA40, isoprenaline or levcromakalim. However, nifedipine prevented the rise in tissue tone and the antagonism of SCA40 and isoprenaline induced by the $K^+$-rich medium. In contrast, nifedipine did not prevent the equivalent antagonism of levcromakalim.

**3** Charybdotoxin (100 nM) increased tracheal tone, antagonized SCA40 (approximately 4 fold) and antagonized isoprenaline (approximately 3 fold). Nifedipine (1 μM) prevented the rise in tissue tone and the antagonism of SCA40 and isoprenaline induced by charybdotoxin.

**4** Quinine (30 μM) caused little or no change in tissue tone and did not modify the relaxant action of isoprenaline. However, quinine antagonized SCA40 (approximately 2 fold). Nifedipine (1 μM) prevented the antagonism of SCA40 induced by quinine.

**5** Tested on spontaneously-beating guinea-pig isolated atria SCA40 (1 nM–10 μM) increased the rate of beating in a concentration-dependent manner. Over the concentration-range 1 μM–10 μM, SCA40 also caused an increase in the force of atrial contraction.

**6** Intracellular electrophysiological recording from guinea-pig isolated trachealis showed that the relaxant effects of SCA40 (1 μM) were often accompanied by the suppression of spontaneous electrical slow waves but no change in resting membrane potential. When the concentration of SCA40 was raised to 10 μM, its relaxant activity was accompanied both by slow wave suppression and by plasmalemmal hyperpolarization.

**7** SCA40 (10 nM–100 μM) more potently inhibited the activity of cyclic AMP phosphodiesterase (PDE) than that of cyclic GMP PDE derived from homogenates of guinea-pig trachealis. Theophylline (1 μM–10 mM) also inhibited these enzymes but was less potent than SCA40 in each case and did not exhibit selectivity for inhibition of cyclic AMP hydrolysis.

**8** Tested against the activity of the isoenzymes of cyclic nucleotide PDE derived from human blood cells and lung tissue, SCA40 proved highly potent against the type III isoenzyme. It was markedly less potent against the type IV and type V isoenzymes and even less potent against the isoenzymes types I and II.

**9** It is concluded that the tracheal relaxant action of SCA40 (1 nM–1 μM) does not involve the activation of β-adrenoceptors or $P_1$ or $P_2$ purinoceptors. Furthermore, this action is unlikely to depend upon the opening of $BK_{Ca}$ channels with consequent cellular hyperpolarization and voltage-dependent inhibition of $Ca^{2+}$ influx. The tracheal relaxant action of SCA40 (up to 1 μM) is more likely to depend upon its selective inhibition of the type III isoenzyme of cyclic nucleotide PDE. At concentrations above 1 μM, SCA40 exerts more general inhibition of the isoenzymes of cyclic nucleotide PDE and may then promote the opening of $BK_{Ca}$ channels.

**Keywords:** Trachealis muscle; cardiac atrial muscle; SCA40; isoprenaline; levcromakalim; $K^+$-rich Krebs solution; charybdotoxin; quinine; nifedipine; cyclic nucleotide phosphodiesterase

## Introduction

The synthesis and antibronchospastic properties of SCA40 (6-bromo-8-methylaminoimidazo[1,2-a]pyrazine-2-carbonitrile) were first described by Bonnet *et al.* (1992). Subsequently, the same group of authors reported studies designed to analyse the mechanism by which SCA40 caused relaxation of guinea-pig isolated trachealis muscle (Laurent *et al.*, 1993). On the basis that SCA40 could be antagonized by a $K^+$-rich (80 mM) medium and by the $K^+$-channel inhibitors, charybdotoxin and quinine, Laurent *et al.* (1993) concluded that the relaxant activity of this agent resided mainly in its ability to promote the opening of high conductance, $Ca^{2+}$-dependent $K^+$-channels ($BK_{Ca}$) in the plasmalemma of the smooth muscle cells. That $BK_{Ca}$ channel opening might explain the activity of SCA40 (<1 μM) received further support from the observation by the same group of workers (Michel *et al.*, 1993) that SCA40 (1 nM–1 μM) exerts negative chronotropic and inotropic effects in rat isolated atria.

As yet the ability of SCA40 (<1 μM) to promote the opening of $BK_{Ca}$ in airways smooth muscle cells awaits direct demonstration by the technique of patch clamp recording.

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Furthermore, the work of Huang *et al.* (1993) has recently called into question the specificity of $K^+$-rich media and $K^+$-channel inhibitors as tools for identifying relaxants of airways smooth muscle that act by promoting the opening of plasmalemmal $K^+$-channels. Huang *et al.* (1993) demonstrated that the relaxant effects of salbutamol acting on guinea-pig isolated trachealis muscle were antagonized by either charybdotoxin or iberiotoxin. However, such antagonism could be offset by nifedipine. This strongly suggests that the antagonism of salbutamol provided by either of the toxins reflects functional antagonism (via toxin-induced promotion of the cellular influx of $Ca^{2+}$) rather than a toxin-salbutamol interaction at the level of the control of $BK_{Ca}$ channel gating mechanisms. Similar considerations apply to the antagonism of salbutamol caused by a $K^+$-rich medium (Huang *et al.*, 1993).

The present experiments were therefore performed to shed more light on the mechanisms underlying the tracheal relaxant action of SCA40. A preliminary account of this work has been communicated to the British Pharmacological Society (Cook *et al.*, 1994).

## Methods

### *Isolation and dissection of tissues used in the mechanical and electrophysiological studies*

Guinea-pigs (300–500 g) of either sex were killed by stunning and bleeding. The hearts were quickly excised from the animals and transferred to a dish of cold Krebs solution where the pericardium was removed. The bulk of the ventricular tissue was then removed from each heart but care was taken not to destroy conduction of excitation between the right and left atria. Tracheae were also excised from the animals and were cleaned of adhering fat and connective tissue. Each trachea was opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis muscle.

### *Tissue bath studies with isolated trachea*

Small segments (containing 4–5 cartilage rings) of guinea-pig trachea were set up for the isometric recording of tension changes essentially as described by Foster *et al.* (1983). At the outset of each experiment, tissues were subjected to an imposed tension of 1.5 g. Approximately 20 min later, aminophylline (1 mM) was added in order to determine the recorder pen position at zero tissue tone. The aminophylline was removed from the tissues (initial wash followed by two further washes at 10 min intervals) and when spontaneous tone subsequently became maximal (40 min after initial wash), study of bronchodilator drugs commenced.

Cumulative log concentration-effect curves were constructed for all relaxant drugs studied. Generally 3.16 fold concentration increments were used. Each concentration of ATP, 5'-N-ethylcarboxamidoadenosine (NECA), isoprenaline, theophylline, SCA40 and levcromakalim was allowed 3, 3, 4, 5, 6 and 8 min tissue contact, respectively. All experiments with ATP were performed in Krebs solution containing S-(4-nitrobenzyl)-6-thioinosine (NBTI; 300 nM). The effects of the various relaxants were expressed in terms of the maximal relaxation induced by aminophylline.

Following construction of initial log concentration-effect curves for the bronchodilator drugs, test tissues were exposed to one of the following modifying agents: charybdotoxin (100 nM), nifedipine (1 μM), propranolol (1 μM), quinine (30 μM), 8-SPT (140 μM), suramin (100 μM) or isosmolar, $K^+$-rich (80 mM) Krebs solution (see below), and combinations of nifedipine (1 μM) with charybdotoxin (100 nM), with quinine (30 μM) or with the isosmolar $K^+$-rich (80 mM) medium. Charybdotoxin was allowed to equilibrate with the tissue for 20 min before proceeding with the experiment. All other modifying agents were allowed to equilibrate with the tissue for at least 40 min. Following equilibration of the modifying agent with the test tissues, log concentration-effect curves for the bronchodilator drugs were reconstructed in the presence of the modifying agent. In all experiments time-matched control tissues were treated identically to test tissues but were not exposed to the modifying agents.

### *Tissue bath studies with isolated atria*

Isolated, spontaneously-beating guinea-pig paired atria were set up in Krebs solution (37.5°C) under an initial resting tension of 1 g. Atrial tension changes were recorded with a force displacement transducer coupled to a Grass 7P1 preamplifier mounted in a Grass model 79D polygraph. The tension change signal was used to trigger a Grass model 7P44 tachograph so that recordings of atrial tension and rate of beating were made simultaneously. Once atrial rate had stabilized, test tissues were treated with SCA40 (1 nM–10 μM). The drug was administered cumulatively, ten-fold concentration increments being made at 15 min intervals. Time-matched control tissues were treated similarly to test tissues, but were exposed to vehicle in place of the SCA40.

### *Intracellular electrophysiological recording from trachealis*

Simultaneous recording of intracellular electrical activity and mechanical changes of a contiguous segment of guinea-pig trachea was performed by use of the technique and tissue holder described by Dixon & Small (1983). In brief, part of the trachealis was immobilized to permit long-term electrical recording while mechanical activity of contiguous muscle bundles was measured under an initial, imposed tension of 1.5 g. The recording microelectrodes were filled with 3 M KCl and were of resistance greater than 40 MΩ.

After impalement of a trachealis cell, several minutes were allowed to elapse to check the stability of the record of electrical activity. SCA40 (1 or 10 μM) was then added to the Krebs solution, superfusing the tissue and the electrical activity of the impaled cell was monitored for 6 min or until the microelectrode became dislodged.

### *Assessment of the ability of SCA40 and theophylline to inhibit the cyclic nucleotide PDE activity of guinea-pig trachea*

Muscle-rich strips of guinea-pig trachea were prepared as previously described (Foster *et al.*, 1983). Tracheal strips from two animals were combined and homogenized with an Ilado laboratory disperser in 20 volumes of cold medium (pH 7.5) containing 100 mM Tris-HCl, 2 mM $MgCl_2$ and 1 mM dithiothreitol. The homogenate was used within 30 min of preparation. Adenosine 3':5'-cyclic monophosphate (cyclic AMP)- and guanosine 3':5'-cyclic monophosphate (cyclic GMP)-PDE activity in the homogenate was measured by a modification of the method of Thomson *et al.*, (1979). The homogenate was diluted 1 in 5 and 25 μl was assayed for enzyme activity in a final volume of 100 μl containing 40 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 3.75 mM mercaptoethanol, 0.1 unit calmodulin (PDE activator), 10 μM $CaCl_2$ and either 1 μM cyclic AMP with 0.2 μCi[$^3$H]-cyclic AMP or 1 μM cyclic GMP with 0.2 μCi[$^3$H]-cyclic GMP. In tests of enzyme inhibition, the reaction mixture contained various concentrations of theophylline (1 μM–10 mM) or SCA40 (10 nM–100 μM).

The reagents and homogenate were mixed on ice and the reaction was initiated by transferring the mixture to a water bath at 37°C. Following 30 min incubation, the reaction was stopped by transferring the reaction vessel to a bath of boiling water for 3 min. After cooling on ice, 20 μl of a 1 mg ml$^{-1}$ solution of *Ophiophagus hannah* venom was added to the reaction mixture and the mixture was incubated at 37°C for 10 min. Unreacted [$^3$H]-cyclic AMP or [$^3$H]-cyclic GMP was removed by the addition of 400 μl of a 1 in 3

suspension of Dowex resin (1 × 8-400) and incubation on ice for 30 min. Each tube was then centrifuged for 2 min and 200 μl of the supernatant was removed for liquid scintillation counting. Less than 10% of the tritiated cyclic nucleotide was hydrolysed in any assay.

### *Assessment of the inhibitory activity of SCA40 against the isoenzymes of cyclic nucleotide PDE isolated from human lung and blood cells*

Platelets and neutrophils were obtained from human blood and were used as the sources of cyclic nucleotide PDE isoenzyme types II, III and IV. Human lung was obtained from patients undergoing surgery and was used as the source of isoenzyme types I and V. The isoenzymes were identified according to the classification of Beavo & Reifsnyder (1990). Cells and tissues were suspended in a medium containing sucrose (250 mM), EDTA (1 mM), dithiothreitol (1 mM), leupeptin (1 mg $ml^{-1}$), pepstatin A (1 mg $ml^{-1}$) and Tris hydrochloride (19 mM), adjusted to pH 7.4. Phenylmethylsulphonylfluoride (PMSF; 0.17 mg $ml^{-1}$) was added to this medium immediately prior to sonication/homogenization of the cells or tissue. Suspensions of platelets and neutrophils were sonicated (4 × 15 s) using a Branson probe. Human lung tissue was homogenized (2 bursts of 30 s) using a Polytron homogenizer. Preparations of PDE isoenzyme types III and IV were derived from low speed supernates of the platelet and neutrophil sonicates, respectively. These preparations selectively hydrolysed cyclic AMP (1 μM) with respect to cyclic GMP (1 μM). The enzyme activity in the supernate from platelets was inhibited by cyclic GMP (10 μM) while that from neutrophils was inhibited by rolipram (10 μM). Other PDE isoenzymes were separated from homogenates of human lung (types I and V) or platelets (type II) by anion exchange chromatography (Q-Sepharose) in a medium similar to that used for tissue homogenization but without sucrose and PMSF. A gradient of NaCl (0 to 0.1 M in 2.5 column volumes) was used to provoke elution of the isoenzymes. PDE I was found in the fraction eluting at 0.17–0.18 M NaCl and its hydrolysis of cyclic AMP (1 μM) could be stimulated by a combination of $Ca^{2+}$ (0.5 mM) and calmodulin (125 nM). PDE II was identified in the fraction eluting at 0.31–0.32 M NaCl. Its hydrolytic activity against cyclic AMP (100 μM) was 15–25 fold higher than that observed against cyclic AMP (1 μM), suggesting an apparent $K_m$ value closer to 100 μM than 1 μM. The hydrolytic activity of this enzyme fraction against cyclic AMP (1 μM) was also stimulated (approximately 2 fold) by cyclic GMP (10 μM). PDE V was identified in the fraction eluting at 0.20–0.24 M NaCl and showed selectivity in hydrolysing cyclic GMP (1 μM) as opposed to cyclic AMP (1 μM).

The activity of the isoenzymes of cyclic nucleotide PDE was assayed essentially according to the batch method of Thompson *et al.*, (1979) using a substrate of cyclic AMP (1 μM) for isoenzyme types I, III and IV. In the case of isoenzyme type I, the assay medium also contained $Ca^{2+}$ (0.5 mM) and calmodulin (125 nM). The substrate for isoenzyme type II was cyclic AMP (100 μM) and that for type V was cyclic GMP (1 μM). In each case the assay medium contained 1.25 mg $ml^{-1}$ bovine serum albumin, 50 μl $ml^{-1}$ dimethylsuphoxide and various concentrations of SCA40 as required. The inhibitory potency of SCA40 was determined by fitting concentration-inhibition curves encompassing the $IC_{50}$ to the two-parameter logistic equation (7 data points).

### *Drugs and solutions/statistical analysis of results*

Drug concentrations are expressed in terms of the molar concentration of the active species. The following substances were used: aminophylline (BDH), the disodium salt of ATP (Sigma), charybdotoxin (Latoxan), 5'-N-ethylcarboxamidoadenosine (NECA; Research Biochemicals Inc.), (−)-isoprenaline hydrochloride (Sigma), levcromakalim (SmithKline Beecham Pharmaceuticals), nifedipine (Bayer), S-(4-nitrobenzyl)-6-thioinosine (NBTI; Research Biochemicals Inc.), (±)-propranolol (ICI), quinine hydrochloride (BDH), SCA40 (6-bromo-8-methylaminoimidazol[1,2-a] pyrazine-2-carbonitrile; Université de Montpellier I), 8-(*p*)-sulphophenyltheophylline (8-SPT; Research Biochemicals Inc.), suramin

**Table 1** The effects of some modifying agencies on the potency of SCA40, isoprenaline, theophylline, levcromakalim and 5'-N-ethylcarboxaminodadenosine

| | *Mean $pD_2$ value for relaxant agonist* | | | | |
|---|---|---|---|---|---|
| *Medium* | *SCA40* | *Isoprenaline* | *Theophylline* | *Levcromakalim* | *NECA* |
| Control | 7.16 ± 0.10 | 7.68 ± 0.06 | – | – | – |
| Propranolol (1 μM) | 7.18 ± 0.07 | 5.52 ± 0.07 | – | – | – |
| Control | 7.11 ± 0.02 | 8.20 ± 0.02 | 3.91 ± 0.04 | – | 5.82 ± 0.14 |
| 8-SPT (140 μM) | 7.14 ± 0.01 | 8.15 ± 0.05 | 3.90 ± 0.04 | – | + |
| Control | 7.10 ± 0.02 | 7.85 ± 0.18 | – | – | – |
| Suramin (100 μM) | 7.30 ± 0.14 | 7.60 ± 0.11 | – | – | – |
| Control | 7.11 ± 0.07 | 8.21 ± 0.12 | – | 6.17 ± 0.05 | – |
| $K^+$-rich (80 mM) | 5.28 ± 0.04* | 6.98 ± 0.11* | – | † | – |
| Nifedipine (1 μM) | 7.01 ± 0.07 | 8.11 ± 0.21 | – | 5.93 ± 0.05 | – |
| $K^+$-rich (80 mM) + nifedipine (1 μM) | 7.17 ± 0.19 | 8.29 ± 0.14 | – | † | – |
| Control | 6.58 ± 0.10 | 8.26 ± 0.11 | – | – | – |
| Charybdotoxin (100 nM) | 5.99 ± 0.08* | 7.71 ± 0.09* | – | – | – |
| Nifedipine (1 μM) | 6.56 ± 0.13 | 8.11 ± 0.21 | – | – | – |
| Charybdotoxin (100 nM) + nifedipine (1 μM) | 6.52 ± 0.13 | 8.15 ± 0.06 | – | – | – |
| Control | 6.85 ± 0.09 | 8.21 ± 0.12 | – | – | – |
| Quinine (30 μM) | 6.57 ± 0.09* | 8.30 ± 0.09 | – | – | – |
| Nifedipine (1 μM) | 6.86 ± 0.12 | – | – | – | – |
| Quinine (30 μM) + nifedipine (1 μM) | 6.73 ± 0.15 | – | – | – | – |

Data represent means ± s.e.mean of determinations made on at least 6 tissues. A dash (–) indicates an interaction not studied. *Significant ($P<0.05$) difference from the time-matched control; †indicates a shift in the position (or depression) of the log concentration-effect curve so marked that a $pD_2$ value was not calculable.

(Bayer), theophylline (Sigma). In the assays of phosphodiesterase activity, radiochemicals were obtained either from Amersham International or from Du Pont NEN. Other reagents were obtained from Fluka AG or from the Sigma Chemical Company.

Stock solutions of most drugs were prepared in twice-distilled water. NBTI was dissolved in dimethylsulphoxide. Stock solutions of levcromakalim and nifedipine were prepared in 70% and absolute ethanol respectively. Stock solutions of isoprenaline and NECA were prepared in 0.1 M HCl. Dilutions of isoprenaline were made using distilled water containing 0.57 mM ascorbic acid.

The Krebs solution used in the tissue bath experiments and for the microelectrode recording of membrane potential changes had the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1. The $K^+$-rich Krebs solution was prepared by the addition of KCl in order to raise the concentration of $K^+$ to 80 mM. The concentration of NaCl was reduced to maintain osmolality. All experiments involving nifedipine were conducted under illumination provided by a sodium vapour lamp to minimize photolysis of the drug.

The significance of differences between means was assessed by means of a two-tailed, unpaired $t$ test. The null hypothesis was rejected when $P<0.05$.

## Results

### *Tissue bath studies with isolated trachea*

ATP (3 μM–1 mM), isoprenaline (1–300 nM), levcromakalim (100 nM–10 μM), NECA (100 nM–100 μM), SCA40 (1 nM–10 μM) and theophylline (1 μM–1 mM) each caused concentration-dependent suppression of the spontaneous tone of the trachea. The maximal relaxant effects of isoprenaline, SCA40 and theophylline were equivalent to that of aminophylline. In contrast, the maximal effects of ATP, levcromakalim and NECA were approximately 75%–85% of that of aminophylline.

Propranolol (1 μM) or 8-SPT (140 μM) or suramin (100 μM) did not themselves alter tracheal tone. However, propranolol (1 μM) markedly (150 fold) antagonized isoprenaline without modifying the relaxant action of SCA40. 8-SPT (140 μM) had no effect against the tracheal relaxant actions of isoprenaline, SCA40 or theophylline but very markedly depressed the log concentration-effect curve of NECA. Suramin (100 μM) failed to modify the relaxant activity of either isoprenaline or SCA40 (Table 1). Suramin (100 μM) also failed to modify the tracheal relaxant action of ATP (data not shown).

Exposure of tracheal segments to an isosmolar, $K^+$-rich (80 mM) Krebs solution increased tissue tone by 49.5 ± 12.4% (mean ± s.e.mean of values from 12 tissues). In the presence of the $K^+$-rich medium, the log concentration-effect curves of isoprenaline and SCA40 were shifted markedly (20 fold and 60 fold respectively) to the right and the log concentration-effect curve for levcromakalim was very profoundly depressed (Figure 1 and Table 1). Nifedipine (1 μM) caused little or no change in tracheal tone and did not significantly alter the tracheal relaxant potencies of isoprenaline, levcromakalim or SCA40. Nifedipine (1 μM) prevented the increase in tissue tone evoked by the $K^+$-rich (80 mM) Krebs solution. In the presence of a combination of nifedipine (1 μM) and the $K^+$-rich medium, the log concentration-effect curves of isoprenaline and SCA40 each lay close to that observed in the control conditions. In other words nifedipine prevented the antagonism of isoprenaline and SCA40 induced by the $K^+$-rich medium. In contrast nifedipine did not prevent the equivalent antagonism of levcromakalim (Figure 1 and Table 1).

Charybdotoxin (100 nM) increased the tone of tracheal segments by 15.7 ± 4.9% (mean ± s.e.mean of values from 12 tissues). The toxin caused approximately 3 fold antagonism of isoprenaline and 4 fold antagonism of SCA40. Nifedipine (1 μM) prevented the increase in tissue tone induced by charybdotoxin. In the presence of a combination of charybdotoxin (100 nM) and nifedipine (1 μM) the log concentration-effect curves of isoprenaline and SCA40 each lay close to that observed under the control circumstance. Nifedipine (1 μM) therefore prevented the antagonism of isoprenaline and SCA40 induced by charybdotoxin (Figure 2 and Table 1).

Quinine (30 μM) caused little or no change in tissue tone and did not modify the relaxant action of isoprenaline. In contrast, quinine (30 μM) caused approximately 2 fold antagonism of SCA40. In the presence of a combination of

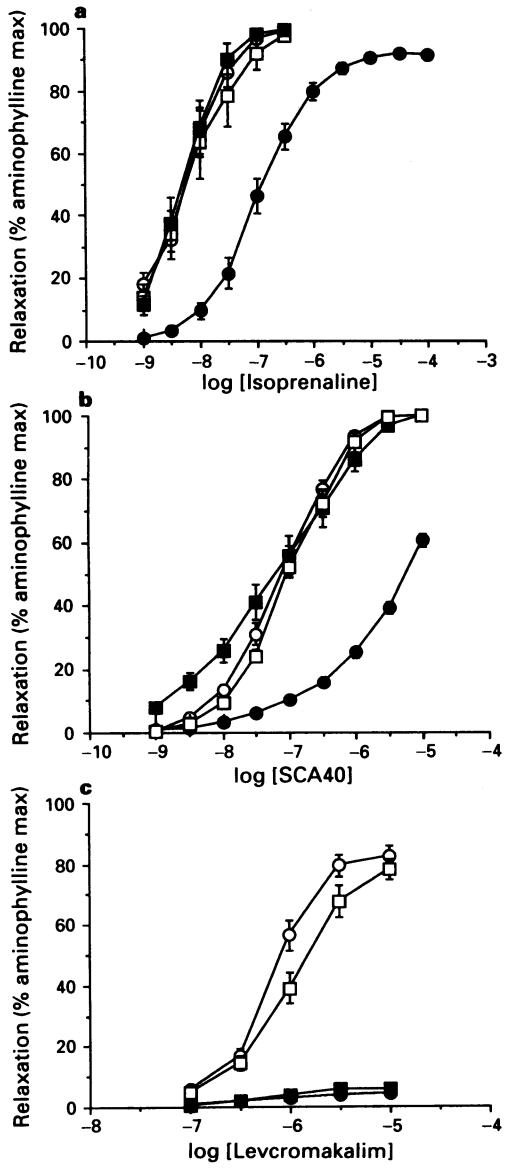


**Figure 1** Guinea-pig isolated trachealis muscle: the effects of nifedipine (1 μM) on the ability of an isosmolar $K^+$-rich (80 mM) medium to inhibit the relaxant actions of isoprenaline, SCA40 and levcromakalim. Abscissae: $\log_{10}$ molar concentration of isoprenaline (a), SCA40 (b) or levcromakalim (c). Ordinates: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In each panel (○) indicates the log concentration-effect curve for the relaxant as observed in normal Krebs solution, (●) the curve obtained in $K^+$-rich (80 mM) Krebs solution, (□) the curve obtained in the presence of nifedipine (1 μM) and (■) the curve obtained in the presence of a combination of the $K^+$-rich (80 mM) medium and nifedipine (1 μM). Data points are the means ± s.e.mean of values from at least 6 tissues.

quinine (30 μM) and nifedipine (1 μM) the log concentration-effect curve of SCA40 was indistinguishable from that observed in control circumstances. Nifedipine therefore prevented the antagonism of SCA40 induced by quinine (Table 1).

### *Tissue bath studies with isolated atria*

At the outset of the experiments the atrial preparations contracted at the rate of approximately 160 beats per min and the force of contraction was generally close to 350 mg. The beating rate of the vehicle-treated control tissues remained relatively constant for the duration (90 min) of the experiment. However, the force of atrial contraction showed some tendency to decline (Figure 3).

Treatment of test tissues with SCA40 (1 nM–10 μM) caused a concentration-dependent increase in the rate of atrial contraction. At concentrations of 100 nM and above, SCA40 significantly increased the rate of atrial beating compared with that observed in the control tissues. Furthermore, at concentrations of 1 and 10 μM, SCA40 clearly and significantly increased the force of atrial contraction (Figure 3). This effect reached a peak approximately 2 min after the appropriate drug concentration increment and thereafter showed some decline.

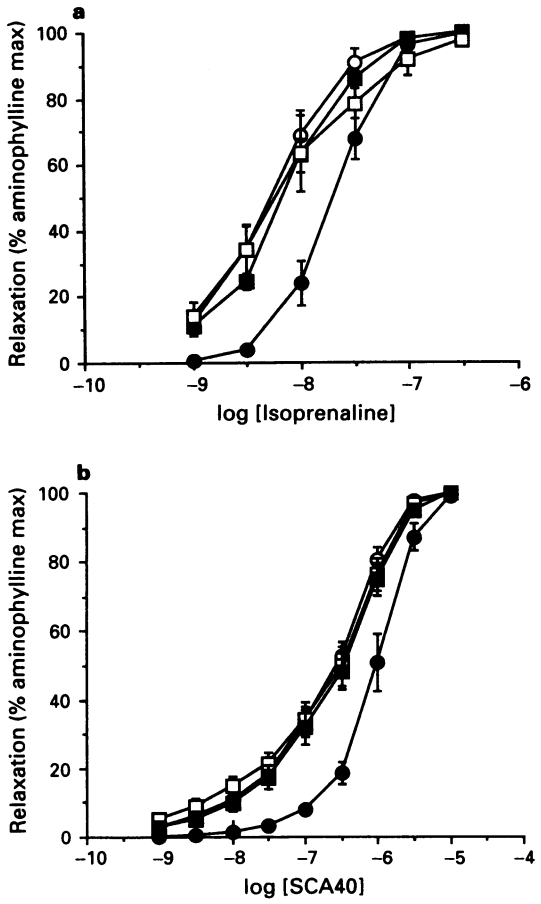


**Figure 2** Guinea-pig isolated trachealis muscle: the effects of nifedipine (1 μM) on the ability of charybdotoxin to inhibit the relaxant actions of isoprenaline and SCA40. Abscissae: $\log_{10}$ molar concentration of isoprenaline (a) or SCA40 (b). Ordinates: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In each panel (○) indicates the log concentration-effect curve for the relaxant as observed in normal Krebs solution, (●) the curve obtained the presence of charybdotoxin (100 nM), (□) the curve obtained in the presence of nifedipine (1 μM) and (■) the curve obtained in the presence of a combination of the charybdotoxin (100 nM) and nifedipine (1 μM). Data points are the means ± s.e.means of values from at least 6 tissues.

### *Intracellular electrophysiological recording from trachealis*

Microelectrode impalement of trachealis cells revealed that, while some cells were electrically quiescent, others exhibited spontaneous oscillations (slow waves) of membrane potential. The addition of SCA40 (1 μM) to the Krebs solution superfusing the tissue caused full suppression of the mechanical tone of the tissue. This mechanical effect was often accompanied by suppression of the electrical slow waves (Figure 4). However, no significant change in resting membrane potential was observed (Table 2). Tested at a concentration of 10 μM, SCA40 suppressed mechanical tone, suppressed slow wave activity and evoked cellular hyperpolarization (Figure 4 and Table 2).

### *Assessment of the ability of SCA40 and theophylline to inhibit the cyclic nucleotide PDE activity of guinea-pig trachea*

SCA40 (10 nM–100 μM) and theophylline (1 μM–10 mM) each caused concentration-dependent inhibition of the hydrolysis of cyclic AMP and cyclic GMP by homogenates of muscle-rich strips of guinea-pig trachea. SCA40 was the more potent PDE inhibitor in each case and, in contrast to theophylline, exhibited selectivity as an inhibitor of cyclic AMP-PDE as opposed to cyclic GMP-PDE (Table 3).

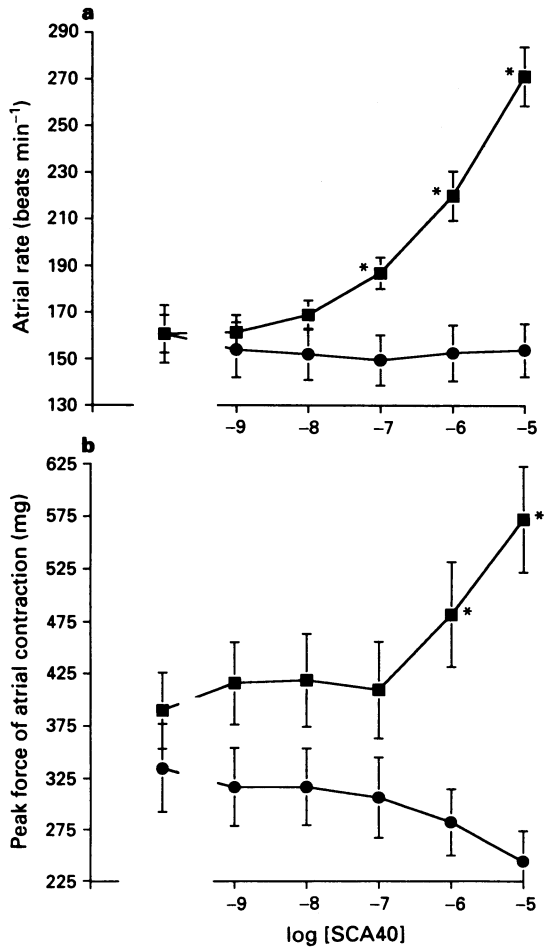


**Figure 3** The effects of SCA40 on the rate (a) and force (b) of beating of the guinea-pig isolated atrial preparation. Abscissae: $\log_{10}$ molar concentration of SCA40. Ordinate scales: rate of beating (a) and peak force of contraction (b). In both (a) and (b), (■) indicates test tissue and (●) time-matched control tissues. The plotted data points represent the means ± s.e.means of values from at least 6 tissues. Significant ($P<0.05$) difference from the corresponding point for the control tissues.

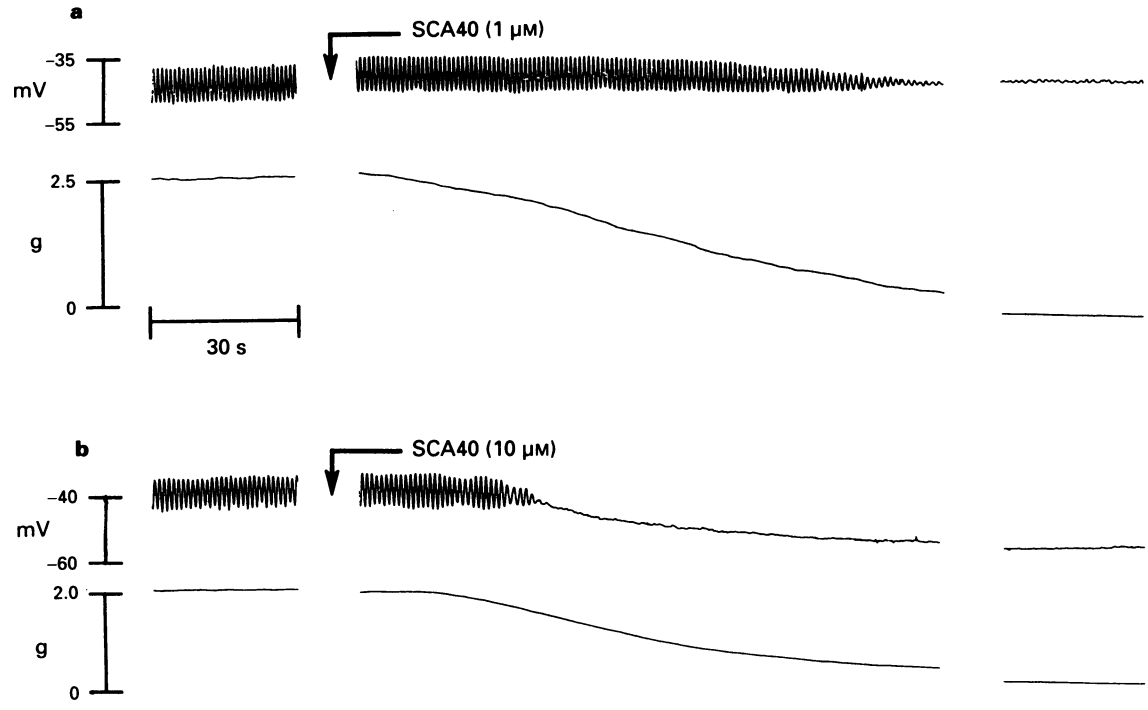


**Figure 4** The effects of SCA40 on the spontaneous electrical and mechanical activity of guinea-pig isolated trachealis. In each row of recordings the upper trace represents membrane potential changes recorded from a single cell while the lower trace represents tension changes recorded from a contiguous segment of trachea. In each row the left hand panel represents control activity recorded immediately prior to the administration of SCA40 (1 μM in (a); 10 μM in (b)). The centre panels show activity recorded 1 min and the right hand panels activity recorded 6 min after the administration of the drug.

**Table 2** The effects of SCA40 on the electrical and mechanical activity of guinea-pig isolated trachealis muscle

| *Concentration of SCA40* | *Membrane hyperpolarization* (mV) | *Relaxation* (g) |
|---|---|---|
| 1 μM | −0.6 ± 0.9† | 1.8 ± 0.39 (6) |
| 10 μM | 19.0 ± 2.7 | 2.3 ± 0.35 (4) |

Data are means ± s.e.mean. The figures in parentheses indicate the number of tissues contributing to the mean. †indicates a value not significantly different from zero.

**Table 3** Inhibition of cyclic AMP (cAMP-PDE) and cyclic GMP (cGMP-PDE) phosphodiesterase activity of homogenates of muscle-rich strips of guinea-pig trachea by SCA40 and by theophylline

| *Inhibitor* | *cAMP-PDE* | *cGMP-PDE* |
|---|---|---|
| SCA40 | 4.67 ± 0.19 (4) | 4.03 ± 0.11* (4) |
| Theophylline | 3.02 ± 0.07 (8) | 2.83 ± 0.10 (7) |

Data indicate mean (± s.e.mean) values of $-\log IC_{50}$ for each inhibitor. The figures in parentheses indicate the number of experiments. *indicates a significant difference from the corresponding value for cAMP-PDE.

**Table 4** Inhibition by SCA40 of the activity of the isoenzymes of cyclic nucleotide phosphodiesterase isolated from human blood cells and lung tissue

| *Isoenzyme type* | | | | |
|---|---|---|---|---|
| *I* | *II* | *III* | *IV* | *V* |
| 3.37 | 3.30 | 6.61 | 4.68 | 4.34 |
| 3.54–3.15 | 3.49–3.06 | 6.79–6.43 | 4.74–4.62 | 4.52–4.15 |

Data indicate $-\log IC_{50}$ values for SCA40 together with their upper and lower 95% confidence limits.

### *Assessment of the inhibitory activity of SCA40 against the isoenzymes of cyclic nucleotide PDE isolated from human lung and blood cells*

SCA40 inhibited each of the isoenzymes of human cyclic nucleotide PDE in a concentration-dependent manner. However, among the various isoenzymes, the drug exhibited differing potencies as an inhibitor. SCA40 was most potent in inhibiting the type III isoenzyme. It was approximately equipotent in inhibiting the type IV and type V isoenzymes and very weak as an inhibitor of the isoenzyme types I and II (Table 4).

## Discussion

### *Does SCA40 cause trachealis muscle relaxation by activating β-adrenoceptors or $P_1$ or $P_2$ purinoceptors?*

The present experiments with propranolol were performed in order to determine whether SCA40 could directly or indirectly activate β-adrenoceptors. Propranolol (1 μM) profoundly antagonized isoprenaline in relaxing guinea-pig isolated trachea but did not modify the relaxant potency of SCA40 (Table 1).

The present experiments with 8-SPT and suramin were prompted by the notion that the molecular structure of SCA40 bears some resemblance to that of the xanthine nucleus. It therefore seemed possible that SCA40 might have some affinity for purinoceptors. 8-SPT (> 1 μM) acts as an antagonist selective for $P_1$ purinoceptors (Gustaffson, 1984; Collis *et al.*, 1987). In the present study, 8-SPT (140 μM) failed to antagonize the tracheal relaxant actions of isoprenaline, theophylline or SCA40 but markedly antagonized NECA. Suramin, at concentrations greater than 10 μM, acts as an antagonist selective for $P_2$ purinoceptors (Dunn & Blakeley, 1988; Hoyle *et al.*, 1990). In the present study suramin (100 μM) did not antagonize isoprenaline, SCA40 (Table 1) or ATP. The failure of suramin to

antagonize the tracheal relaxant action of ATP confirms the findings of Kelley & Hollingsworth (1994) and indicates that $P_2$ purinoceptors may not be expressed in guinea-pig trachealis muscle.

In summary, the present experiments using propranolol, 8-SPT and suramin collectively suggest that the relaxant action of SCA40 in guinea-pig trachealis muscle does not involve the activation of β-adrenoceptors, $P_1$ purinoceptors of $P_2$ purinoceptors.

### *Role of $BK_{Ca}$ channel opening in the tracheal relaxant action of SCA40*

The rationale behind using an isosmolar, $K^+$-rich (>40 mM) medium to identify smooth muscle relaxants that act by opening plasmalemmal $K^+$-channels is that the $K^+$-rich medium moves the $K^+$ equilibrium potential to a value that closely approaches the membrane potential. In this circumstance, $K^+$-channel opening cannot evoke sufficient hyperpolarization to ensure the closure of voltage-dependent $Ca^{2+}$-channels. Voltage-dependent inhibition of $Ca^{2+}$ influx does not therefore occur and the mechano-inhibitory effects of the $K^+$-channel opener are markedly reduced or abolished (Small *et al.*, 1992). However, the $K^+$-rich medium itself promotes $Ca^{2+}$ influx and increases tissue tone. It may therefore functionally antagonize relaxant agents that have no component of action attributable to $K^+$-channel opening (Huang *et al.*, 1993; Small *et al.*, 1993).

The present observations (Figure 1 and Table 1) that an isosmolar $K^+$-rich medium antagonizes the tracheal relaxant action of an agonist at β-adrenoceptors (isoprenaline), an opener of ATP-sensitive $K^+$-channels (levcromakalim) and SCA40 confirm the results of earlier studies (Allen *et al.*, 1985; 1986; Huang *et al.*, 1993; Laurent *et al.*, 1993). In the case of agonists at β-adrenoceptors such antagonism is prevented by the presence of nifedipine (Huang *et al.*, 1993; present study). Nifedipine also prevented the antagonism of SCA40 provided by a $K^+$-rich medium (present study). In contrast, nifedipine did not affect the antagonism of levcromakalim provided by a $K^+$-rich medium (Figure 1 and Table 1). These findings suggest that the interaction between the $K^+$-rich medium and agonists at β-adrenoceptors or SCA40 represents functional antagonism attributable to the effect of the $K^+$-rich medium in promoting $Ca^{2+}$ influx through nifedipine-sensitive channels. The failure of nifedipine to prevent the antagonism of levcromakalim provided by the $K^+$-rich medium suggests that the action of levcromakalim depends upon its ability to hyperpolarize the plasmalemma but not on an ability to cause voltage-dependent inhibition of $Ca^{2+}$ influx through nifedipine-sensitive channels. How membrane hyperpolarization is transduced into the mechano-inhibitory effects of levcromakalim remains a challenge for further experimentation.

The present observations (Figure 2; Table 1) that charybdotoxin (an inhibitor of $BK_{Ca}$ channels) antagonizes the tracheal relaxant actions of an agonist at β-adrenoceptors and that of SCA40 confirm the results of earlier studies (Jones *et al.*, 1990; Murray *et al.*, 1991; Huang *et al.*, 1993; Laurent *et al.*, 1993). Since charybdotoxin converts the spontaneous electrical slow waves of guinea-pig trachealis into regenerative action potentials and increases the mechanical tone of the tissue (Murray *et al.*, 1991), it is likely that this agent promotes the cellular influx of $Ca^{2+}$. The finding that nifedipine can offset the antagonism of agonists at β-adrenoceptors and that of SCA40 provided by charybdotoxin (Huang *et al.*, 1993; present study) suggests that such antagonism represents not an interaction between the toxin and the relaxant drugs at the level of $BK_{Ca}$ channel gating but rather functional antagonism attributable to the toxin promoting $Ca^{2+}$ influx.

Quinine is a relatively non-selective inhibitor among $Ca^{2+}$-dependent $K^+$-channels (Haylett & Jenkinson, 1990). Laurent *et al.*, (1993) showed that quinine could antagonize SCA40 in relaxing guinea-pig trachea bathed by a $K^+$-rich (20 mM) medium. Similar antagonism was demonstrated in the present experiments (Table 1), but this could be offset by nifedipine. It therefore seems likely that, as for charybdotoxin, the antagonism of SCA40 provided by quinine represents functional antagonism resulting from quinine-induced promotion of $Ca^{2+}$ influx.

The abilities of a $K^+$-rich medium, charybdotoxin and quinine each to antagonize SCA40 prompted Laurent *et al.*, (1993) to suggest that the opening of $BK_{Ca}$ channels underlies the tracheal relaxant action of this agent. However, the present observation that nifedipine can, in each case, prevent the antagonism casts doubt on the usefulness of each of the three tools for identifying agents that act to promote $K^+$-channel opening in trachealis muscle. That $K^+$-channel opening is not an important feature of the tracheal relaxant action of SCA40 is also suggested by the present electrophysiological experiments in which a concentration (1 μM) of SCA40 causing full suppression of the spontaneous tone of the trachea failed to hyperpolarize the trachealis cells (Figure 4; Table 2). Further support for this idea comes from patch clamp experiments performed on bovine trachealis where SCA40 (0.1–10 μM) failed to modify the activity of $BK_{Ca}$ channels recorded from either inside-out, outside-out or cell-attached plasmalemmal patches (MacMillan *et al.*, 1994).

That $K^+$-channel opening is not an important feature of the action of SCA40 is also suggested by the present experiments with guinea-pig isolated atrial preparations. Were the promotion of $K^+$-channel opening to be a fundamental action of SCA40 (1 nM–1 μM), we would have expected this agent to have exerted negative chronotropic and negative inotropic effects. However, this proved not to be the case (Figure 3). The present failure of SCA40 (1 nM–1 μM) to reduce the rate and force of atrial beating contrasts with the observations of Michel *et al.* (1993) in atria isolated from the rat. The reasons for this difference are currently unclear. It is possible that rat and guinea-pig atrial tissues may exhibit species differences with respect to their sensitivity to SCA40. However, the report of Michel *et al.* (1993) did not include data from vehicle-treated, time-matched control tissues. Hence, it is currently difficult to assess whether the reductions in atrial rate and force observed (Michel *et al.*, 1993) in rat tissue represented time-dependent changes in the activity of the tissue rather than effects attributable to the action of SCA40.

We conclude that the suggestion of Laurent *et al.* (1992) that SCA40 (<10 μM) relaxes guinea-pig trachea by opening $BK_{Ca}$ channels is unlikely to be correct. As discussed below, this agent is more likely to promote tracheal relaxation by virtue of its activity as an inhibitor of cyclic AMP-PDE.

### *Role of cyclic nucleotide phosphodiesterase inhibition in the tracheal relaxant action of SCA40*

Using an enzyme preparation derived from bovine heart (Sigma PO134), Bonnet *et al.* (1992) demonstrated that SCA40 was able to inhibit cyclic nucleotide PDE with an $IC_{50}$ of 70 μM. In the present study SCA40 inhibited the total cyclic AMP-PDE and the total cyclic GMP-PDE of guinea-pig trachea with $IC_{50}$ values of approximately 20 μM and 100 μM respectively (Table 3). At first sight the relatively low potency of SCA40 as an inhibitor of total cyclic AMP-PDE in guinea-pig trachea might suggest that inhibition of cyclic AMP-PDE cannot explain the tracheal relaxant activity of SCA40, for the relaxant effects of this agent are observed over the concentration-range 10 nM–8 μM (Laurent *et al.*, 1993; present study). However, in guinea-pig trachea, the cyclic AMP hydrolysing isoenzyme that mainly regulates mechanical tone is the type III isoenzyme with the type IV isoenzyme perhaps playing a supportive role (Harris *et al.*, 1989; Berry *et al.*, 1991). Since these isoenzymes account for only a fraction of the total cyclic AMP hydrolytic activity of

a homogenate of the trachea (Berry *et al.*, 1991), experiments involving the measurement of total cyclic AMP-PDE may mask the true potency of any selective inhibitor of the isoenzymes that are the principal regulators of the mechanical tone of the tissue.

The fact that, in homogenates of guinea-pig trachea, SCA40 proved more potent as an inhibitor of total cyclic AMP-PDE than as an inhibitor of total cyclic GMP-PDE suggests that it may selectively inhibit one or more of the isoenzymes (i.e. types III andIV) that preferentially hydrolyse cyclic AMP. The cyclic nucleotide PDE isoenzyme that principally regulates the mechanical activity of guinea-pig cardiac muscle is the isoenzyme type III (Gristwood *et al.*, 1985; Gristwood & Owen, 1986; Reeves *et al.*, 1987; Berry *et al.*, 1991; Elliott *et al.*, 1991). In view of this, the ability of SCA40 (1 nM–10 μM; Figure 3) to increase the beating rate of guinea-pig isolated atria suggests that SCA40 selectively inhibits this isoenzyme of cyclic nucleotide PDE. This suggestion receives direct support from the present studies of SCA40 as an inhibitor of the isoenzymes of cyclic nucleotide PDE derived from human blood cells and lung tissue (Table 4).

Since the $IC_{50}$ (approximately 250 nM; Table 4) of SCA40 as an inhibitor of isoenzyme type III lies within the effective concentration range of SCA40 in relaxing guinea-pig trachea, we conclude that inhibition of this isoenzyme represents the principal mechanism by which SCA40 relaxes the trachea. Towards the upper end of the effective concentration-range of SCA40, the inhibition of isoenzyme types IV and/or V may also have a role to play. Plasmalemmal $K^+$-channel opening with consequential cellular hyperpolarization (Figure 4) is only observed in guinea-pig trachealis with concentrations of SCA40 in excess of 1 μM and may perhaps be mediated by marked inhibition of various of the isoenzymes of cyclic nucleotide PDE and therefore an elevation in the cellular content of cyclic AMP (Small *et al.*, 1993).

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# Functional characterization of the adenosine receptor mediating inhibition of intestinal secretion

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**1** Previous studies have shown that the mixed $A_1/A_2$ adenosine agonist 5'-N-ethylcarboxamidoadenosine (NECA) inhibits intestinal fluid secretion which is thought to contribute to its antidiarrhoeal effect in the rat. The aim of this study was to characterize the adenosine receptor mediating this antisecretory effect via functional studies using a range of selective agonists and antagonists and by applying the pharmacological criteria of relative agonist and antagonist potencies.

**2** Adenosine agonists and antagonists were administered i.v. to anaesthetized rats. Intestinal secretion was then stimulated by i.a. infusion of vasoactive intestinal peptide (VIP, 0.8 $\mu g\ min^{-1}$) and the net fluid transport across the wall of the jejunum was measured by a recirculation technique.

**3** The rank order of agonist potency to reduce the response to VIP was: NECA > $N^6$-cyclopentyladenosine (CPA) > R-$N^6$-(2-phenylisopropyladenosine) (R-PIA) > S-PIA > chloroadenosine (2-CADO) > 2-phenylaminoadenosine (CV-1808). This order best complies with the rank order of agonist potency that represents activation of the recently described $A_{2B}$ receptor: NECA > 2-CADO > R-PIA = CHA > S-PIA > = CV-1808 > = CGS-21680. The most potent agonists (NECA, CPA and R-PIA) had $ED_{50}$ values in the low microgram range.

**4** The anitsecretory action of NECA (submaximal dose of 40 $\mu g\ kg^{-1}$) was antagonized equally (approximately 50%) by the selective adenosine antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.1 $mg\ kg^{-1}$) and 8-phenyltheophylline (8-PT, 0.1 $mg\ kg^{-1}$). This equipotent activity indicates the presence of an $A_2$ and not an $A_1$ receptor.

**5** It is suggested that adenosine $A_{2B}$ receptor agonists could be evaluated for potential use as antidiarrhoeal drugs.

**Keywords:** Adenosine receptors; rat intestine; antisecretory; antidiarrhoeal; vasoactive intestinal peptide (VIP); *in vivo*; fluid transport; NECA; $A_{2B}$ receptor.

## Introduction

Two subclasses of adenosine receptors were initially defined on the basis of inhibition ($A_1$) or stimulation ($A_2$) of adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation (Van Calker *et al.*, 1979). However, it is now known that adenosine receptors are not linked to adenylyl cyclase activity in all cases (Jacobson, 1990). Structure-activity relationship studies have led to the development of selective agonist and antagonists for $A_1$ and $A_2$ receptors (Jacobson *et al.*, 1992) which together with radioactive ligands have created a definitive receptor classification based on agonist potency order and antagonist affinity order (Collis & Hourani, 1993). There is also increasing evidence from biochemical, functional and receptor cloning studies supporting the existence of further receptor subtypes: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (Girdlestone & Watson, 1994).

The $A_1$ receptor is differentiated from the $A_2$ receptor by the selective $A_1$ agonist, $N^6$-cyclopentyladenosine (CPA) (Lohse *et al.*, 1988) and the selective $A_1$ antagonist 1,3 -dipropyl-8-cyclopentylxanthine (DPCPX) (Bruns *et al.*, 1987a). The existence of the $A_1$ receptor is further established since it has been cloned from bovine (Olah *et al.*, 1992), rat (Mahan *et al.*, 1991) and human (Libert *et al.*, 1992) brain. The selective $A_2$ receptor ligand, CGS-21680, is used to characterize $A_2$ receptor-mediated tissue responses (Collis & Hourani, 1993).

The possible existence of $A_2$ receptor subtypes was first introduced by Daly *et al.*, (1983) on the basis of regional differences in affinity orders of adenosine analogues and high and low affinity binding sites for adenosine in the rat brain. Bruns *et al.* (1986) later proposed that the high affinity $A_2$ receptor be designated $A_{2A}$ and the low affinity receptor be designated $A_{2B}$. Further studies found that the agonist CGS-21680 (Jarvis *et al.*, 1989) and the antagonist PD-115199 (Bruns *et al.*, 1987b) have high affinity at the $A_{2A}$ compared to the $A_{2B}$ receptor. Unfortunately, selective ligands specific for $A_{2B}$ receptors have not yet been developed. At present the $A_{2B}$ receptor is identified by the low affinity of several $A_{2A}$-selective ligands where the agonist potency order is: NECA > 2-CADO > R-PIA = CHA > S-PIA > = CV-1801 > = CGS-21680 and antagonist affinity order is DPCPX = 8-PT > = PD-115199 (Collis & Hourani, 1993). Molecular cloning and expression of proteins which correspond to $A_{2A}$ (Furlong *et al.*, 1992) and $A_{2B}$ receptors (Pierce *et al.*, 1992) provides further evidence for the existence of $A_2$ receptor subtypes.

In 1986, Riberio and Sebastio first suggested a third class of adenosine receptor, the $A_3$ receptor. However, the existence of this receptor has been challenged (Carruthers & Fozard, 1993). The more readily accepted $A_3$ receptor is that proposed by Zhou and colleagues in 1992, which has been cloned from rat striatum and is characterized by similar binding affinities of NECA and R-PIA. However, the alkylxanthine antagonists, such as DPCPX at concentrations as high as 100 $\mu M$ did not significantly bind to this receptor.

Identification of receptor subtypes provides an opportunity to develop therapeutic drugs with selective action. However, functional studies have fallen behind the biochemical studies to date. For instance, studies to determine the therapeutic potential of adenosine receptor agonists in the treatment of diarrhoea would be of value. This is highlighted by the finding that the non-selective adenosine agonist, NECA, is a potent inhibitor of morphine withdrawal diarrhoea in rats (Dionyssopoulos *et al.*, 1992). The diarrhoea that is produced on opiate with-

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drawal in this species is associated with a decrease in intestinal fluid absortion (Chang *et al.*, 1984) and increased intestinal motility (Brown *et al.*, 1988). Recently we have established that NECA exerts its antidiarrhoeal effect by inhibiting both prostaglandin and vasoactive intestinal peptide (VIP)-induced intestinal fluid secretion as well as inhibiting peristalsis (Coupar & Hancock, 1994). The specific aim of this study was to characterize the receptor mediating one of these effects, namely inhibition of intestinal fluid secretion, using a range of metabolically stable adenosine analogues with different potencies and degrees of specifity for adenosine receptor subtypes.

Some of these results have been communicated to the Australian Society of Clinical and Experimental Pharmacologists and Toxicologists (Coupar & Hancock, 1993).

## Methods

The method of measuring fluid transport by the rat jejunum follows that described in detail by Coupar in 1985. A brief outline is given below.

### *Surgical procedure*

Hooded Wistar rats of either sex (200–290 g weight) were fasted over night. They were anaesthetized with pentobarbitone sodium (60 mg $kg^{-1}$ s.c.), which has been shown to be appropriate for intestinal secretion experiments (Coupar, 1985). A cannula was introduced into the left jugular vein for administration of the adenosine agonist and/or antagonist in saline (0.1 ml 100 $g^{-1}$) or in the case of the controls, saline or vehicle. Another cannula was introduced into the left common carotid artery for constant intra-arterial (i.a.) infusion of VIP (0.8 $\mu g\ min^{-1}$) in saline to induce near maximum fluid secretion (Coupar, 1985), or saline as the control to measure the basal absorptive value. The rates of infusion were 40 $\mu l\ min^{-1}$. Mean systemic blood pressure was recorded to monitor the condition of the rats from a side-arm off the carotid cannula by means of a Statham pressure transducer connected to a Neomedix Neotrace recorder.

A recirculation technique was used to measure the net fluid transport rate of the jejunum. A 25 to 30 cm section of the jejunum, starting distal from the Ligament of Trietz, was continuously perfused with 8 ml of an iso-osmotic solution containing (mM): NaCl 148, KCl 5, dextrose 5.5 and phenol red 0.05, as a non-absorbable water marker. The solution was contained in a reservoir maintained at 37°C and recirculated through the lumen of the jejunum for 20 min by a gas-lift column of moistened 5% $CO_2$ in $O_2$.

The drug administration sequence for testing adenosine agonists commenced with the i.v. injection of the adenosine agonist or vehicle as the control. This was followed at 5 min with i.a. infusion of VIP, or saline as the control with mean systemic blood pressure recorded. The segment of jejunum was washed before intestinal perfusion was started at 10 min. At 30 min the fluid from the intestine was collected for analysis and the weight of the intestine perfused was measured. The testing of the adenosine antagonists followed a similar order to that of the above, except that the antagonist or its vehicle as the control was injected i.v. 5 min prior to injecting a submaximal dose of NECA (40 $\mu g\ kg^{-1}$) or saline as the control.

VIP was chosen to induce intestinal secretion in the present studies, since it is present in intrinsic enteric nerves that project to the mucosa and thought to be involved in fluid transport control. In addition it is associated with certain neuroendocrine-related diarrhoeas such as pancreatic cholera (Brown & Miller, 1991).

### *Analytical procedure*

Samples were centrifuged and then diluted with buffer and peak absorbances were measured at 560 nm as well as 520 and 600 nm to correct for non-specific interferences as described by Miller & Schedl (1972). The results were expressed as the amount absorbed(+) or secreted(−) in $\mu l\ g^{-1}$ weight of tissue during the 20 min perfusion.

### *Secretory potency*

The secretory potency of VIP was monitored routinely since it showed a slight variation in its effect over the 6 months of experiments. Consequently, separate secretory base lines to VIP were established for each adenosine agonist and the antagonists tested.

### *Statistical analysis*

The rates of water transport are given as means ± s.e.mean. The potency of each adenosine agonist is expressed as an $ED_{50}$ value, which is the concentration that elicits a half-maximal effect on the net water transport between the range of the VIP-induced secretion to the basal level of absorption with a 95% confidence interval using linear regression analysis (Tallarida & Murray, 1987).

Pairs of means were compared for statistical differences using Student's two tailed *t* test and series of means were compared to common controls using Dunnett's t test (Tallarida & Murray, 1987). The criterion for statistical significance was set at $P < 0.05$.

### *Drugs*

The following were used: pentobarbitone sodium (Nembutal, Boehringer Ingelheim, Artarmon, Australia), vasoactive intestinal peptide (VIP; AUSPEP, Melbourne, Australia).

Agonists: chloroadenosine (2-CADO, mol. wt. = 301.7), $N^6$-cyclopentyladenosine (CPA, mol. wt. = 335.4), 2-phenylaminoadenosine (CV-1808, mol. wt. = 358.4), 5'-N-ethylcarboxamidoadenosine (NECA, mol. wt. = 308.3), **R**-(−)-$N^6$-(2-phenylisopropyl) adenosine (**R**-(−)-PIA, mol. wt. = 385.4) and **S**-(+)-$N^6$-(2-phenylisopropyl) adenosine (**S**-(+)-PIA, mol. wt. = 385.4). All were obtained from RBI, Natick, U.S.A.

All agonists and VIP were dissolved in 0.9% w/v saline except CV-1808 and CPA which were dissolved in 1:1 ethanol:saline solution and then diluted with saline to give the required dose. The higher dose of CV-1808 (3125 $\mu g\ kg^{-1}$) was dissolved in 90% ethanol and diluted with saline. The vehicle had no significant effect on VIP-induced secretion ($P > 0.05$, Student's two tailed *t* test).

Antagonists: 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, RBI, Natick, U.S.A.) and 8-phenyltheophylline (8-PT, RBI, Natick, U.S.A.) were dissolved in 1% v/v dimethylsulphoxide 0.75% v/v 1 M NaOH in saline. This vehicle had no significant effect of VIP-induced secretion.

## Results

The rate of fluid absorption from the jejunum of rats infused with saline i.a. as a control was 239 ± 24 $\mu l\ g^{-1}$ wet weight in 20 min ($n = 6$) and this was reversed by the i.a. infusion of VIP which induced a large net secretion of fluid into the lumen. The secretory response to VIP was monitored for each drug tested. All adenosine agonists tested, except CV-1808, produced a dose-related reversal of the VIP-induced secretory response (see Figure 1). The most potent agonist, NECA displayed a high efficacy at 100 $\mu g\ kg^{-1}$ but this dose did not fully restore fluid transport to the basal level of absorption (Figure 1, $P = 0.03$, two tailed *t* test). A dose of CV-1808 (750 $\mu g\ kg^{-1}$) failed to inhibit VIP-induced secretion ($P = 0.08$, two tailed *t* test) and even at a higher dose (3125 $\mu g\ kg^{-1}$), CV-1808 caused only a small inhibition (Figure 1, $P = 0.04$, two tailed *t* test). The potency of each adenosine agonist is shown in Table 1.

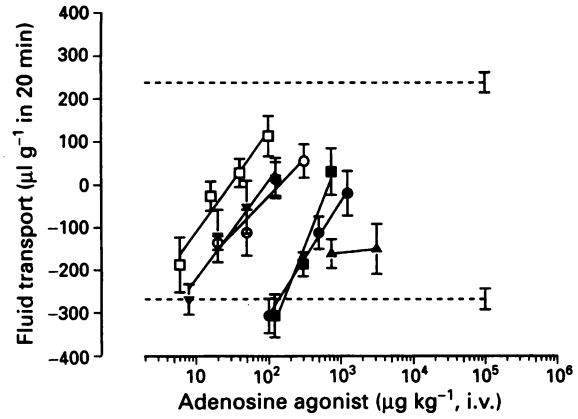


**Figure 1** Inhibition by NECA (□), CPA (▼), R-PIA (○) S-PIA (■), 2-CADO (●) and CV-1808 (▲) of intestinal secretion induced by vasoactive intestinal peptide (VIP). The lower non-continuous line indicates the overall net fluid secretion rate (µl $g^{-1}$ wet tissue in 20 min, $n = 21$) in response to i.a. infusion of VIP (0.8 µg $min^{-1}$). Basal rates of secretion for each adenosine agonist tested are not shown for clarity. The top non-continuous line indicates the absorption rate (i.a. infusion and i.v. injection of saline as controls); s.e.means are indicated. For abbreviations, see text.

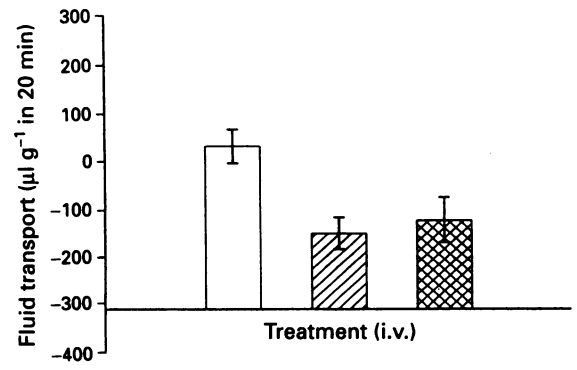


**Figure 2** Effect of the adenosine antagonists (0.1 mg $kg^{-1}$), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (hatched column) and 8-phenyltheophylline (8-PT) (cross hatched column) on the antisecretory action of 5'-N-ethylcarbox-amidoadenosine (NECA, 40 µg $kg^{-1}$, open column); s.e. means are indicated. The bottom continuous line represents the secretion rate ($-316 \pm 40$ µl $g^{-1}$) in response to i.a. infusion of VIP (0.8 µg $min^{-1}$). Both antagonists caused a significant inhibition of NECA ($P < 0.05$, Dunnett's $t$ test).

Adenosine antagonists, 8-PT (0.1 mg $kg^{-1}$, i.v.) and DPCPX (0.1 mg $kg^{-1}$, i.v.) significantly inhibited the antisecretory response of NECA (40 µg $kg^{-1}$, i.v. Figure 2, $P < 0.05$, Dunnett's $t$ test). Neither antagonist affected the basal secretory response to VIP (control secretory response = $-316 \pm 40$ ($n = 7$), DPCPX = $-273 \pm 54$ ($n = 5$), 8-PT = $-376 \pm 74$ ($n = 5$), $P > 0.05$, Dunnett's $t$ test).

## Discussion

The potencies and efficacies of both adenosine agonists and antagonists reported in this *in vivo* study suggest that the $A_{2B}$ adenosine receptor is involved in controlling intestinal antisecretory activity in the rat jejunum. This is based on the classification table outlined in the review by Collis & Hourani (1993) which is a proposed framework to aid in the classification of functional adenosine receptors.

Dionyssopoulos *et al.* (1992) found that the adenosine agonists, R-PIA, NECA but not CV-1808 were potent inhibitors of opiate withdrawal diarrhoea in rats. These

**Table 1** The potencies of adenosine agonists at inhibiting vasoactive intestinal peptide (VIP)-induced intestinal secretion

| *Agonist* | $ED_{50}$ (µg $kg^{-1}$) | *95% C.I.*, n | *Basal VIP-induced secretion* (µl $g^{-1}$) ± s.e.mean, n |
|---|---|---|---|
| NECA | 21 | 1.4, 20 | $-327 \pm 22$, 7 |
| CPA | 74 | 2.4, 15 | $-275 \pm 31$, 10 |
| R-PIA | 107 | 2.3, 25 | $-285 \pm 33$, 9 |
| S-PIA | 588 | 1.6, 12 | $-302 \pm 40$, 7 |
| 2-CADO | 1184 | 2.2, 8 | $-282 \pm 30$, 4 |
| CV-1808 | >3125 | -, 14 | $-297 \pm 33$, 9 |

For abbreviations, see text.

authors concluded that the antidiarrhoeal response was due to the activation of $A_1$ receptors since at the time R-PIA was classified as a selective $A_1$ agonist, NECA as a non-selective $A_1/A_2$ agonist and CV-1808 a selective $A_2$ agonist (Burnstock & Buckley, 1985; Stone, 1985; Bruns *et al.*, 1986). These results correlate with those of the present study, where NECA had the most potent antisecretory effect and high doses of CV-1808 were ineffective at restoring VIP-induced intestinal secretion to the control level of absorption. An additional feature of the present study is that a greater range of agonists as well as antagonists was tested since the modern classification of adenosine receptors is based on the rank order of both agonist potency and antagonist affinity.

The agonists used in this study inhibited VIP-induced secretion in the order of NECA > CPA > R-PIA > S-PIA > 2-CADO > CV-1808 which best complies with the rank order of agonist potency that represents activation of the recently described $A_{2B}$ receptor: NECA > 2-CADO > R-PIA = CHA > S-PIA > = CV-1808 > = CGS-21680 (see review by Collis & Hourani, 1993). The low potency of 2-CADO in this study could be due to its poor chemical stability (Jacobson, 1990) and the above classification order being derived mainly from *in vitro* techniques. CPA displayed potent antisecretory activity closely following that of NECA. Unfortunately CPA does not appear in the $A_{2B}$ receptor classification but there is no evidence in the literature to suggest that CPA is without activity at the $A_{2B}$ receptor.

The rank order of agonist potency reported in this study next best complies with the $A_1$ receptor classification where the agonist potency order is: CPA > R-PIA = CHA = > NECA > 2-CADO > S-PIA > CV-1808 > = CGS-21680 (Collis & Hourani, 1993). Both S-PIA and CV-1808 are the least potent, with S-PIA the stronger of the two in both the $A_1$ and $A_{2B}$ receptor classification which agrees with the results of this study. CPA does appear in the $A_1$ but not the $A_{2B}$ receptor classification where it is more potent than NECA. However, the results from this study show the reverse of this order. In addition, R-PIA is equipotent or more potent than NECA in the $A_1$ receptor classification, which is not true for this study since NECA was significantly more potent than R-PIA.

The agonist potency order of the $A_{2A}$ receptor is: CGS21680 = NECA > CV-1808 > = 2-CADO > R-PIA = CHA = CPA > S-PIA (Collis & Hourani, 1993). This order is almost the reverse of the order of the agonists as antisecretory agents, except for NECA which was the most potent. For instance, the least potent agonist in this study CV-1808 is second in line to that of NECA in the $A_{2A}$ receptor classification. The potency of the selective $A_{2A}$ agonist, CGS-21680 was not investigated in this study since the agonists studied did not follow the $A_{2A}$ order of potency but rather the $A_{2B}$ or $A_1$ where CGS-21680 is the least potent in both classifications. The $A_3$ receptor was discounted because the agonists R-PIA and NECA are equipotent in the $A_3$ classification (Collis & Hourani, 1993) whereas NECA inhibits VIP-induced secretion to a significantly greater extent

than R-PIA in this study. In addition, xanthine antagonists, such as those used in this study have low affinity at the $A_3$ receptor.

The classification of adenosine receptors is also dependent on antagonist affinity order (Collis & Hourani, 1993). The adenosine antagonists, 8-PT and DPCPX were specifically investigated to reinforce the receptor classification based on agonist potencies in this study, since they are able to differentiate between $A_1$ and $A_2$ receptors. For instance, binding studies (see review by Van Galen *et al.*, 1992) have established that 8-PT shows a small degree of selectivity for $A_1$ compared to $A_2$ sites ($K_i$ = 86 nM at $A_1$ and 850 nM at $A_2$) while DPCPX in contrast is highly selective for $A_1$ sites ($K_i$ = 0.5 nM at $A_1$ and 340 nM at $A_2$). The difference between the affinities and selectivity ratios of the two antagonists is also apparent in intact tissues where 8-PT is non-selective for $A_1$ and $A_2$ receptors, whilst DPCPX has a 30–50 fold greater affinity for $A_1$ but is equi-effective with 8-PT at $A_2$ receptors (Collis *et al.*, 1989). Even though much of the data on these antagonists is derived from *in vitro* studies their use should be valid *in vivo* since in the conscious rat the duration of action of DPCPX is 3–5 h (Collis *et al.*, unpublished results) and 8-PT is up to 5 h (Bowmer *et al.*, 1986). In this study a dose of 0.1 mg kg$^{-1}$ of DPCPX was investigated since both Kellett *et al.* (1989) and Collis *et al.* (1991) established that this low dose was effective at inhibiting the adenosine-mediated responses of bradycardia and diuresis in the rat. DPCPX was thought to block $A_1$ receptors since a much higher dose of 8-PT was required to achieve a similar inhibition of adenosine-induced responses. In the present study, NECA, the most potent agonist, was inhibited by approximately 50% by both DPCPX and 8-PT at doses of 0.1 mg kg$^{-1}$. This finding discounts the possibility that the adenosine agonists are inhibiting VIP-induced secretion via the $A_1$ receptor, since DPCPX failed to exert a more potent inhibitory effect than 8-PT. This equipotent antagonist activity suggests the presence of an $A_2$ receptor (Collis *et al.*, 1989) and together with the agonist potency order, more precisely indicates the presence of an $A_{2B}$ receptor subtype (Collis & Hourani, 1993). However, at this stage a definitive receptor characterization cannot be completed until the tissue location of these receptors is determined and *in vitro* studies are performed.

An incidental finding of the study was that both DPCPX and 8-PT were free of intrinsic pro-secretory activity at the doses employed. This suggests that endogenous adenosine is not released to inhibit secretion under the conditions of the presently described experiments. However, the possibility that the antagonist doses were not high enough to unmask the presence of endogenous adenosine activity cannot be discounted.

The clinically used opiate antidiarrhoeal drugs such as difenoxin and loperamide have $ED_{50}$ values of 0.23 mg kg$^{-1}$ and 0.5 mg kg$^{-1}$ respectively for inhibiting VIP-induced secretion in the rat jejunum (De Luca & Coupar, 1993). However, in this study the adenosine agonists NECA ($ED_{50}$ = 21 μg kg$^{-1}$) and CPA ($ED_{50}$ = 74 μg kg$^{-1}$) prove to be more potent antisecretory drugs since they have $ED_{50}$ values in the microgram range. In addition, De Luca & Coupar (1993) found that the highest dose of both difenoxin (3.1 mg kg$^{-1}$) and loperamide (1.5 mg kg$^{-1}$) tested were unable to restore fluid transport further than zero net fluid transport. However, this study shows that the highest dose of NECA investigated (100 μg kg$^{-1}$) had a higher efficacy compared to the clinically used antidiarrhoeal drugs.

We suggest that adenosine $A_{2B}$ receptor agonists could be evaluated for potential use as antidiarrhoeal drugs. This proposal is based on the present results where selectivity of the $A_{2B}$ receptor was achieved with agonist analogues displaying high potency and efficacy in inhibiting intestinal fluid secretion in the rat.

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# Examination of the role of inhibition of cyclic AMP in $\alpha_2$-adrenoceptor mediated contractions of the porcine isolated palmar lateral vein

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**1** We have examined the effect of elevation of cellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) on $\alpha_1$- and $\alpha_2$-adrenoceptor-mediated contraction of the isolated palmar lateral vein of the pig. Cellular cyclic AMP was increased by either inhibition of phosphodiesterase by rolipram, or direct activation of adenylyl cyclase by forskolin.

**2** Noradrenaline (1 nM–10 μM) caused concentration-dependent contractions of the porcine isolated palmar lateral vein ($pD_2$ 7.32 ± 0.07, $n = 10$). The selective $\alpha_1$-adrenoceptor antagonist, prazosin (0.1 μM) and the selective $\alpha_2$-adrenoceptor antagonist, rauwolscine (1 μM) caused a 10 fold rightward displacement of the concentration-response curve and a combination of the two antagonists caused a 200 fold rightward displacement of the concentration-response curve. The selective $\alpha_2$-adrenoceptor agonist, UK-14304, also produced concentration-dependent contractions of the palmar lateral vein ($pD_2$ 7.70 ± 0.15, $n = 5$), but the maximum response was 55.5 ± 7.6% ($n = 5$) of that produced by noradrenaline. Prazosin (0.1 μM) failed to affect responses to UK-14304 but rauwolscine, 1 μM, caused a 200 fold rightward displacement. The estimated $pK_B$ value for rauwolscine (8.28 ± 0.19, $n = 10$) is consistent with inhibition of $\alpha_2$-adrenoceptors. Thus, the porcine isolated palmar lateral vein has a population of $\alpha_1$- and $\alpha_2$-adrenoceptors capable of producing a contraction.

**3** Rolipram, 10 μM, and forskolin, 1 μM, caused a 2–3 fold rightward displacement of the noradrenaline concentration-response curve (CRC), but 1,9-dideoxyforskolin, 1 μM, a forskolin analogue which does not activate adenylyl cyclase, failed to produce a significant inhibition of noradrenaline-induced contractions. The combination of forskolin (1 μM) and rolipram (10 μM) were additive, producing a 20 fold rightward displacement of the noradrenaline CRC.

**4** Responses to noradrenaline were similarly affected by a combination of rolipram (10 μM) and prazosin (0.1 μM) (isolation of $\alpha_2$-adrenoceptors) and the combination of rolipram (10 μM) and rauwolscine (1 μM) (isolation of $\alpha_1$-adrenoceptors), resulting in a 100 fold rightward displacement of the noradrenaline CRC. Although forskolin inhibited both $\alpha_1$- and $\alpha_2$-adrenoceptor-mediated contractions, the effects produced were not similar. In particular, noradrenaline, 0.3–3 μM, produced a significant contraction in the presence of forskolin (1 μM) and prazosin (0.1 μM) (an $\alpha_2$-adrenoceptor-mediated response) but not in the presence of forskolin (1 μM) and rauwolscine (1 μM) (an $\alpha_1$-adrenoceptor-mediated response).

**5** Five minute exposure to either rolipram (10 μM) or forskolin (1 μM) elevated [$^3$H]-cyclic AMP of the porcine isolated palmar lateral vein by approximately 70% and 150–200%, respectively. Neither noradrenaline (1 nM–100 μM) nor UK-14304 (1 nM–100 μM) affected basal levels of [$^3$H]-cyclic AMP, but both produced a concentration-dependent inhibition of forskolin-stimulated [$^3$H]-cyclic AMP accumulation with a $pK_i$ of 7.43 ± 0.1 ($n = 3$) and 7.97 ± 0.18 ($n = 3$), respectively. The effect of noradrenaline against forskolin-stimulated [$^3$H]-cyclic AMP accumulation was reversed by rauwolscine (1 μM) but not by prazosin (0.1 μM). In contrast, $\alpha_2$-adrenoceptor activation did not affect rolipram-induced elevation of [$^3$H]-cyclic AMP.

**6** These findings indicate that $\alpha_2$-adrenoceptor contractions of the porcine isolated palmar lateral vein are not produced by reduction in cellular cyclic AMP *per se*. It is proposed that this response involves a novel signal transduction mechanism. However, when cellular cyclic AMP has been elevated by agents that stimulate adenylyl cyclase, rather than through inhibition of phosphodiesterase, the ability of $\alpha_2$-adrenoceptors to inhibit cyclic AMP formation may be of functional importance in vascular smooth muscle.

**Keywords:** Forskolin; rolipram; $\alpha_1$-adrenoceptors; $\alpha_2$-adrenoceptors; contractions; inhibition of cyclic AMP

## Introduction

The existence of post-junctional $\alpha_2$-adrenoceptors mediating contractions of vascular smooth muscle has been known for 15 years. However, compared to $\alpha_1$-adrenoceptor-mediated responses, there is surprisingly little information detailing the events between receptor stimulation and the end-organ response (McGrath *et al.*, 1989). Part of the problem has been that although $\alpha_2$-adrenoceptor-mediated pressor responses are readily demonstrated *in vivo*, there are few examples of isolated blood vessels that exhibit responses to noradrenaline with the appropriate pharmacological characteristics; resistant to prazosin but sensitive to either yohimbine or rauwolscine (McGrath, 1982).

Several studies have shown that $\alpha_2$-adrenoceptor-mediated pressor responses in pithed and anaesthetized animals are sensitive to pertussis toxin (Boyer *et al.*, 1983; Nichols *et al.*, 1989) and organic calcium channel antagonists (Ruffolo *et al.*, 1984; Timmermans *et al.*, 1985), which indicates the involvement of a guanine nucleotide binding protein and the

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entry of extracellular calcium via voltage-dependent calcium-channels. This particular view is also supported by studies in some isolated blood vessels. For example, $\alpha_2$-adrenoceptor-mediated contraction in the rabbit isolated saphenous vein (Daly *et al.*, 1990) and human isolated blood vessels (Arner *et al.*, 1988; Nielsen *et al.*, 1992) are practically abolished by removal of extracellular calcium ions. However, since responses of the rabbit isolated saphenous vein (Dunn *et al.*, 1991) and human isolated dorsal metacarpal veins (Arner *et al.*, 1988) are relatively unaffected by nifedipine and nimodipine, compared to the effect of removal of extracellular calcium, this raises the possibility that dihydropyridine-insensitive calcium channels are implicated. The involvement of other pathways coupled to vascular $\alpha_2$-adrenoceptor is also suggested by reports that activation of $\alpha_2$-adrenoceptors in the canine isolated saphenous vein (Guan *et al.*, 1989) and rabbit isolated ear vein (Daly *et al.*, 1990) can elicit contractions even in the absence of extracellular calcium. Finally, recent evidence suggests that $\alpha_2$-adrenoceptor activation might also sensitize the contractile proteins to intracellular calcium ions (Arburto *et al.*, 1993).

It is generally acknowledged that in non-vascular tissue, activation of $\alpha_2$-adrenoceptors is associated with an inhibition of adenylyl cyclase activity (see McGrath *et al.*, 1989); usually measured as an inhibition of forskolin- or agonist-stimulated cyclic AMP. To date, however, $\alpha_2$-adrenoceptor-mediated modulation of cyclic AMP has been reported only in two isolated blood vessels; the cat isolated basilar artery (Fredholm *et al.*, 1985) and the rat isolated femoral vein (Stubbs *et al.*, 1988). Thus, the potential for this pathway to provide a link between the receptor and either calcium channels or intracellular stores of calcium has not been investigated.

We recently reported that the selective $\alpha_2$-adrenoceptor agonist, UK-14304, produced a concentration-dependent inhibition of forskolin-stimulated cyclic AMP in the porcine isolated palmar lateral vein (Wright *et al.*, 1993). This has provided us with a basis to examine the effect of a direct stimulant of cellular cyclic AMP, forskolin (Seamon & Daly, 1986), and an indirect elevator of cellular cyclic AMP, the phosphodiesterase inhibitor rolipram (Donaldson *et al.*, 1988), against $\alpha_1$- and $\alpha_2$-adrenoceptor-mediated responses, to gain insights into the coupling mechanisms for post-junctional $\alpha_2$-adrenoceptors. Some of these results were presented at the Leicester Meeting of the Physiological Society (Harling & Wilson, 1993).

## Methods

### *Preparation of the blood vessel*

Porcine trotters from the forelimbs were obtained with 30 min of the death of the animal, placed on ice and transported to the laboratory. A 3–4 cm length of the palmar lateral vein was dissected out and stored overnight at 4°C in modified Krebs-Henseleit saline containing 2% Ficoll. The composition of the modified Krebs-Henseleit saline was (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, $KH_2PO_4$ 1.2 and glucose 11.1.

### *Isometric tension recordings*

On the following day, the vein was carefully cleaned of connective tissue and divided into 5 mm ring segments. Stainless steel wire (0.2 mm thick) supports were then inserted into the lumen and each segment was suspended in an isolated organ bath containing modified Krebs-Henseleit saline with cocaine, 10 μM (to inhibit uptake$_1$), propranolol, 1 μM (to inhibit β-adrenoceptors) and $Na_2EDTA$ (23 μM) to prevent the oxidative degradation of noradrenaline. The Krebs-Henseleit saline was maintained at 31°C and gassed with 95% $O_2$ and 5% $CO_2$. No attempt was made to remove the endothelium. The lower support was fixed, and the upper support was connected to a Grass FT-03 transducer linked to a Grass Polygraph. After 30 min equilibration in the Krebs-Henseleit saline, approximately 4 g wt. tension was slowly applied and the tissue allowed to relax to a final resting tension of 0.5–0.7 g wt. tension. After 60 min equilibration each preparation was exposed to (−)-noradrenaline (NA), 1 μM, and allowed to contract for 5 min. Following complete washout, the preparation was again challenged with noradrenaline (1 μM), washed and an additional 1 h equilibration period allowed before starting the experiment. This procedure was found to minimize changes in the sensitivity of the preparation to further addition of noradrenaline or UK-14304.

In all experiments cumulative concentration-response curves (CRC) were determined by plotting the contractile response of the tissue to increasing concentrations (0.5 log unit increments) of the agonist. For the majority of preparations, contractile responses to the α-adrenoceptor agonists were not well maintained over a period greater than 90 s. Therefore, the addition of the next concentration of the agonist was made as close to the peak response as possible. Successive CRC were separated by 60 min, as measured from the time following washout and complete relaxation of the preparation and only two CRCs were constructed in each preparation.

*The effect of α-adrenoceptor antagonists* Cumulative CRCs to noradrenaline and UK-14304 were constructed and the agonists compared on the basis of potency ($pD_2 = -\log EC_{50}$ = the negative logarithm of the concentration required to produce 50% of maximum response interpolated from the CRC) and the maximum response relative to noradrenaline (1 μM). Prazosin (0.1 μM), rauwolscine (1 μM) or a combination of prazosin (1 μM) and rauwolscine (1 μM) were added at least 40 min prior to the construction of a second CRC. The agonist concentration-ratio (i.e. $EC_{50}$ of the agonist in the presence of the antagonist divided by the control $EC_{50}$ value) produced by the antagonists was determined in each preparation, and the negative logarithm of the dissociation constant ($pK_B$) determined by the method of Furchgott (1972).

*The effect of rolipram and forskolin* CRCs to noradrenaline were constructed in the presence and absence of either forskolin (0.1–10 μM), rolipram (0.1–10), 1,9-dideoxyforskolin (1 μM) or a combination of forskolin (1 μM) and rolipram (10 μM). These agents were added 20 min before the 2nd CRC to noradrenaline. The agonist concentration-ratio was calculated at the level of 40% of the maximum control response to noradrenaline, because the maximum responses in the presence of an α-adrenoceptor antagonist and either rolipram or forskolin (see below) were often significantly less than the control maximum response.

*The effect of rolipram and forskolin after α-adrenoceptor blockade* CRCs to noradrenaline were constructed in the presence and absence of rolipram (10 μM) with prazosin (0.1 μM), rolipram (10 μM) with rauwolscine (1 μM), forskolin (1 μM) with prazosin (0.1 μM) and forskolin (1 μM) with rauwolscine (1 μM). The antagonists were added 40 min, and forskolin and rolipram 20 min, before the onset of the 2nd CRC to noradrenaline. The agonist concentration-ratio was calculated at the level of 40% of the maximum control response to noradrenaline.

In all experiments, one preparation was run in parallel with the experimental tissues, but received no antagonist, and was used to determine time-dependent changes in agonist sensitivity (Furchgott, 1972). All responses are expressed as a percentage of the maximum response and given as the mean ± s.e.mean. Differences between means were considered statistically significant if $P<0.05$ for unpaired or paired observations (Student's $t$ test). The logarithm of the concentration of noradrenaline or UK-14304 producing 50% of the

maximum response ($pD_2$) or agonist-concentration ratios were calculated using the logistic equation described by DeLean *et al.* (1978) with Kalidegraph software (Synergy) on a MacIntosh LC II computer.

### *Second messenger studies*

Vessels were cut into 5 mm lengths and then incubated for 60 min at 37°C in a shaking waterbath. After this period of time segments of vessel were incubated with [$^3$H]-adenine (specific activity = 851 GBq mmol$^{-1}$; 74 kBq ml$^{-1}$), again for a period of 60 min at 37°C in a shaking waterbath. After incubation the segments of vessel were washed 3 times by resuspension and then each segment was transferred into a flat-bottomed plastic incubation vial containing Krebs solution to a final assay volume of 300 µl. Segments were allowed to equilibrate for 20 min, all experiments were carried out in quadruplicate. Agonists (NA or UK14304) were added 10 min prior to forskolin (antagonists added 10 min prior to agonists). Forskolin (1 µM) or rolipram (10 µM) was added for 5 min. Incubation tubes were resealed under $O_2$:$CO_2$ (95%:5%) after each addition. Incubations were stopped by the addition of 200 µl 1 M HCl, followed by 750 µl of distilled water.

[$^3$H]-cyclic AMP was separated from [$^3$H]-adenine and other $^3$H-products by sequential Dowex/alumina chromatography (Salomon *et al.*, 1974), adjusting for variation in recovery by the addition of [$^{14}$C]-cyclic AMP (c. 30 Bq per tube). [$^3$H]-cyclic AMP and total $^3$H in the tissue were determined by liquid scintillation counting. [$^3$H]-cyclic AMP levels were corrected for the recovery from the dowex/alumina chromatography and the amount of total tritium taken up into each individual tissue.

### *Data analysis*

NA and UK14304 inhibition curves of forskolin-stimulated cyclic AMP accumulation were analysed by iterative fitting to a least squares non-linear equation (Kaleidagraph, MacIntosh) to generate $IC_{50}$ values (concentration of drug at the 50% value in the inhibition curve of forskolin-stimulated cyclic AMP accumulation).

### *Drugs*

The following compounds were used: prazosin HCl (Pfizer); rauwolscine HCl (Roth); UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer); (−)-noradrenaline bitartrate (Sigma); propranolol HCl (Sigma); cocaine HCl (MacCarthys), forskolin (Sigma), rolipram (Smith Kline Beecham); Ficoll 70, 000 (Sigma) and 1,9,dideoxyforskolin (Sigma). Stock solutions of noradrenaline were prepared in distilled water with 23 µM $Na_2EDTA$, while forskolin (1 mM), 1,9-dideoxyforskolin (1 mM) and rolipram (1 mM) were dissolved in absolute ethanol. Prazosin (1 mM) was dissolved in 0.1 M lactic acid and dilutions made in distilled water. All other drugs were dissolved in distilled water and added to the organ baths in a volume of 0.1 ml or less.

## Results

### *Pharmacological characteristics of contractile responses to noradrenaline*

Noradrenaline and UK-14304 produced concentration-dependent contractions of the porcine isolated palmar lateral vein. UK-14304 ($pD_2$ 7.70 ± 0.15, $n$ = 5) was approximately 3 fold more potent than noradrenaline ($pD_2$ 7.32 ± 0.07, $n$ = 10) but the maximum response was 55.5 ± 7.6% ($n$ = 5) of that produced by noradrenaline (1 µM).

Prazosin (0.1 µM) and rauwolscine (1 µM) caused a 10 fold rightward displacement of the noradrenaline concentration-response curve (CRC) and a combination of the antagonists produced more than a 200 fold rightward displacement of the noradrenaline CRC (Figure 1a; Table 1). In contrast, prazosin (0.1 µM) failed to inhibit UK-14304-induced contractions, while rauwolscine (1 µM) produced more than a 200 fold rightward displacement of the UK-14304 CRC (Figure 1b; Table 1). The estimated log $K_B$ value for rauwolscine against UK-14304 was 8.28 ± 0.19 ($n$ = 10), which is significantly greater ($P<0.05$) than that determined against noradrenaline (7.14 ± 0.1, $n$ = 9). Taken together, these observations indicate that prazosin (0.1 µM) and rauwolscine (1 µM) are selective for $\alpha_1$- and $\alpha_2$-adrenoceptors, respectively, and that both receptors are activated by noradrenaline.

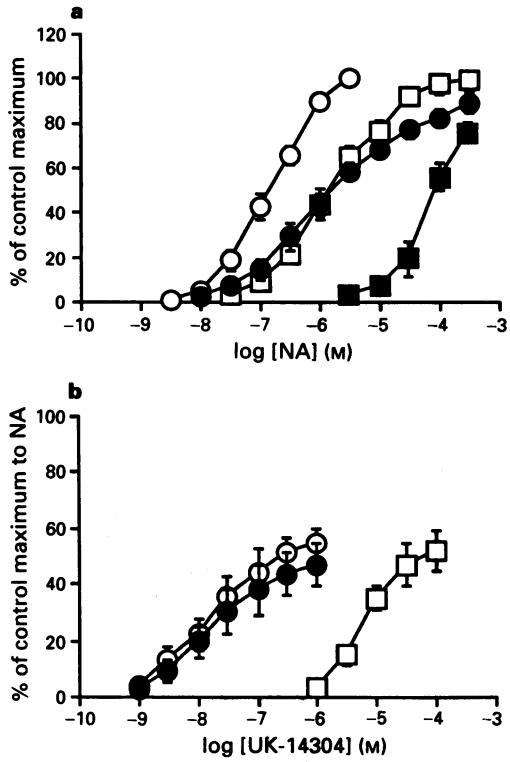


**Figure 1** The effect of prazosin, 0.1 µM (●), rauwolscine, 1 µM, (□) and a combination of prazosin (0.1 µM) and rauwolscine (1 µM) (■) against (a) noradrenaline (NA) and (b) UK-14304-induced contraction of the porcine isolated palmar lateral vein. Responses to the agonists in the absence of the antagonists is shown by (○). All responses have been determined as a percentage of the maximum control response to noradrenaline and expressed as the mean ± s.e.mean of observations made in 5–10 separate experiments.

**Table 1** The effect of prazosin and rauwolscine against noradrenaline and UK-14304-induced contractions of the porcine isolated palmar lateral vein

| | *Mean log agonist concentration-ratio* | *Estimated $pK_B$* |
|---|---|---|
| *Noradrenaline* | | |
| Prazosin 0.1 µM | 1.06 ± 0.12 ($n$ = 10) | 8.00 ± 0.13 |
| Rauwolscine 1 µM | 1.17 ± 0.08 ($n$ = 9) | 7.14 ± 0.10 |
| Prazosin 0.1 µM and rauwolscine 1 µM | 2.67 ± 0.20 ($n$ = 6) | NA |
| *UK-14304* | | |
| Prazosin 0.1 µM | 0.15 ± 0.09 ($n$ = 5) | >7 |
| Rauwolscine 1 µM | 2.28 ± 0.20 ($n$ = 5) | 8.28 ± 0.19 |

*Mean log agonist concentration-ratio determined at 50% of the maximum response.
NA – not applicable.

### *The effect of rolipram and forskolin against noradrenaline-induced contractions*

Rolipram (10 μM) and forskolin (1 μM) caused a small reduction in the resting tone (0.3–0.4 g wt.) in approximately 50% of preparations ($n = 11-16$) while forskolin (10 μM) caused a similar relaxation in all preparations ($n = 6$) of the porcine isolated palmar lateral vein. Figure 2 shows the effect of various concentrations of rolipram and forskolin and a combination of a single concentration of rolipram and forskolin against noradrenaline-induced contractions of the porcine isolated palmar lateral vein. Rolipram (1 μM–10 μM) produced a 2–3 fold rightward displacement of the noradrenaline CRC (Figure 2a; Table 2), while forskolin (1 μM–10 μM) produced a 4–10 fold rightward displacement (Figure 2b; Table 2). With the exception of rolipram (10 μM), neither agent caused a significant reduction in the maximum response to noradrenaline. In contrast to rolipram and for-

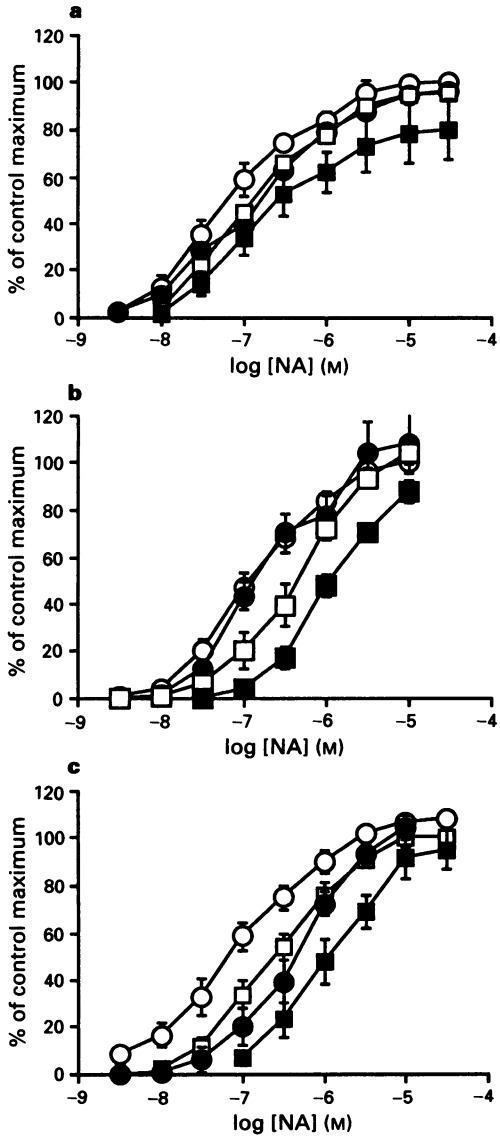


**Figure 2** The effect of rolipram and forskolin against noradrenaline (NA)-induced contractions of porcine isolated palmar lateral vein. (a) The effect of (●) 0.1 μM rolipram; (□) 1 μM rolipram and (■) 10 μM rolipram against noradrenaline-induced contractions. (b) The effect of (●) 0.1 μM forskolin; (□) 1 μM forskolin and (■) 10 μM forskolin against noradrenaline-induced contractions. (c) The effect of (□) 10 μM rolipram; (●) 1 μM forskolin and (■) 10 μM rolipram and 1 μM forskolin against noradrenaline contractions. In all three graphs responses to noradrenaline under control conditions are shown by (○). All responses have been determined as a percentage of the maximum control response to noradrenaline and expressed as the mean ± s.e.mean of observations made in 6–10 separate experiments.

**Table 2** The effect of forskolin and rolipram against noradrenaline induced contractions of the porcine isolated palmar lateral vein

| *Condition* | n | *log Agonist concentration-ratio* | |
|---|---|---|---|
| Control | 12 | − 0.02 ± 0.09 | NS |
| Forskolin 0.1 μM | 4 | 0.23 ± 0.15 | NS |
| Forskolin 1 μM | 6 | 0.66 ± 0.14[a] | ** |
| Forskolin 10 μM | 6 | 0.99 ± 0.09 | ** |
| 1,9-Dideoxyforskolin 1 μM | 6 | 0.16 ± 0.12 | NS |
| Rolipram 10 μM | 6 | 0.40 ± 0.06 | ** |
| Rolipram 10 μM + Forskolin 1 μM | 6 | 1.27 ± 0.12[b] | ** |

**Indicates that the mean log agonist concentration-ratio is significant ($P<0.05$) when compared with corresponding controls; NS, indicates that the mean log agonist concentration-ratio is not significantly different from the corresponding controls. [a]Represents a significant difference ($P<0.05$) between the mean shifts produced by 1 μM forskolin and 1 μM 1,9-dideoxyforskolin. [b]Represents a significant difference ($P<0.05$) between the mean shift produced by 1 μM forskolin and 1 μM forskolin with 10 μM rolipram.

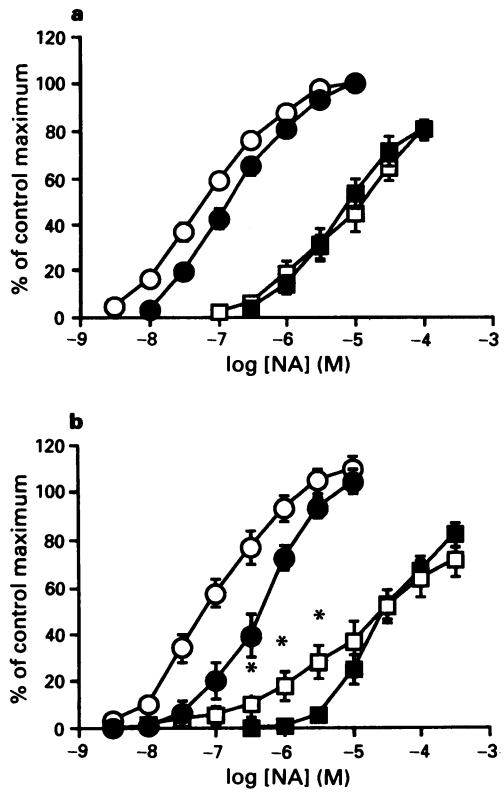


**Figure 3** The effect of rolipram and forskolin, in the presence of either prazosin (0.1 μM) or rauwolscine (1 μM), against noradrenaline (NA)-induced contractions of the porcine isolated palmar lateral vein. (a) The effect of (●) rolipram 10 μM; (□) prazosin 0.1 μM with rolipram, 10 μM; and (■) rauwolscine 1 μM with rolipram 10 μM against noradrenaline-induced contractions. (b) The effect of (●) forskolin 1 μM; (□) prazosin 0.1 μM with forskolin 1 μM and (■) rauwolscine 1 μM with forskolin 1 μM against noradrenaline-induced contractions. Responses to noradrenaline under control conditions are shown by (○). All responses have been determined as a percentage of the maximum control response to noradrenaline and expressed as the mean ± s.e.mean of observation made in 6–10 separate experiments. *Significant difference ($P<0.05$) between the effect of prazosin (0.1 μM) with forskolin (1 μM) compared to the effect of rauwolscine (1 μM) with forskolin (1 μM).

skolin, 1,9 dideoxyforskolin (1 μM) failed to produce a significant rightward displacement of noradrenaline CRC (Table 2). As shown in Figure 2c, the combination of rolipram (10 μM) and forskolin (1 μM) produced a significantly greater rightward displacement of the noradrenaline CRC (20 fold; Table 2) than either agent alone (approximately 3–4 fold), which was equivalent to that produced by 10 μM forskolin alone.

### *The effect of prazosin (0.1 μM) and rauwolscine (1 μM) against noradrenaline-induced contractions in the presence of either rolipram (10 μM) or forskolin (1 μM)*

The combination of rolipram (10 μM) with either prazosin (0.1 μM) or rauwolscine (1 μM) caused a rightward displacement of the noradrenaline CRC which was parallel to the CRC in the presence and absence of rolipram (10 μM) (Figure 3a). In this series of experiments rolipram (10 μM) did not reduce the maximum response to noradrenaline. Although the magnitude of the displacement produced by the combination of rolipram and the antagonists were not significantly different from each other (Table 3), i.e. the two CRCs were superimposable, the effects were greater than that produced by the antagonists alone (compare appropriate values in Tables 1 and 3).

The combination of forskolin (1 μM) with either prazosin (0.1 μM) or rauwolscine (1 μM) also caused a rightward displacement of the noradrenaline CRC (Figure 3b). At the 40% level of the control maximum response, the displacement produced by the combination of forskolin and the antagonists were not significantly different from each other (Table 3), but the noradrenaline CRC in the presence of forskolin (1 μM) and prazosin (0.1 μM) was not parallel to those observed under control conditions or in the presence of either forskolin (1 μM) or forskolin (1 μM) and rauwolscine (1 μM). Thus, noradrenaline (0.3 μM–3 μM) produced significantly greater responses in the presence of prazosin (0.1 μM) and forskolin (1 μM) (<30% of the control maximum) than in the presence of rauwolscine (1 μM) and forskolin (1 μM), i.e. the two CRCs were not superimposable (cf. Figure 3a).

**Table 3** Comparison of the effect of rauwolscine and prazosin against noradrenaline-induced contractions of the porcine isolated palmar lateral vein in presence of either rolipram or forskolin

| | *Mean log agonist concentration-ratio** | |
|---|---|---|
| Prazosin 0.1 μM + rolipram 10 μM | 2.01 ± 0.21 | ($n$ = 11) |
| Rauwolscine 1 μM + rolipram 10 μM | 1.96 ± 0.16 | ($n$ = 10) |
| Prazosin 0.1 μM + forskolin 1 μM | 1.91 ± 0.30 | ($n$ = 6) |
| Rauwolscine 1 μM + forskolin 1 μM | 2.34 ± 0.09 | ($n$ = 6) |

*Mean values determined at the 40% level of the control maximum response to noradrenaline.

### *Modulation of cellular cyclic AMP*

Basal levels of [$^3$H]-cyclic AMP (after correction for recovery from the Dowex/alumina columns and uptake into the vessels) in 4–5 mm segments of the porcine isolated palmar lateral vein were 1935 ± 326 d.p.m. ($n$ = 8). Neither noradrenaline (1 nM–100 μM), UK-14304 (1 nM–100 μM), rauwolscine (1 μM) nor prazosin (1 μM) affected basal levels of [$^3$H]-cyclic AMP. In contrast, rolipram (10 μM) produced a 1.7 fold increase in [$^3$H]-cyclic AMP (basal: 1823 ± 57 d.p.m.; 10 μM rolipram 3088 ± 229, $n$ = 3) and forskolin a 2–3 fold increase in [$^3$H]-cyclic AMP (Figure 4). In the absence of the phosphodiesterase inhibitor, rolipram, the forskolin-stimulated elevation of [$^3$H]-cyclic AMP peaked after 5 min (unpublished observations), hence in all subsequent experiments reactions were stopped after this time.

As shown in Figure 4, noradrenaline (1 nM–10 μM) and UK-14304 (1 nM–10 μM) produced a concentration-depen-

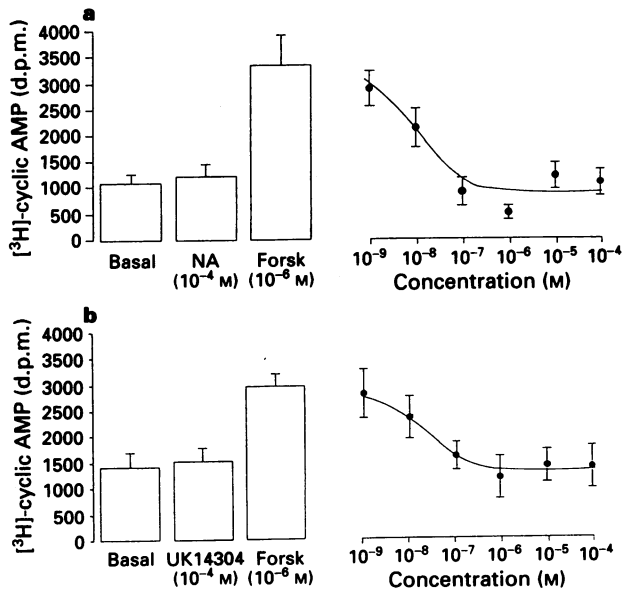


**Figure 4** Effect of (a) noradrenaline (NA) and (b) UK-14304 on basal and forskolin (Forsk) stimulated [$^3$H]-cyclic AMP accumulation in the porcine isolated palmar lateral vein. NA was added 10 min prior to forskolin which was incubated for a 5 min period. The figures represent the results from single experiments carried out in quadruplicate, with data expressed as mean ± s.e.mean for that particular experiment (to illustrate intra-assay variation).

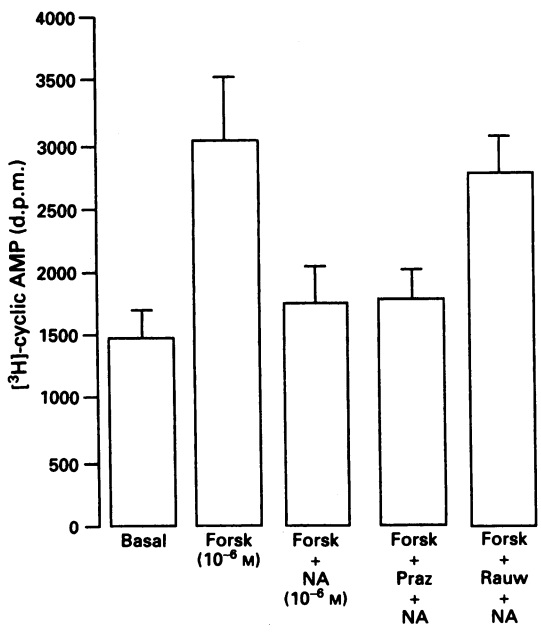


**Figure 5** Effect of prazosin (Praz) and rauwolscine (Rauw) on noradrenaline (NA)-induced inhibition of forskolin (Forsk)-stimulated [$^3$H]-cyclic AMP accumulation in the porcine isolated palmar lateral vein. Preparations were exposed to NA for 10 min prior to the addition of forskolin, and the experiment terminated 5 min later. Antagonists were added 10 min prior to NA. The figure represents the results from a single experiment carried out in quadruplicate, with data expressed as mean ± s.e.mean for that particular experiment (to illustrate intra-assay variation). This experiment was repeated on two other occasions with similar results.

dent inhibition of forskolin (1 μM)-stimulated [$^3$H]-cyclic AMP, with a p$K_i$ of 7.43 ± 0.10 ($n$ = 3) and 7.97 ± 0.18 ($n$ = 3), respectively. Both agonists completely reversed the effect of forskolin. Rauwolscine (1 μM), but not prazosin (0.1 μM), inhibited the effect of noradrenaline (1 μM) on forskolin-induced elevation of [$^3$H]-cyclic AMP (Figure 5). In contrast, UK-14304 failed to reverse rolipram-induced elevation of [$^3$H]-cyclic AMP formation (Figure 6).

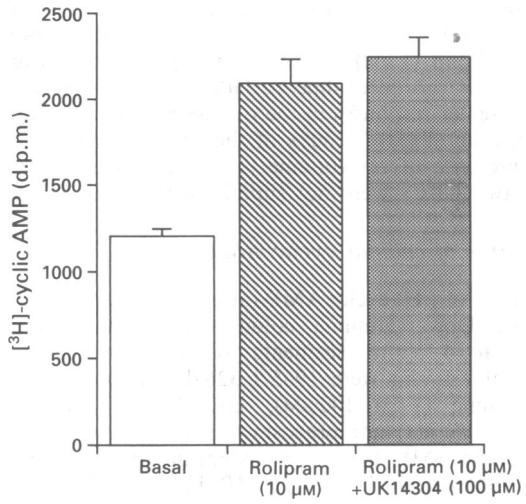


**Figure 6** The effect of UK-14304 (100 μM) on rolipram-stimulated [$^3$H]-cyclic AMP accumulation in the porcine isolated palmar lateral vein. Preparations were exposed to UK-14304 for 10 min prior to the addition of rolipram, and the experiment terminated 5 min later. The figure represents the results from a single experiment carried out in quadruplicate, with data expressed as mean ± s.e.mean for that particular experiment (to illustrate intra-assay variation). This experiment was repeated on two other occasions with similar results.

## Discussion

In the present study several observations indicate the presence of vasoconstrictor $\alpha_1$- and $\alpha_2$-adrenoceptors on smooth muscle cells of the porcine isolated palmar lateral vein. First, prazosin (0.1 μM) and rauwolscine (1 μM), antagonists selective for $\alpha_1$-adrenoceptors and $\alpha_2$-adrenoceptors, respectively (McGrath *et al.*, 1989), produced a 10 fold rightward displacement of the noradrenaline CRC, while the effect of the combination of the two receptor antagonists was greater than predicted for antagonists acting at a common site (see Shankley *et al.*, 1988). Secondly, the selective $\alpha_2$-adrenoceptor agonist, UK-14304, was 3 fold more potent than noradrenaline but produced a maximum response equivalent to 50% of the maximum response to noradrenaline. Thirdly, prazosin (0.1 μM) failed to affect contractile responses to UK-14304 and rauwolscine (1 μM) was more potent against responses to UK-14304 than those to noradrenaline (see Table 1). The estimated log $K_B$ value for rauwolscine against UK-14304 (8.29) is consistent with an effect at $\alpha_2$-adrenoceptors (Wilson *et al.*, 1991). Finally, it is noteworthy that a similar profile for the effects of the antagonists against noradrenaline-induced contractions has been reported for human isolated superficial epigastric veins (Sjöberg *et al.*, 1987), rabbit isolated plantaris veins (Daly *et al.*, 1988a) and human isolated skin arteries (Borbujo *et al.*, 1989): preparations with a heterogeneous population of vasoconstrictor α-adrenoceptors. Thus, noradrenaline-induced contraction in the presence of prazosin (0.1 μM) and rauwolscine (1 μM) represent responses mediated via $\alpha_2$- and $\alpha_1$-adrenoceptors, respectively.

Although this study was primarily concerned with coupling mechanisms for post-junctional $\alpha_2$-adrenoceptors, the use of noradrenaline (in the presence of either 0.1 μM prazosin or 1 μM rauwolscine) affords an opportunity to compare the effects of interventions thought to elevate cyclic AMP against contractions via each α-adrenoceptor subtype. Most previous

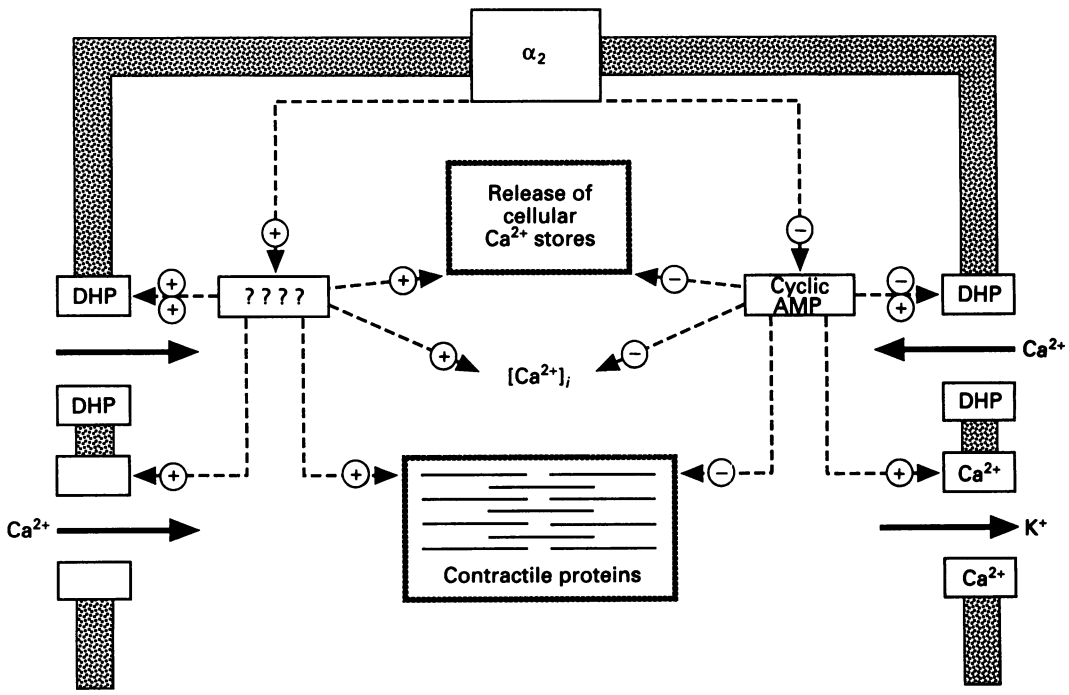


**Figure 7** Schematic diagram for possible pathways associated with $\alpha_2$-adrenoceptor-mediated contractions of vascular smooth muscle. Since $\alpha_2$-adrenoceptors in the porcine isolated palmar lateral vein failed to affect basal levels of cyclic AMP, it is unlikely that this pathway is involved in the initiation of contractile responses *in vitro*. Another signalling pathway is proposed to account for the pharmacological characteristics of $\alpha_2$-adrenoceptors mediated in variety of isolated blood vessels, i.e. the involvement of cellular bound calcium (Guan *et al.*, 1989; Daly *et al.*, 1990), dihydropyridine-sensitive and dihydropyridine-resistant $Ca^{2+}$ channels (Arner *et al.*, 1988; Dunn *et al.*, 1991) and modulation of the sensitivity of the contractile proteins to free intracellular calcium ions (Arburto *et al.*, 1993). In this proposed model, the inhibitory effect of $\alpha_2$-adrenoceptors on cyclic AMP formation would become important for contractile events only after elevation of this cyclic nucleotide (perhaps by β-adrenoceptor activation).

studies using agents affecting cellular cyclic AMP have involved pre-constriction of the blood vessel followed by exposure to increasing concentrations of the dilator (Jones *et al.*, 1984; Suzuki *et al.*, 1988; Hessen & DeMey, 1990). However, submaximal $\alpha_1$- and $\alpha_2$-adrenoceptor-mediated contractions in the porcine isolated palmar lateral vein were not sustained and were subject to a slow fade. Similar observations have been reported for α-adrenoceptor-mediated contractions for the rabbit isolated saphenous vein (Daly *et al.*, 1988b). Thus, in order to make accurate measurement of contractile responses, it was necessary to examine the effect of increasing concentrations of noradrenaline in the presence and absence of a single concentration of the agent affecting cellular cyclic AMP. The agents employed to investigate the effect of elevating cellular cyclic AMP were rolipram, an inhibitor of $Ca^{2+}$/calmodulin-independent, low $K_m$ phosphodiesterase insensitive to cyclic GMP inhibition (Challis & Nicholson, 1990) and forskolin, a direct activator of adenylyl cyclase (Seamon & Daly, 1986).

Rolipram (10 μM) produced a 2–3 fold rightward displacement of the noradrenaline CRC. In one series of experiments this was associated with a reduction in the maximum response, but in a later (and larger) series of experiments the maximum response was unaffected. The basis of this difference is not known. A similar effect was observed with forskolin (1 μM), although this was not associated with a reduction in the maximum response to noradrenaline. Both agents caused a reduction in resting tone of segments of the palmar lateral vein, probably related to the 2–3 fold elevation in cellular cyclic AMP, which demonstrates the presence of myogenic tone in this venous preparation. Support for the involvement of changes in cellular cyclic AMP in the inhibitory effect of rolipram and forskolin was provided by two other observations. First, 1,9-dideoxyforskolin, an analogue of forskolin which does not activate adenylyl cyclase (Seamon & Daly, 1986), failed to relax the blood vessel in the resting state or produce a significant displacement of the noradrenaline CRC. Secondly, forskolin (1 μM) and rolipram (10 μM) produced a greater inhibitory effect than either agent alone; an effect consistent with activation of adenylyl cyclase and inhibition of phosphodiesterase activity.

Rolipram (10 μM) and forskolin (1 μM) caused a similar (2–3 fold) increase in [$^3$H]-cyclic AMP, indicating that this pool of the cyclic nucleotide is of functional importance. Significantly, noradrenaline and the selective $\alpha_2$-adrenoceptor agonist, UK-14304, inhibited forskolin-stimulated cyclic AMP, and the potency for the two agonists was consistent with an effect at $\alpha_2$-adrenoceptors. Confirmation of the adrenoceptor subtype implicated was provided by the finding that the inhibitory effect of noradrenaline was reversed by rauwolscine but not by prazosin. In marked contrast to the effects observed with forskolin, elevation of [$^3$H]-cyclic AMP accumulation by rolipram was not reduced by $\alpha_2$-adrenoceptor activation. Since the effect of the phosphodiesterase inhibitor is probably a reflection of the basal turnover of cyclic AMP, these observations raise the possibility that basal turnover of [$^3$H]-cyclic AMP and forskolin-stimulated [$^3$H]-cyclic AMP represent different pools of the cyclic nucleotide. Furthermore, since neither noradrenaline nor UK-14304 affected basal accumulation of cellular [$^3$H]-cyclic AMP, this suggests that inhibition of adenylyl cyclase *per se* is not implicated in the initiation of $\alpha_2$-adrenoceptors *in vitro*. It should be noted, however, that our findings contrast with those of Stubbs *et al.* (1988), who reported that activation of $\alpha_2$-adrenoceptors in the rat isolated femoral artery caused a 60–70% reduction in total cyclic AMP. One explanation for the difference between the two studies is that we employed a pre-labelling assay for [$^3$H]-cyclic AMP, which tends to label preferentially fast-turnover (and presumably physiologically-important) pools of cyclic AMP, while Stubbs *et al.* (1988) used a method to measure total tissue cyclic AMP. Experiments are in progress to examine the possibility that a slow-turnover pool of cyclic AMP makes a significant contribution to the regulation of tone in the porcine isolated palmar lateral vein.

Interestingly, significant differences were detected in the functional consequences of elevation of cellular cyclic AMP. Although $\alpha_1$- and $\alpha_2$-adrenoceptor-mediated contractions (in the presence of 1 μM rauwolscine and 0.1 μM prazosin, respectively) were similarly sensitive to rolipram, $\alpha_2$-adrenoceptors-mediated contractions were less affected by forskolin than those produced by $\alpha_1$-adrenoceptors. These observations are consistent with the biochemical findings that $\alpha_2$-adrenoceptors can inhibit forskolin-mediated elevation in cyclic AMP but not that produced by rolipram, and demonstrates that, under the appropriate circumstances, inhibition of cyclic AMP formation by $\alpha_2$-adrenoceptors may be functionally important. Interestingly, the $\alpha_2$-adrenoceptor agonist, UK-14304, has been shown to reverse the vasodilator effects of low concentrations of forskolin ($<0.1$ μM) against agonist-induced tone in the rat isolated tail artery (Aidulis *et al.*, 1992) and bovine isolated pulmonary artery (Clayton *et al.*, 1993). However, direct comparison with the present study is not possible because neither the biochemical measurements nor the corresponding experiment with an $\alpha_1$-adrenoceptor agonist were undertaken.

Clearly, the physiological significance of this intracellular signal will be critically dependent upon the prevailing level of cellular cyclic AMP, which has the potential to influence vascular tone by affecting plasma membrane ion channels, the contractile protein or sarcoplasmic reticulum calcium stores (Bülbring & Tomita, 1987 and see Figure 7). For example, Sadoshima *et al.* (1989) reported that forskolin, isoprenaline and dibutyryl cyclic AMP increased the probability of opening of $Ca^{2+}$-activated $K^+$ channels in cultured smooth muscle cells of the rat aorta, even at resting levels of intracellular $Ca^{2+}$ ion concentration. This effect could account for the small relaxations produced by rolipram and forskolin in resting segments of the porcine isolated palmar lateral vein. Also, Shafiq *et al.* (1992) have shown that NKH 477 (0.1 μM), a water-soluble derivative of forskolin, produced only a 5 fold increase in cyclic AMP but reduced the sensitivity of porcine isolated coronary artery contractile proteins to calcium ions. This effect explains the finding that elevation in cyclic AMP increases the intracellular calcium ion concentration elicited by KCl, probably by enhancing dihydropyridine-sensitive $Ca^{2+}$ channels (Fukumitsu *et al.*, 1990; Ishikawa *et al.*, 1993), but reduces the magnitude of contractile responses (Shafiq *et al.*, 1992). Finally, the ability of cyclic AMP to reduce the release of $Ca^{2+}$ ions from intracellular stores (Itoh *et al.*, 1982; Xuan *et al.*, 1991; Ito *et al.*, 1993) would preferentially inhibit $\alpha_1$-adrenoceptor-mediated contractions, particularly if, as observed in other isolated blood vessels (Daly *et al.*, 1990), contractions via this subtype are more dependent upon the release of intracellular calcium. A detailed examination of the role of intracellular and extracellular calcium in $\alpha_1$- and $\alpha_2$-adrenoceptor-mediated contractions of the porcine isolated palmar lateral vein may indicate whether this latter effect is important in the action of forskolin (see Figure 7).

The observations in the present study also provides insight into the excitation-contraction coupling mechanism for vascular neuropeptide Y (NPY) receptors (Fredholm *et al.*, 1985) and 5-$HT_1$-like receptors (Sumner *et al.*, 1992). Sumner and coworkers (1992) demonstrated that 5-$HT_1$ receptor-mediated vasoconstriction in the dog isolated saphenous vein is entirely dependent upon the presence of extracellular calcium ions and is associated with a reduction in both basal and forskolin-stimulated cyclic AMP. However, since dideoxyadenosine also reduced forskolin-stimulated cyclic AMP without evoking a contractile response, these authors concluded that inhibition of cyclic AMP formation is not directly responsible for the contractile responses. As illustrated by our results with $\alpha_1$- and $\alpha_2$-adrenoceptor-mediated contractions, it remains possible that contractions via 5-$HT_1$ receptors are more resistant to interventions that elevate

cyclic AMP than are responses produced by 5-$HT_2$ receptor activation; a receptor system directly coupled to phosphoinositide metabolism.

In conclusion we have demonstrated that $\alpha_2$-adrenoceptors in the porcine isolated palmar lateral vein are negatively coupled to cyclic AMP formation and produce an overt constrictor response. However, since basal levels of cyclic AMP were unaffected by $\alpha_2$-adrenoceptor agonists, inhibition of adenylyl cyclase does not appear to be responsible for the initiation of the contractile response. Elevation of cyclic AMP following inhibition of phosphodiesterase failed to discriminate between $\alpha_1$- and $\alpha_2$-adrenoceptor-mediated contractions, but direct activation of adenylyl cyclase with forskolin resulted in greater inhibition of $\alpha_1$-adrenoceptor responses. It seems likely, therefore, that the basis of this difference in the effect of forskolin is due to the ability of $\alpha_2$-adrenoceptors to reduce adenylyl cyclase activity. Future experiments will be directed towards identifying the signal responsible for initiating $\alpha_2$-adrenoceptor-mediated contractions and determining its role in regulating the release of cellular-bound calcium ions and the entry of extracellular calcium ions (see Figure 7).

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# Contractility of urinary bladder and vas deferens after sensory denervation by capsaicin treatment of newborn rats

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1 Capsaicin, a selective sensory neurotoxin, was given to newborn rats and at the age of 3 months the contractile activity of the urinary bladder detrusor muscle and vas deferens evoked by either electrical field stimulation (EFS) or exogenous adenosine 5'-triphosphate (ATP) and carbachol (urinary bladder), or ATP and noradrenaline (vas deferens) were tested.

2 EFS of the urinary bladder evoked contractions which consisted of cholinergic and purinergic components, since they could be partially blocked by either the muscarinic cholinoceptor antagonist, atropine (0.3 μM) or by desensitization of $P_{2X}$-purinoceptors with α,β-methylene ATP (10 μM). In capsaicin-treated rats, contractions of the urinary bladder evoked by EFS were significantly larger than those of control (vehicle-treated) animals, and this difference remained after the purinergic component of the contractions was blocked by desensitization of $P_{2X}$-purinoceptors with α,β-methylene ATP. However, when the cholinergic component of the contractions was blocked with atropine, the difference between the groups at 8 Hz and 16 Hz was abolished; EFS caused significantly larger contractions of the capsaicin-treated rat bladder only at frequencies of 2 Hz and 4 Hz.

3 EFS evoked contractions of the vas deferens consisted of adrenergic and purinergic components since they could be partially blocked by either the α-adrenoceptor antagonist, phentolamine (3 μM) or by α,β-methylene ATP (10 μM). The contractions of the vas deferens were significantly larger than in the capsaicin-treated rats only at a frequency of 16 Hz. There were no differences between vas deferens contractions of the two groups either after desensitization of $P_{2X}$-purinoceptors by α,β-methylene ATP or in the presence of phentolamine.

4 Contractions of the capsaicin-treated rat urinary bladder evoked by exogenous carbachol (0.1–100 μM) were not significantly different from those of controls, the $pD_2$ values being 1.78 ± 0.23 μM and 1.90 ± 0.20 μM respectively. There was also no significant difference between the groups in contractions of the bladder evoked by ATP (10 μM–3 mM).

5 Contractions of the vas deferens evoked by either ATP (10 μM–3 mM) or noradrenaline (1–1000 μM) in the capsaicin-treated group showed no significant difference between control and capsaicin-treated rats.

6 In conclusion, the present results indicate that chronic capsaicin treatment increases the amplitude of contractions of the rat urinary bladder, an effect which preferentially involves the cholinergic component of the response; since the response to carbachol is unaffected, the change involves prejunctional mechanisms. In contrast, both the purinergic and adrenergic components of contraction in the vas deferens are unaffected by capsaicin. It is suggested that sensory nerves have a trophic influence on the development of parasympathetic nerves in the rat bladder; removal of sensory nerves shortly after birth results in an increase mainly in the cholinergic, and to a lesser extent purinergic component.

**Keywords:** Capsaicin; ATP; carbachol; purinoceptors; cholinoceptors; electrical field stimulation; rat urinary bladder; rat vas deferens

## Introduction

There is increasing evidence showing that changes may occur in the expression of transmitters in the autonomic nerves that remain following selective autonomic denervation, illustrative of the plasticity of the autonomic nervous system (see Burnstock, 1990; 1991). For example, after selective denervation of sympathetic nerves by chronic guanethidine treatment, there is a marked increase in expression of calcitonin gene-related peptide (CGRP) in sensory nerves in rat mesenteric arteries (Aberdeen *et al.*, 1990) and in neuropeptide Y (NPY) in parasympathetic nerves in rat cerebral arteries and iris (Mione *et al.*, 1990). The innervation of the urinary bladder is complex; however, contractions of the bladder to electrical field stimulation (EFS) are mediated by a combination of acetylcholine and adenosine 5'-triphosphate (ATP) released probably as cotransmitters from parasympathetic nerves (Burnstock *et al.*, 1978; Hoyle & Burnstock, 1993). Contractions of the vas deferens to EFS are mediated by a combination of noradrenaline and ATP released as cotransmitters from sympathetic nerves (see Hoyle & Burnstock, 1991). Sensory innervation of the urinary bladder and vas deferens have been described (see Maggi & Meli, 1988; Anderson, 1993). Chemical denervation is one approach which has been used to study trophic interactions between these populations of nerves.

Capsaicin is widely used as a neurotoxic agent selective for primary afferent (sensory) neurones (Maggi & Meli, 1988; Holzer, 1991). The final effect depends on the dose and duration of capsaicin administration as well as the age of the animal. In newborn rats capsaicin treatment at a dose of 50 mg $kg^{-1}$ causes lifelong degeneration of primary afferent neurones which is associated with sensory deficit (Jancsó *et al.*, 1977; Nagy, 1982). Since the neuropeptides, substance P (SP) and CGRP, are depleted by capsaicin-treatment of newborn rats they have been ascribed a sensory neurotransmitter role (Geppetti *et al.*, 1988; Holzer, 1991). Capsaicin treatment of newborn rats significantly impairs the micturition reflex (Holzer-Petsche & Lembeck, 1984) and it is believed

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that capsaicin-sensitive nerves in the bladder are involved in regulation of this reflex (Maggi & Meli, 1988).

In the present study we have investigated the effect of selective chronic denervation of sensory nerves by systemic capsaicin administration on contractions of rat urinary bladder mediated by the parasympathetic nervous system and of the vas deferens mediated by the sympathetic nervous system.

## Methods

Male Sprague-Dawley rats were treated with capsaicin, 50 mg $kg^{-1}$ subcutaneously, dissolved in a vehicle of 80% saline, 10% Tween 80 and 10% ethanol on days 2, 3, 4, 7 and 14 of life. Control animals under the same conditions were treated only with the vehicle. At the age of 3 months rats were killed by inhalation of di-ethyl ether. The urinary bladder and vasa deferentia were removed and strips, approximately 15 mm long, were prepared. The strips were suspended vertically in 10 ml organ baths for isometric recording of mechanical activity. An initial load of 1 g was applied to the strips which were then allowed to equilibrate for at least 60 min, with the Krebs solution being changed every 15 min. The Krebs solution had the following composition (mM): NaCl 133, KCl 4.7, $NaHCO_3$ 16.4, $MgSO_4$ 0.6, $NaH_2PO_4$ 1.4, $CaCl_2$ 2.5, glucose 7.7, and was gassed with 95% $O_2$ and 5% $CO_2$ (pH 7.3–7.4) and maintained at 36 ± 1°C. Contractions were recorded by either a Dynamometer UF1 or Grass FTOC3 force-displacement transducer, and displayed on a Grass 79D ink-writing oscillograph.

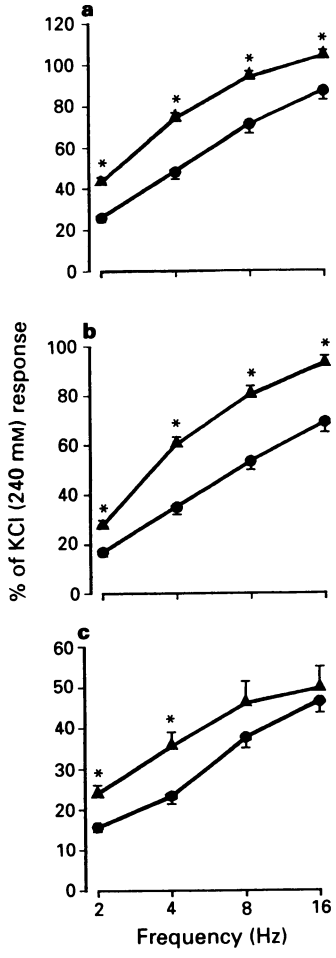


**Figure 1** Frequency-response relationships for electrical field stimulation in the urinary bladder of control (●) and capsaicin-treated (▲) rats. Responses obtained before adding any drugs (a; $n = 12$) and after incubation with either α,β-methylene ATP (10 μM, b; $n = 6$) or atropine (0.3 μM, c; $n = 6$). Responses are expressed as a percentage of contraction evoked by KCl, 240 mM. Points show mean with s.e.mean. $^*P < 0.05$.

EFS was applied via two platinum-wire rings 2.5 mm in diameter 10 mm apart through which the strip was threaded. Frequency-response relationships for urinary bladder were examined before and after incubation with either α,β-methylene ATP (10 μM) or atropine (1 μM) for at least 20 min. Frequency-response relationships for vas deferens were tested before and after incubation with either α,β-methylene ATP (10 μM) or phentolamine (3 μM) for at least 20 min. EFS was provided by a Grass S9 stimulator and applied at a given frequency (2–16 Hz) with a pulse width of 0.5 ms and supramaximal voltage, until a maximum contraction had been reached and the tone had declined by about a third (usually 2–3 s).

In experiments with exogenous agonists, single doses of ATP (10 μM–3 mM), carbachol (0.1–100 μM) or noradrenaline (1–1000 μM) were added directly to the organ bath and were washed out after a maximum contraction had been reached. Intervals of at least 10 min were allowed between consecutive applications of agonists for washout.

Only the frequency- or concentration-response relationship for one of the agonists (ATP, carbachol or noradrenaline) was obtained on one preparation before and after incubation with either α,β-methylene ATP, atropine or phentolamine.

All responses were calculated as a percentage of the contraction evoked by KCl at a concentration of 240 mM, which was added to all preparations at the end of each experiment. In preliminary experiments we tested only effects of KCl and expressed them as g per mg tissue used.

Means were compared by paired and unpaired $t$ tests as indicated in the figures. A probability of less than or equal to 0.05 was considered as significant. Data are presented as mean ± s.e.mean.

### *Drugs used*

Atropine sulphate was obtained from Phoenix Pharmaceuticals Ltd., phentolamine mesylate (Rogitine) was from Ciba, all other compounds were from Sigma.

## Results

There was no significant difference in the weight of capsaicin-treated compared to control rats: 448.5 ± 17.7 g ($n = 19$) and 455 ± 22.2 g ($n = 16$) respectively.

### *Contractions to KCl*

KCl at a concentration of 240 mM caused contractions of capsaicin-treated rat urinary bladder which were not significantly different from those of control rats, being 0.70 ± 0.07 g $mg^{-1}$ wet tissue ($n = 4$) and 0.63 ± 0.09 g $mg^{-1}$ wet tissue ($n = 4$) respectively. In vas deferens, contractions evoked by KCl at 240 mM were 0.12 ± 0.01 g $mg^{-1}$ wet tissue ($n = 4$) in control and 0.14 ± 0.01 g $mg^{-1}$ wet tissue ($n = 4$) in capsaicin-treated rats and these figures were also not significantly different ($P > 0.05$, unpaired $t$ test).

### *Urinary bladder*

EFS caused frequency-dependent contractions of the rat urinary bladder which were significantly larger in capsaicin-treated rats than in controls at any given frequency (Figure 1a, $P < 0.005$ or 0.001, unpaired $t$ test). Desensitization of $P_{2X}$-purinoceptors by α,β-methylene ATP (10 μM) significantly inhibited contractions of the bladder in both groups at all frequencies tested ($P < 0.01$, paired $t$ test). However, the contractions of capsaicin-treated rat urinary bladder remained significantly larger than those of the controls at any given

frequency (Figure 1b, $P<0.05$, unpaired $t$ test). Although the blockade of the cholinoceptors by atropine (1 μM) also decreased the contractions of the urinary bladder in both groups at all used frequencies, at the two higher frequencies (8 and 16 Hz) there were no significant differences between the two groups (Figure 1c, $P>0.05$, unpaired $t$ test). At frequencies of 2 Hz and 4 Hz the contractions of capsaicin-treated rat urinary bladders were significantly larger than those of the controls, being 23.8 ± 2.2% ($n=7$) and 15.7 ± 1.1% ($n=6$) at 2 Hz and 35.6 ± 3.4% ($n=7$) and 23.3 ± 2.0% ($n=6$) at 4 Hz, respectively ($P<0.01$ and $P<0.05$, unpaired $t$ test).

Carbachol (0.1–100 μM) caused concentration-dependent contractions of urinary bladder, which at concentrations of carbachol of 1 μM and above were not significantly different in control and capsaicin-treated animals (Figure 2a). At concentrations of 0.1 and 0.3 μM carbachol-evoked contractions of urinary bladder were significantly larger in capsaicin-treated rats than in controls ($P<0.05$, unpaired $t$ test). However, $pD_2$ values (negative log $EC_{50}$) for carbachol were not significantly different being 1.90 ± 0.20 μM in control and 1.78 ± 0.23 μM in the capsaicin-treated group.

ATP (10 μM–3 mM) caused concentration-dependent contractions of capsaicin-treated rat urinary bladder which were not significantly different at any concentration from those of controls (Figure 2b).

*Vas deferens*

EFS caused frequency-dependent contractions of vas deferens in both capsaicin- and vehicle-treated animals. At frequencies of 2–8 Hz there was no difference between the two groups, but at a frequency of 16 Hz contractions of the capsaicin-treated rat vas deferens were significantly larger than those of the controls being 84.8 ± 3.1% ($n=16$) and 71.8 ± 3.1% ($n=12$), respectively (Figure 3a, $P<0.01$, unpaired $t$ test). After incubation with α,β-methylene ATP, there was a significant decrease in contractions evoked by EFS at all frequencies in both groups ($P<0.001$, paired $t$ test). There was no significant difference in contractions of the vas deferens in the presence of α,β-methylene ATP between the groups at any given frequency (Figure 3b, $P>0.05$, unpaired $t$ test). In both the control and capsaicin-treated groups, incubation with the α-adrenoceptor blocker, phentolamine (3 μM), caused a significant inhibition of the vas deferens contraction at frequencies of 2 and 4 Hz and potentiation of the contraction at a frequency of 16 Hz compared with contractions obtained before incubation with phentolamine. However, at all the frequencies there was no difference between the two groups (Figure 3c, $P>0.05$, unpaired $t$ test). It should be noted that, at a frequency of 16 Hz, after incubation with either α,β-methylene ATP or phentolamine, the contractions of capsaicin-treated rat vas deferens tended to be larger than those of control ones.

Contractions of capsaicin-treated rat vas deferens evoked by either noradrenaline (1–1000 μM) or ATP (10 μM–3 mM) were not significantly different from those of controls (Figure 4a and b; $P>0.05$, unpaired $t$ test).

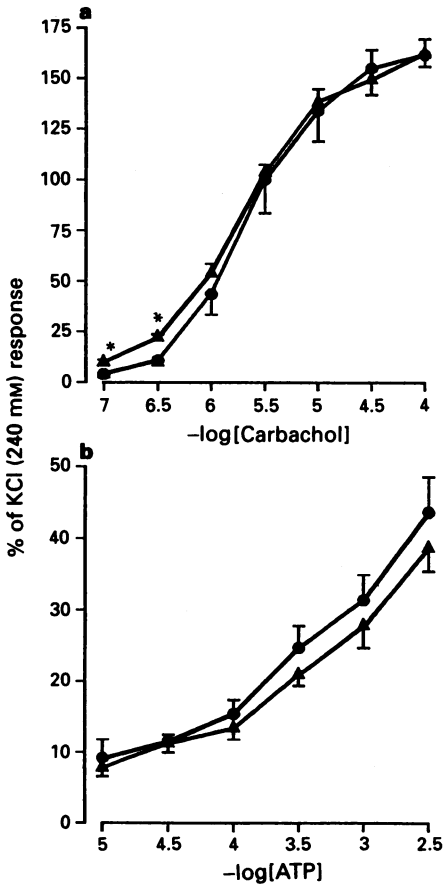


**Figure 2** Concentration-response relationships for carbachol (a) and ATP (b) in the urinary bladder of control (●) and capsaicin-treated rats (▲). Responses are expressed as a percentage of contractions evoked by KCl, 240 mM. Points show mean with s.e.mean ($n=5$). *$P<0.05$.

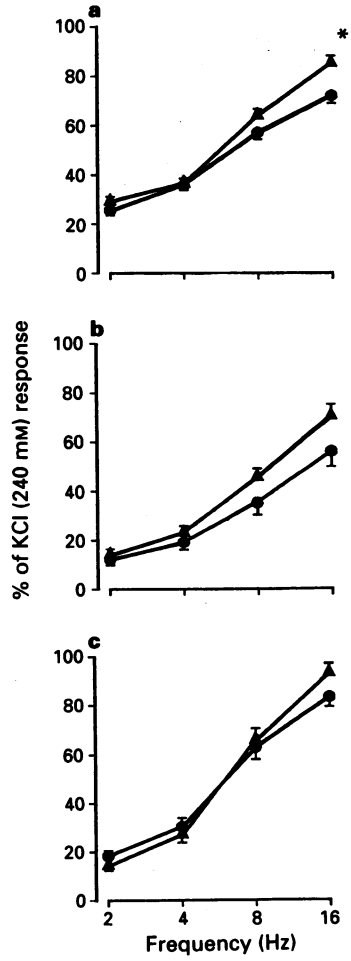


**Figure 3** Frequency-response relationships for electrical field stimulation in the vas deferens of control (●) and capsaicin-treated (▲) rats. Responses obtained before adding any drugs (a; $n=12$) and after incubation with either α,β-methylene ATP (10 μM, b; $n=6$) or phentolamine (3 μM, c; $n=6$). Responses are expressed as a percentage of contraction evoked by KCl, 240 mM. Points show mean with s.e.mean. *$P<0.05$.

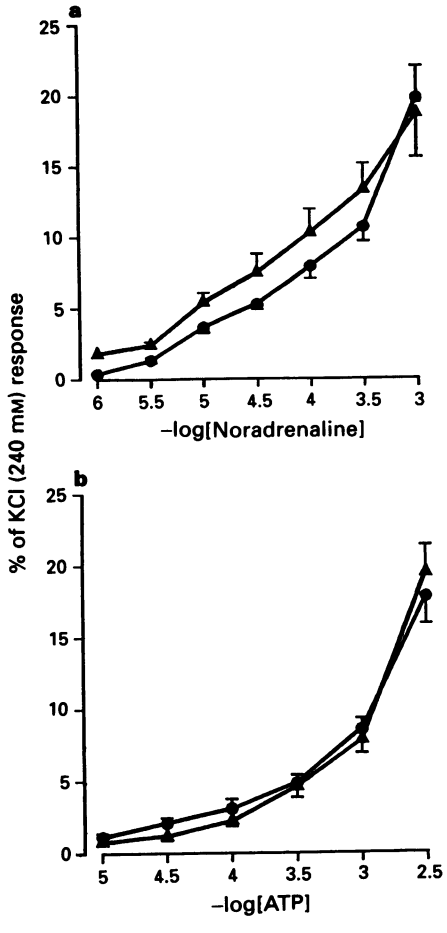


**Figure 4** Concentration-response relationships for noradrenaline (a) and ATP (b) in the vas deferens of control (●) and capsaicin-treated rats (▲). Responses are expressed as a percentage of contractions evoked by KCl, 240 mM. Points show mean with s.e.mean. ($n = 4$).

## Discussion

The results presented here show that capsaicin treatment of newborn rats to destroy selectively sensory afferents enhances the contractility of the urinary bladder at all frequencies of EFS, potentiates contractions only to low concentrations of carbachol and has no effect on contractions to ATP. Contractions of vas deferens evoked by exogenous ATP and noradrenaline, as well as by EFS (except at 16 Hz), are not affected by capsaicin.

Capsaicin treatment did not significantly affect contractile responses of urinary bladder and vas deferens to KCl, which indicates that there was no direct effect on smooth muscle cells. We have shown that contractions to EFS were significantly larger in capsaicin-treated rat urinary bladder. When we examined the two main components of these contractions, we found that enhancement of EFS-evoked contractions by capsaicin clearly involved the cholinergic component, since after desensitization of $P_{2X}$-purinoceptors by $\alpha,\beta$-methylene ATP the difference between control and capsaicin-treated groups remained the same at all the frequencies tested. Further, the purinergic component was also involved in an increase of contractility at frequencies of 2 and 4 Hz, since in the presence of atropine there were significant differences between the groups at these two frequencies. However, the concentration-response curve to either carbachol or ATP was not significantly shifted by capsaicin treatment, which indicates that potentiation of the contractile response is predominantly a prejunctional effect. Our results are consistent with those of Lee & Wong (1989) who found potentiation of responses to neurogenic stimulation, but not to carbachol, in the rat urinary bladder after newborn capsaicin treatment. On the other hand, Malmgren *et al.* (1990) showed that, after ten days capsaicin treatment of adult rats, not only the frequency-response curve to EFS but also the concentration-response curve to carbachol were significantly shifted to the left in the urinary bladder.

Similar responses to exogenous carbachol and ATP in control and capsaicin-treated rats excludes an involvement of postjunctional effects in capsaicin-potentiation of bladder contractions. At least three mechanisms may be involved in prejunctional augmentation of EFS-evoked contractions: (1) An increase in the number of parasympathetic nerves; (2) An increase in neurotransmitter(s) synthesis, storage, perhaps resulting in an increase of total amount of neurotransmitter released after nerve stimulation; (3) A change in prejunctional autoregulatory mechanisms. While the study did not address the mechanisms involved, it is intriguing that the change should mostly involve one of two coexisting transmitters, namely acetylcholine and ATP. The increase of the cholinergic component of the contractile response in rat bladder after capsaicin treatment may be regarded as a compensatory effect to replace the lack of sensory innervation.

Although the present study did not examine this, potentiation of bladder contraction by capsaicin may also involve a third, non-cholinergic, non-purinergic, component of neurogenic contraction. It has been shown that in the presence of atropine, indomethacin and desensitization of $P_{2X}$-purinoceptors, there is still some neurogenic contractile response to EFS in the rat urinary bladder, which is more prominent at lower frequencies of EFS than at higher frequencies (Luheshi & Zar, 1990). The nature of this component is as yet unclear, however, it is suggested that possibly neither substance P (SP) nor CGRP can be considered as excitatory neurotransmitters in this third component since both these peptides are depleted by capsaicin treatment (Holzer, 1991). Further studies in the presence of both atropine and $\alpha,\beta$-methylene ATP are required to determine the precise involvement, if any, of this third component in potentiation of bladder contractions after long-term sensory denervation.

The physiological implications of increased responses to EFS after sensory-motor denervation are as yet unclear. It has been shown that parasympathetic cholinergic innervation of the rat urinary bladder takes part in the rat voiding efficiency (Maggi *et al.*, 1989). A significant role of capsaicin-sensitive afferent nerves in the micturition reflex has also been observed (Holzer-Petsche & Lembeck, 1984; Maggi & Meli, 1988). However, while the involvement of SP and other tachykinins in this reflex had been proposed (Sharkey *et al.*, 1983; Maggi *et al.*, 1991), CGRP had no significant effect on rat urinary bladder motility (Maggi *et al.*, 1987b). Moreover, when capsaicin was given to adult rats there were no or very few changes in function of the urinary bladder (Maggi *et al.*, 1987a; 1989; Malmgren *et al.*, 1990), which is believed to be due to a different sensitivity of newborn and adult rat afferents to capsaicin. Thus, capsaicin-induced bladder dysfunction can be the consequence of multi-source disorders of sensory-motor innervation, and an involvement of cholinergic neurotransmission is very possible.

It has been shown that capsaicin treatment of newborn rats causes a significant depletion of CGRP-like immunoreactivity and SP-like immunoreactivity in the vas deferens (Geppetti *et al.*, 1988). In mammalian vas deferens the predominant excitatory neurotransmitters are noradrenaline and ATP (Hoyle & Burnstock, 1991). We were interested to see if there is any change in contractile activity of the vas deferens *in vitro* after capsaicin treatment, and, to our knowledge, the present study is the first to investigate the effect of systemic capsaicin of newborn rats on contractility of the vas deferens. We found that only at the highest frequency used (16 Hz), was contraction of capsaicin-treated rat vas deferens significantly greater than that of controls. This enhanced contraction was likely to involve both puriner-

gic and adrenergic components, since after either blockade of α-adrenoceptors by phentolamine or desensitization of $P_{2X}$-purinoceptors by α,β-methylene ATP, the contractions at 16 Hz tended to be larger in the capsaicin-treated group than in controls. There were no significant differences between the groups at any other frequency. Thus, capsaicin does not significantly affect contractility of the rat vas deferens, and in particular has no effect on responses mediated via α-adrenoceptors or $P_{2X}$-purinoceptors. The marked difference in the effect of capsaicin on the two tissues studied may be because sensory innervation is substantially higher in the bladder than in the vas deferens (Maggi & Meli, 1988; Anderson, 1993).

In conclusion, the present study shows that sensory denervation by systemic capsaicin treatment of newborn rats causes a marked potentiation of contractile responses of the urinary bladder (predominantly due to an increase in the cholinergic component of the response), but not the vas deferens. Sensory nerves may have a trophic role in the development of other autonomic nerve types, influencing mechanisms of synthesis, storage and/or release of transmitter.

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# Endothelin-1 and endothelin-3 regulate differently vasoconstrictor responses of smooth muscle of the porcine coronary artery

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**1** Using front-surface fluorometry of fura-2 and medial strips of the porcine coronary artery, we investigated mechanisms by which endothelin-1 (ET-1) and ET-3 function as vasoconstrictors.

**2** In the presence of extracellular $Ca^{2+}$(1.25 mM), ET-1 ($10^{-10}$–$10^{-7}$ M) increased cytosolic $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) and tension, in a concentration-dependent manner. ET-1, at concentrations greater than $10^{-8}$ M, induced an abrupt elevation of $[Ca^{2+}]_i$ which reached a transient peak (the first component, $[Ca^{2+}]_i$-rising phase) and subsequently declined ($[Ca^{2+}]_i$-declining phase) to reach a lower sustained phase (the second component, steady-state phase), while the tension rose monotonically to reach a peak and then slightly and gradually declined. ET-1, at concentrations lower than $10^{-8}$ M, induced slowly developing and sustained increases in $[Ca^{2+}]_i$ and tension ($[Ca^{2+}]_i$-rising phase followed by steady-state phase). All concentrations of ET-1 increased tension more slowly than $[Ca^{2+}]_i$.

**3** In the presence of extracellular $Ca^{2+}$, ET-3 ($10^{-8}$–$10^{-5}$ M) induced concentration-dependent increases in $[Ca^{2+}]_i$ and tension. However, the maximal elevations of $[Ca^{2+}]_i$ and tension induced by ET-3 were substantially smaller than those induced by ET-1, indicating the involvement of an $ET_A$ receptor subtype. ET-3, at concentrations greater than $6 \times 10^{-7}$ M, caused biphasic slowly developing increases in $[Ca^{2+}]_i$ and tension. At concentrations lower than $10^{-6}$ M, ET-3 caused monophasic increases in $[Ca^{2+}]_i$ and tension. At all concentrations of ET-3, the time courses of increases in $[Ca^{2+}]_i$ and tension were similar.

**4** The biphasic increases in $[Ca^{2+}]_i$ and tension induced by $10^{-5}$ M ET-3 and by $10^{-7}$ M ET-1 were significantly inhibited by pretreatment with $10^{-5}$ M of the $Ca^{2+}$ entry blocker, diltiazem, although the inhibition of the first component of ET-1-induced $[Ca^{2+}]_i$ increase was partial.

**5** In the absence of extracellular $Ca^{2+}$, ET-1 induced a concentration-dependent transient increase in $[Ca^{2+}]_i$, possibly due to release of $Ca^{2+}$ from intracellular stores, and a sustained contraction. In contrast, ET-3 ($\geq 10^{-6}$ M) caused little, if any, transient increase in $[Ca^{2+}]_i$ and a small sustained contraction.

**6** Temporal changes in the relationships between $[Ca^{2+}]_i$ and tension ($[Ca^{2+}]_i$-tension relationship) during contractions induced by ET-1 and ET-3 were compared with the $[Ca^{2+}]_i$-tension relationship of $Ca^{2+}$-induced contractions ($Ca^{2+}$-contractions) obtained by cumulative applications of extracellular $Ca^{2+}$ (0–7.5 mM) to tissues depolarized in the presence of 118 mM $K^+$. In the $[Ca^{2+}]_i$-rising phase, ET-1 increased tension more slowly than $[Ca^{2+}]_i$, thereby shifting the $[Ca^{2+}]_i$-tension relation to the right from that for $Ca^{2+}$-contractions. In the $[Ca^{2+}]_i$-declining and the steady-state phases, ET-1, at concentrations higher than $10^{-9}$ M, produced greater tension development than that expected from a given change in $[Ca^{2+}]_i$, resulting in a leftward shift of the $[Ca^{2+}]_i$-tension relation. During ET-3-induced contractions, ($[Ca^{2+}]_i$-rising, $[Ca^{2+}]_i$-declining and steady-state phases), the $[Ca^{2+}]_i$-tension relation was similar to that of $Ca^{2+}$-contractions.

**7** BQ-123, a selective $ET_A$ receptor antagonist, completely inhibited the increases in $[Ca^{2+}]_i$ and tension induced by ET-1 and ET-3.

**8** These results suggest: (1) That ET-1 elicits vasoconstriction by increasing $[Ca^{2+}]_i$ through the activation of $Ca^{2+}$ influx from the extracellular space and $Ca^{2+}$ release from intracellular storage sites, and by increasing the $Ca^{2+}$ sensitivity of the contractile apparatus, whereas ET-3 induces vasoconstriction by increasing $[Ca^{2+}]_i$ mainly through $Ca^{2+}$ influx from the extracellular space. (2) Distinct mechanisms of time-dependent modulation of the $Ca^{2+}$ sensitivity function in the vasoconstrictor responses to ET-1 and ET-3. (3) That both ET-1- and ET-3-induced contractions seem to be mediated via $ET_A$-receptors in porcine coronary artery, and that the $ET_A$-receptor-mediated effects of ET-1 and ET-3 can be dissociated at the sub-receptor levels of the signal transduction pathway.

**Keywords:** Endothelin-1, endothelin-3; cytosolic calcium concentration; vascular smooth muscle

## Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide synthesized by the vascular endothelium (Yanagisawa *et al.*, 1988) and its isopeptides also exist in mammalian tissues (Inoue *et al.*, 1989). ET-3, which differs from ET-1 at positions 2, 4, 5, 6, 7 and 14, is not expressed by vascular endothelial cells and its role in vascular tone regulation, especially in relation to vasoconstriction, is uncertain.

ET-1 elicits vasoconstriction mainly through three distinct signal transduction pathways; (1) An increase in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) through activation, either direct or indirect, of L-type $Ca^{2+}$ channels (Van Renterghem *et al.*, 1988; Yanagisawa *et al.*, 1988; Goto *et al.*, 1989; Kasuya *et al.*, 1989) or of a non-selective cationic channel that is perm-

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eable to $Ca^{2+}$ (Van Renterghem *et al.*, 1988), (2) Mobilization of stored $Ca^{2+}$ (Miasiro *et al.*, 1988) through activation of phospholipase C (Resinik *et al.*, 1988; Takuwa *et al.*, 1990), and (3) $Ca^{2+}$-independent mechanisms (Kodama *et al.*, 1989) involving enhancement of $Ca^{2+}$ sensitivity of the contractile apparatus (Sakata *et al.*, 1989; Nishimura *et al.*, 1992). The effectors of these pathways are coupled to the receptors via G proteins (Takuwa *et al.*, 1990l; Kasuya *et al.*, 1992). Intracellular signalling pathways through which ET-3 induces smooth muscle contraction are not well understood.

Two distinct ET receptors have been identified and cloned, one type, termed $ET_A$ (Arai *et al.*, 1990) has a higher affinity for ET-1 than for ET-3. The other, termed $ET_B$ (Sakurai *et al.*, 1990) has a similar affinity for ET-1 and ET-3. In intact vascular strips, activation of the $ET_A$ receptor on vascular smooth muscle cells (Arai *et al.*, 1990) induces vasoconstriction whereas activation of the $ET_B$ receptor on vascular endothelial cells (Sakurai *et al.*, 1990) releases endothelium-derived relaxing factor, resulting in vasorelaxation (Ushio-Fukai *et al.*, 1992). However, using BQ-123, a selective antagonist for the $ET_A$ receptor subtype (Ihara *et al.*, 1992), it was found that non-$ET_A$ (probably $ET_B$) receptors might also mediate vasoconstriction. These reports raise questions as to which receptor subtypes are actually involved in ET-1 and ET-3-induced contractions in the coronary artery of the pig.

$[Ca^{2+}]_i$ plays a major role in excitation-contraction coupling in vascular smooth muscle (Kuriyama *et al.*, 1982). However, some agonists can increase the $Ca^{2+}$ sensitivity of the contractile apparatus, which is shown by a greater than expected tension development for given changes in $[Ca^{2+}]_i$ when compared to the changes following membrane depolarization with solutions enriched in $K^+$ (Morgan & Morgan, 1984; Rembold & Murphy, 1988; Kodama *et al.*, 1989; Abe *et al.*, 1990). Using vascular smooth muscle permeabilized with Staphylococcal α-toxin (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989) and β-estin (Kobayashi *et al.*, 1989), it was demonstrated that enhanced $Ca^{2+}$ sensitivity is mediated by receptor coupled G-protein activation. In addition to the steady-state relation between $[Ca^{2+}]_i$ and tension, the relationship between $[Ca^{2+}]_i$ and tension ($[Ca^{2+}]_i$-tension relationship) during contractions varies with time, because of the presence of a time-dependent activation and inactivation of myosin light chain phosphatase (Kamm & Stull, 1985), an 'economic' low phosphorylation-high force state ('latch') (Murphy, 1989), and cooperative re-attachment of unphosphorylated crossbridges (Somlyo & Somlyo, 1990), in vascular smooth muscle. The extent of these changes depends on the agonist (Himpens *et al.*, 1990), the intensity of stimulation (Himpens & Casteels, 1990), and on the speed of increase in $[Ca^{2+}]_i$ (Yagi *et al.*, 1988). Thus, analyses of temporal changes in the $[Ca^{2+}]_i$-tension relationship during contractions (Himpens *et al.*, 1990; Hirano *et al.*, 1991) may be a useful way to characterize the mode of time-dependent modulation of $Ca^{2+}$-sensitivity during ET-1 and ET-3-induced contractions.

We report here characteristics of the contractile actions of both ET-1 and ET-3. Using front-surface fluorometry of the fura-2-loaded porcine coronary arterial strip, we examined the effects of ET-1 and ET-3 on $[Ca^{2+}]_i$ and tension in the presence and absence of extracellular $Ca^{2+}$, and determined the time-dependent changes in the $[Ca^{2+}]_i$-tension relationship during ET-1 and ET-3-induced contractions.

## Methods

### *Tissue preparation and fura-2 loading*

Hearts from adult pigs of either sex were obtained from a local slaughterhouse immediately after the animals had been killed. The hearts were placed in ice-cold physiological salt solution (PSS) and transferred to our laboratory. Left circumflex coronary arteries were isolated and longitudinal segments excised 2–3 cm from the origin. The endothelium was rubbed off with a cotton swab. After removal of the adventitia, medial preparations were cut into circular strips (1 × 5 mm).

The strips were loaded with the $Ca^{2+}$ indicator dye, fura-2, by incubating in oxygenated (a mixture of 95% $O_2$ and 5% $CO_2$) Dulbecco's modified Eagle's medium containing 25 μM fura-2/AM (an acetoxymethyl ester form of fura-2) and 2.5% foetal bovine serum for 3–4 h at 37°C. The strips were then rinsed with normal PSS for at least 60 min at 37°C to remove the dye remaining in the extracellular space and to equilibrate the strips before starting the measurements. Loading the strips with fura-2 did not affect contractility (Hirano *et al.*, 1990).

### *Simultaneous measurement of force and $[Ca^{2+}]_i$*

Isometric force developed by the strips mounted vertically in a quartz organ bath was measured via a strain gauge transducer (TB-612-T, Nihon Kohden, Japan), simultaneously with $[Ca^{2+}]_i$. During 1 h of the equilibration period, the strips were stimulated with 118 mM $K^+$ every 15 min and the resting tension was increased stepwise. After equilibration, the resting tension was adjusted to 250 mg. Reproducible contractions of each strip to 118 mM $K^+$-depolarization were obtained before starting the experiment. The developed tension was expressed as a percentage, assuming the steady-state values in normal (5.9 mM $K^+$) and 118 mM $K^+$ PSS to be 0% and 100%, respectively.

Changes in fluorescence intensity of the fura-2-$Ca^{2+}$ complex were monitored with front-surface fluorometry (Abe *et al.*, 1990), using a fluoremeter specifically designed for fura-2 fluorometry, developed by us in collaboration with Japan Spectroscopic Co., Tokyo, Japan.

In brief, strips were illuminated by guiding the alternating (400 Hz) 340 and 380 nm excitation light from a xenon light source through quartz optic fibres arranged in a circle (diameter = 3 mm). Surface fluorescence of strips was collected by glass optic fibres arranged in an outer circle (diameter = 7 mm) and introduced into a photomultiplier through a 500 nm bandpass filter (bandwidth = 10 nm). The ratio of the fluorescence intensities at 340 nm excitation to those at 380 nm excitation were monitored and expressed as a percentage, assuming the steady-state values in normal (5.9 mM $K^+$) and 118 mM $K^+$ PSS to be 0% and 100%, respectively. Response of $[Ca^{2+}]_i$ to 118 mM $K^+$-depolarization was obtained before starting the experiment. When the absolute values of $[Ca^{2+}]_i$ in normal and 118 mM $K^+$ PSS were determined separately, using the equation of Grynkiewicz *et al.* (1985) with minor modification and a $K_d$ value of 224 nM at 37°C, the mean values of $[Ca^{2+}]_i$ at normal PSS (at rest; 0% and 118 mM $K^+$-depolarization (100%) were 105.5 ± 3.8 nM ($n$ = 10) and 718.2 ± 3.2 nM ($n$ = 10), respectively. Since it is necessary to permeabilize the cell membrane destructively with Ionomycin in this procedure to obtain the absolute $[Ca^{2+}]_i$ value, percentage changes of the fluorescence ratio were used to express $[Ca^{2+}]_i$ levels, taking the response to 118 mM $K^+$-depolarization to be 100%.

### *Effects of diltiazem on ET-1 and ET-3-induced contractions in normal PSS*

After recording the control response to 118 mM $K^+$-depolarization in normal PSS, diltiazem, a blocker of voltage-operated $Ca^{2+}$ channels was applied 10 min before and during the application of ET-1 or ET-3. Ten min is sufficient to achieve equilibration of diltiazem at its binding sites.

### *Cumulative applications of external $Ca^{2+}$ (0-7.5 mM) in $Ca^{2+}$-free 118 mM $K^+$ solution*

After recording the control response to 118 mM $K^+$-depolarization in normal PSS, the vascular strips were incubated in $Ca^{2+}$-free PSS containing 2 mM EGTA for 10 min, followed by a 5 min exposure to $Ca^{2+}$-free PSS without EGTA. The solution was then changed to a $Ca^{2+}$-free-118 mM $K^+$ solution and cumulative applications of extracellular $Ca^{2+}$ were initiated.

### *Drugs and solutions*

The millimolar composition of the normal physiological salt solution (normal PSS) was: NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, $KH_2PO_4$ 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. The $Ca^{2+}$-free solution ($Ca^{2+}$-free PSS) contained 2 mM EGTA instead of 1.25 mM $CaCl_2$. High $K^+$ PSS was made by equimolar substitution of KCl for NaCl. All solutions were gassed with a mixture of 5% $CO_2$ and 95% $O_2$ (pH 7.4 at 37°C). Synthetic ET-1 and ET-3 were obtained from Peptide Institute Co. Ltd. (Osaka, Japan), BQ-123 (cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-)) was kindly donated by Banyu Seiyaku Co. (Tokyo, Japan), fura-2/AM was purchased from DOJINDO (Kumamoto, Japan). Fura-2/AM was dissolved in dimethyl sulphoxide (DMSO) as a stock solution and diluted in the medium just before loading the dye. The final concentration of DMSO was 5%.

### *Statistical analysis*

Values are expressed as means ± standard error. Statistical analysis of the shift of $[Ca^{2+}]_i$-tension curves was carried out by an analysis of covariance. $P<0.05$ was considered to be significant.

The $EC_{50}$ values, the concentrations that increased fluorescence ratio or tension to 50% of the maximum response, were determined from concentration-response curves fitted by a four-parameter logistic model (De Lean *et al.*, 1987).

## Results

### *Effects of ET-1 and ET-3 on $[Ca^{2+}]_i$ and tension development in the presence of extracellular $Ca^{2+}$*

Figure 1 shows the averaged time courses of increases in $[Ca^{2+}]_i$ and tension induced by various concentrations of ET-1 and ET-3 in normal PSS. The maximal elevations of $[Ca^{2+}]_i$ and tension elicited by $10^{-7}$ M ET-1 ($[Ca^{2+}]_i$, 109.3 ± 8.3%, $n = 10$, and tension 133.8 ± 3.0%, $n = 10$) were much greater than those elicited by $10^{-5}$ M ET-3 ($[Ca^{2+}]_i$, 74.2 ± 2.5%, $n = 5$; and tension, 86.7 ± 4.7%, $n = 5$). As shown in Figure 1a, changes in $[Ca^{2+}]_i$ and tension induced by $10^{-10}$ to $10^{-7}$ M ET-1 were concentration-dependent. At each concentration of ET-1, tension increased

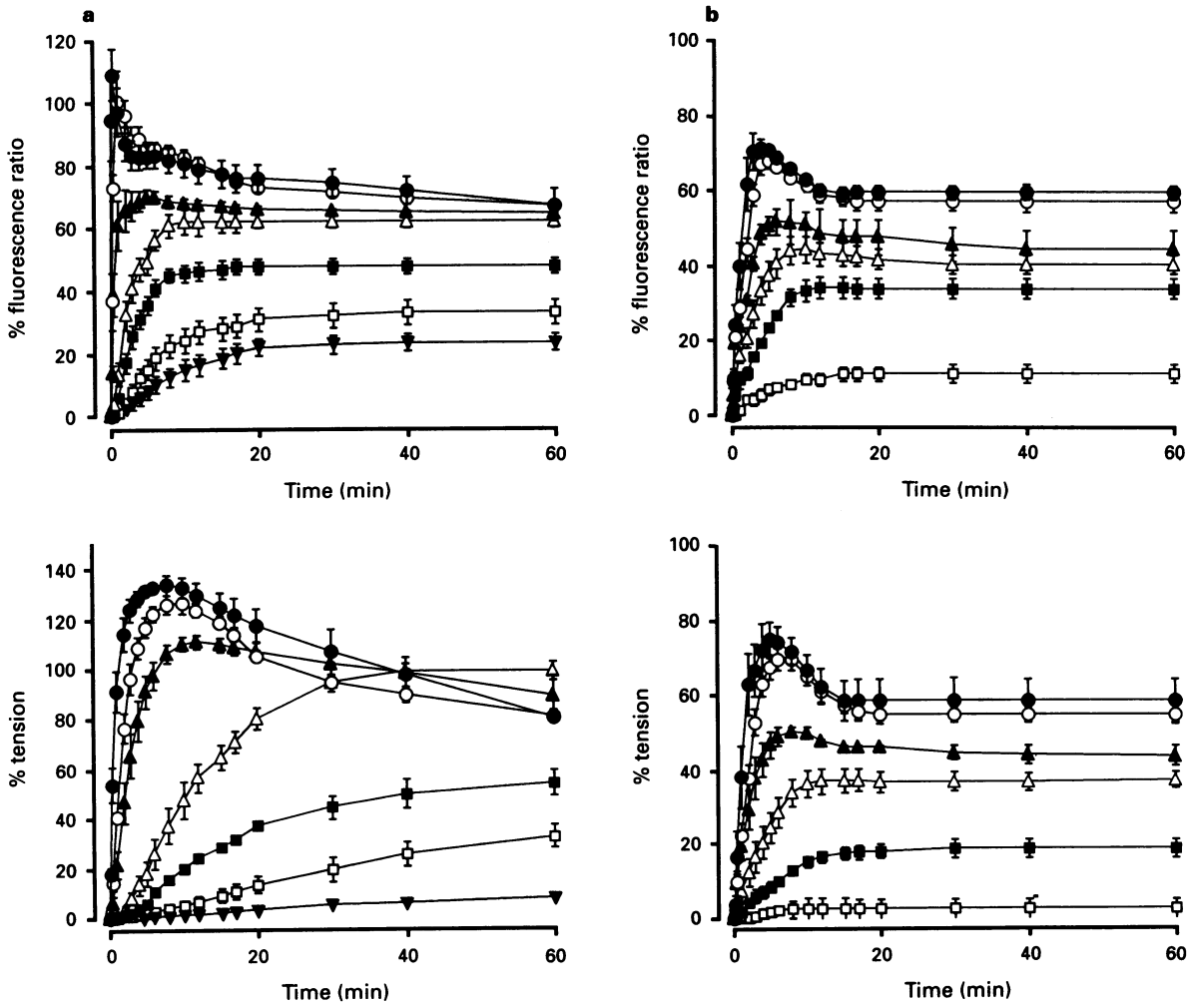


**Figure 1** Time courses of changes in fluorescence ratio ($[Ca^{2+}]_i$) and tension development induced by $10^{-10}$ M (▼), $3 \times 10^{-10}$ M (□), $6 \times 10^{-10}$ M (■), $10^{-9}$ M (△), $10^{-8}$ M (▲), $3 \times 10^{-8}$ M (○) and $10^{-7}$ M (●) endotheline-1 (ET-1) (a) and by $3 \times 10^{-8}$ M (□), $10^{-7}$ M (■), $3 \times 10^{-7}$ M (△), $6 \times 10^{-7}$ M (▲), $10^{-6}$ M (○) and $10^{-5}$ M (●) ET-3 (b). The abscissa scales indicate the time (in min) after the application of endothelin. Data are means ± s.e.mean ($n \geq 5$).

more slowly than did $[Ca^{2+}]_i$. For example, applications of $3 \times 10^{-10}$ M and $10^{-9}$ M ET-1 increased $[Ca^{2+}]_i$, gradually and monotonically, with peaks at 20 min (30.9 ± 3.8%, $n = 6$) and 15 min (65.4 ± 3.9%, $n = 7$), respectively, and these levels were maintained for at least 60 min. The tension induced by $3 \times 10^{-10}$ M and $10^{-9}$ M ET-1 increased much more slowly than $[Ca^{2+}]_i$, reaching peaks at 60 min (32.4 ± 4.8%, $n = 6$) and 40 min (99.0 ± 3.0%, $n = 7$), respectively, and these levels were maintained for at least 60 min. When $10^{-7}$ M ET-1 was applied, $[Ca^{2+}]_i$ rapidly rose to reach a transient peak (the first component; at 30 sec) and then declined to a lower steady-state level (the second component that lasted longer than 30 min) in 20 min. The $[Ca^{2+}]_i$ levels associated with the first and the second components (20 min) amounted to 109.3 ± 8.3% ($n = 10$), and 76.0 ± 4.3% ($n = 10$), respectively. The tension developed more slowly than $[Ca^{2+}]_i$ to reach a peak at 8 min and then gradually declined to a steady-state level. The extent of tension development at the time of the initial $[Ca^{2+}]_i$ maximum and at 20 min after application (the second component) were 133.9 ± 3.0% ($n = 10$) and 117.5 ± 6.1% ($n = 10$), respectively. Thus, 20 min after application, the tension development induced by $10^{-7}$ M ET-1 was greater than that induced by 118 mM $K^+$ for a given $[Ca^{2+}]_i$ level.

As shown in Figure 1b, changes in $[Ca^{2+}]_i$ and tension induced by ET-3 were also dependent on concentration ($10^{-8}$–$10^{-5}$ M). In contrast to the effects of ET-1, the time courses of ET-3-induced changes in $[Ca^{2+}]_i$ and tension were similar at all concentrations. For example, applications of $10^{-7}$ M ET-3 caused gradual monophasic increases in $[Ca^{2+}]_i$ with a peak at 16 min (34.2 ± 2.9%, $n = 6$) and this level was maintained for at least 60 min. After $10^{-7}$ M ET-3, tension increased gradually and monotonically, with a similar time course of change in $[Ca^{2+}]_i$, to reach a peak at 19 min (17.8 ± 1.9%, $n = 6$), and this level was maintained for at least 60 min. When vascular strips were exposed to $10^{-5}$ M ET-3 in normal PSS, biphasic increases in $[Ca^{2+}]_i$ and tension were observed; $[Ca^{2+}]_i$ and tension gradually increased to reach a peak at 5 min (69.1 ± 2.0%, $n = 9$) and at 7 min (71.7 ± 2.9%, $n = 9$), respectively, and both declined to a lower steady-state level (54.9 ± 2.2%, $n = 9$ and 55.9 ± 2.2%, $n = 9$, respectively) by 20 min.

The effects of diltiazem ($10^{-5}$ M, a blocker of voltage-operated $Ca^{2+}$ channels on the time courses of increases in $[Ca^{2+}]_i$ and tension induced by $10^{-7}$ M ET-1 and $10^{-5}$ M ET-3 are shown in Figure 2. In normal PSS, application of $10^{-5}$ M) diltiazem caused a significant decrease in resting $[Ca^{2+}]_i$ to −5.5 ± 0.7% ($n = 5$), but did not affect the resting tension at 10 min. As shown in Figure 2a, diltiazem ($10^{-5}$ M) significantly inhibited both the first and the second components of increases in $[Ca^{2+}]_i$ and tension induced by $10^{-7}$ M ET-1. In the presence of $10^{-5}$ M diltiazem, the levels of

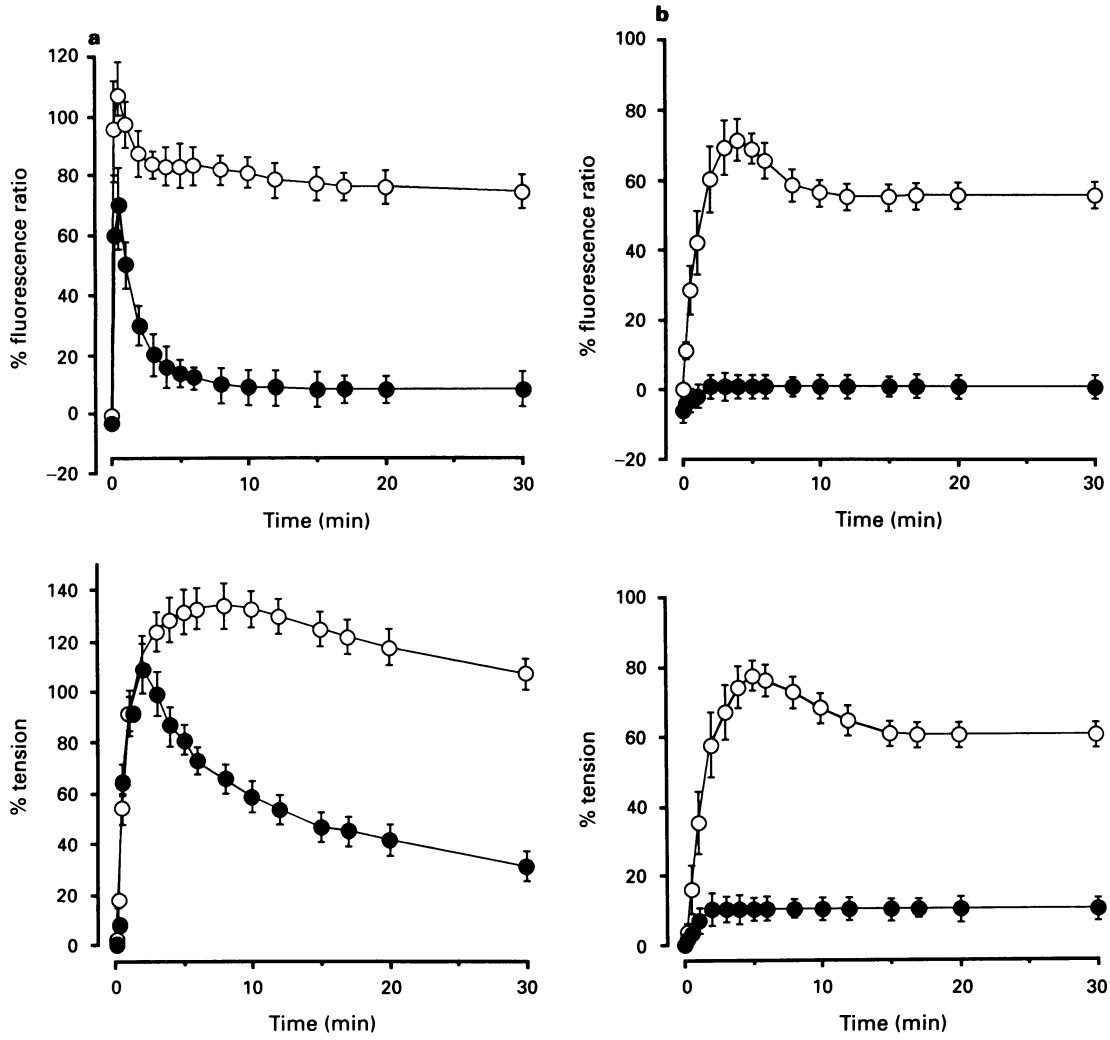


**Figure 2** Effects of $10^{-5}$ M diltiazem on the elevations of fluorescence ratio ($[Ca^{2+}]_i$) and tension development induced by $10^{-7}$ M endothelin-1 (ET-1) (a) and by $10^{-5}$ M ET-3 (b). Time courses of changes in $[Ca^{2+}]_i$ and tension development induced by $10^{-7}$ M ET-1 (a) and by $10^{-5}$ M ET-3 (b), with (●) or without (○) treatment with $10^{-5}$ M diltiazem. Diltiazem was applied 10 min before and during the application of endothelin. The abscissa scales indicate the time (in min) after the application of endothelin. Data are means ± s.e.mean ($n \geq 5$).

$[Ca^{2+}]_i$ and tension of the first component were 70.0 ± 10.2% ($n = 5$) and 109.5 ± 9.9% ($n = 5$), respectively and those obtained 20 min after ET-1 application (the second component) were 8.0 ± 3.3% ($n = 5$) and 41.4 ± 6.0% ($n = 5$), respectively. In contrast, biphasic increases in $[Ca^{2+}]_i$ and tension induced by $10^{-5}$ M ET-3 were markedly and significantly inhibited by $10^{-5}$ M diltiazem to 1.0 ± 1.9% ($n = 5$) and to 10.3 ± 2.7% ($n = 5$), respectively (Figure 2b). In the case of contractions induced by 118 mM $K^+$, $10^{-5}$ M diltiazem completely inhibited the increases in $[Ca^{2+}]_i$ and tension.

### *Effects of ET-1 and ET-3 on $[Ca^{2+}]_i$ and tension development in the absence of extracellular $Ca^{2+}$*

The effects of ET-1 and ET-3 on $[Ca^{2+}]_i$ and tension development in $Ca^{2+}$-free PSS containing 2 mM EGTA are summarized in Figure 3. When the vascular strips were exposed to $Ca^{2+}$-free PSS, $[Ca^{2+}]_i$ gradually declined to reach a steady-state (−22.1 ± 2.2% $n = 10$), while the tension remained unchanged. After 15 min incubation in $Ca^{2+}$-free PSS, application of $10^{-7}$ M ET-1 induced transient elevations of $[Ca^{2+}]_i$ and tension with peaks at 30 s (48.6 ± 4.8%, $n = 10$) and at 2 min (66.6 ± 6.8%, n = 10), respectively. $[Ca^{2+}]_i$ then rapidly declined to the pre-stimulation level in 5 min (−21.0 ± 1.2%, $n = 10$), while the tension declined gradually, but remained slightly but significantly higher than the pre-stimulation level (18.0 ± 1.5%, $n = 10$) for at least 30 min. Changes in $[Ca^{2+}]_i$ and tension induced by $10^{-9}$ M to $10^{-7}$ M ET-1 were concentration-dependent and the $EC_{50}$ values for ET-1 to increase $[Ca^{2+}]_i$ and tension were $9.8 \pm 0.4 \times 10^{-9}$ M and $5.9 \pm 0.3 \times 10^{-9}$ M, respectively. In contrast, even $10^{-5}$ M ET-3, sufficient to induce maximal elevations of $[Ca^{2+}]_i$ and tension in normal PSS, elicited little mobilization of intracellular $Ca^{2+}$. For example, after 15 min incubation in Ca2+-free PSS, application of $10^{-5}$ M ET-3 caused, if anything, only small elevations of $[Ca^{2+}]_i$ and tension with peaks at 1 min and at 2 min, respectively. $[Ca^{2+}]_i$ then rapidly declined to the pre-stimulation level, while the tension remained slightly but significantly higher than the pre-stimulation level for at least 30 min. The peak levels of $[Ca^{2+}]_i$ and tension induced by $10^{-5}$ M ET-3 were −16.1 ± 3.6% ($n = 6$) and 15.0 ± 6.4% ($n = 6$), respectively.

### *Temporal changes in $[Ca^{2+}]_i$-tension relationship during ET-1- and ET-3-induced contraction*

Figure 4 shows the summarized data of changes in fluorescence ratio ($[Ca^{2+}]_i$) and tension development induced by cumulative applications of external $Ca^{2+}$ (0–7.5 mM) during depolarization induced by 118 mM $K^+$ solution (Abe *et al.*, 1990). Both $[Ca^{2+}]_i$ and tension increased stepwise with eleva-

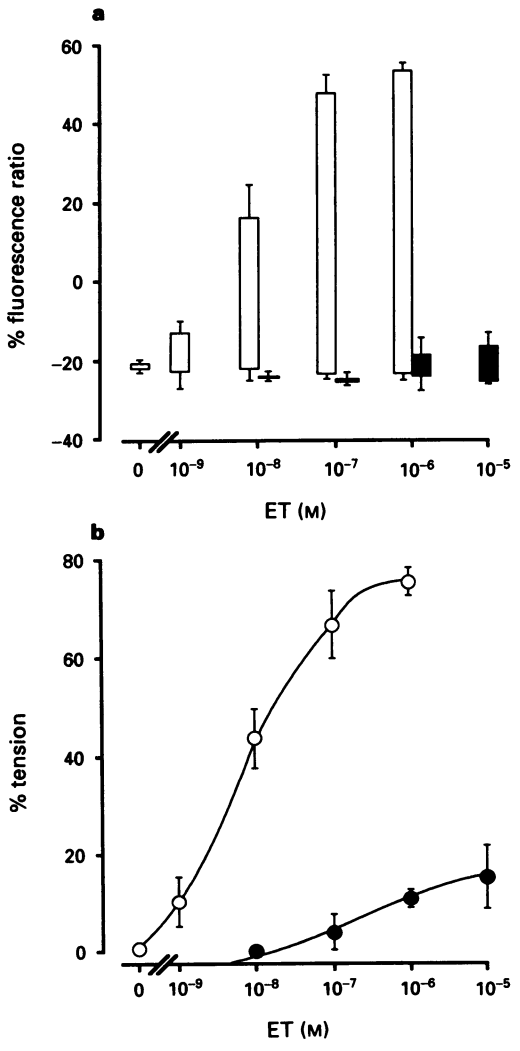


**Figure 3** Concentration-response relationships for the effects of various concentrations of endothelin-1 (ET-1) (□,○) and ET-3 (■,●) on fluorescence ratio ($[Ca^{2+}]_i$) (a) and on tension development (b) in $Ca^{2+}$-free PSS (2 mM EGTA). ET-1 and ET-3 were applied 5 min after the normal PSS had been changed to $Ca^{2+}$-free PSS. In (a), the bottom and the top of each column indicate the $[Ca^{2+}]_i$ just before and at the peak levels obtained by the application of endothelin, respectively. Data are means ± s.e.means ($n \geq 6$).

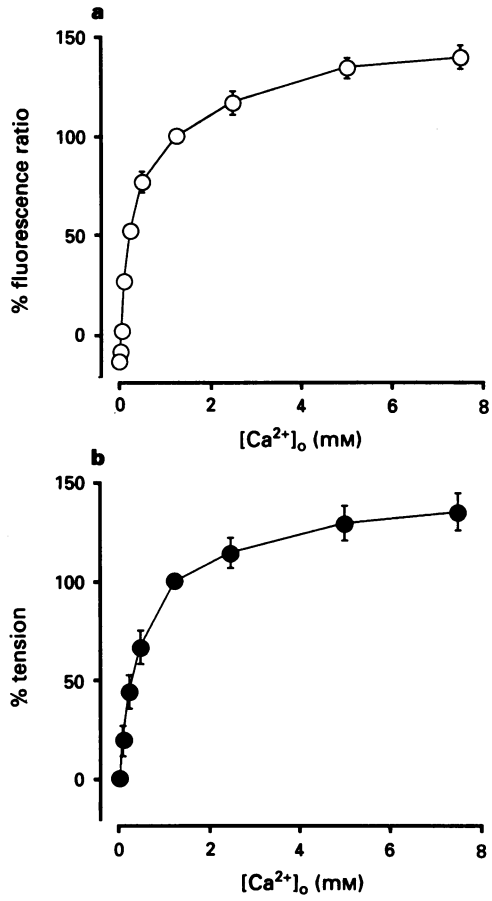


**Figure 4** Summary of data on changes in fluorescence ratio ($[Ca^{2+}]_i$) (a) and tension development (b) in response to cumulative applications of extracellular $Ca^{2+}$ (0–7.5 mM) during 118 mM $K^+$-depolarization. Data were obtained at the time of maximal tension development at each application of extracellular $Ca^{2+}$. The abscissa scales indicate concentrations of extracellular $Ca^{2+}$ (in mM). Data are means ± s.e.mean ($n = 10$).

tions of extracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_o$). $[Ca^{2+}]_i$ increased from $-19.5 \pm 3.3\%$ (at 0 mM $[Ca^{2+}]_o$) to $140.5 \pm 6.1\%$ (at 7.5 mM $[Ca^{2+}]_o$), and the tension increased from 0% (at 0 mM $[Ca^{2+}]_o$) to $135.5 \pm 9.5\%$ (at 7.5 mM $[Ca^{2+}]_o$). The $[Ca^{2+}]_i$ (abscissa)-tension (ordinate) relationship obtained from these data was named the 'basic' $[Ca^{2+}]_i$-tension relationship of $Ca^{2+}$-induced contractions ($Ca^{2+}$-contractions).

The $[Ca^{2+}]_i$-tension relationship that changes with time for any given concentration of ET-1 and ET-3 was examined (Figure 5). In these figures, the 'basic' $[Ca^{2+}]_i$-tension relationship of $Ca^{2+}$-contractions is also presented as a reference. As shown in Figure 5a, for concentrations of ET-1 of $10^{-10}$ to $10^{-9}$ M, the tension increased much more slowly than did $[Ca^{2+}]_i$ over the first few min of agonist contact but thereafter tension increased more rapidly than did $[Ca^{2+}]_i$ and even increased without an obvious change in $[Ca^{2+}]_i$. In the presence of $3 \times 10^{-8}$ and $10^{-7}$ M ET-1, there was also a slower increase in tension than of $[Ca^{2+}]_i$ but sometime thereafter $[Ca^{2+}]_i$ decreased while tension increased and this was followed by a stage where both tension and $[Ca^{2+}]_i$ decreased. Thus, in the $[Ca^{2+}]_i$-rising phase, the $[Ca^{2+}]_i$-tension relation of ET-1 shifted significantly to the right ($P<0.05$) from that for $Ca^{2+}$-contractions. However, in the $[Ca^{2+}]_i$-declining and steady-state phases, ET-1 ($\geqslant 10^{-9}$ M) produced a greater tension than that expected from given changes in $[Ca^{2+}]_i$, the result being a leftward shift of the $[Ca^{2+}]_i$-tension curve, as compared with that for $Ca^{2+}$-contractions ($P<0.05$).

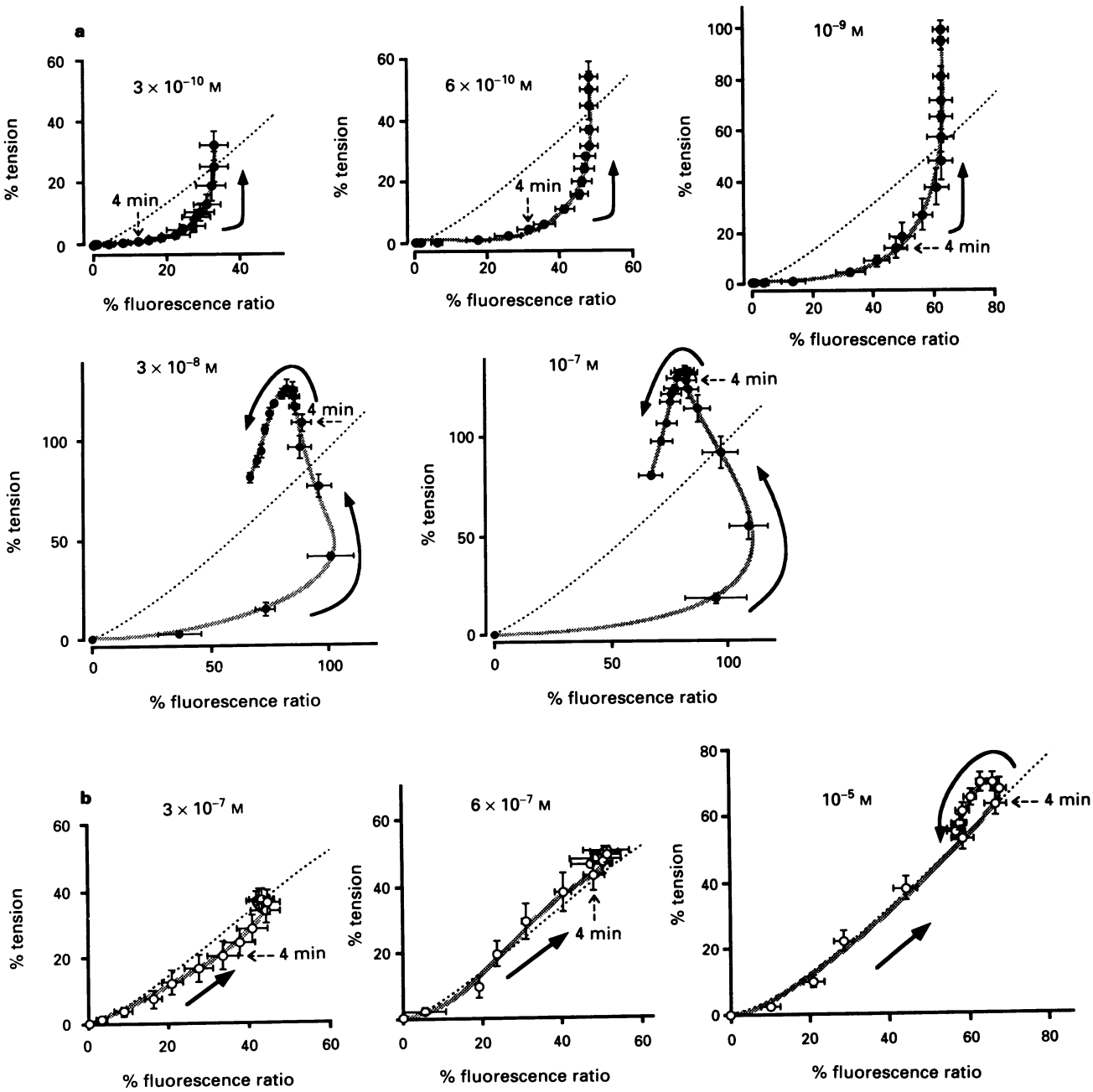


**Figure 5** Temporal changes in the $[Ca^{2+}]_i$-tension relationship during contractions induced by $3 \times 10^{-10}$, $6 \times 10^{-10}$, $10^{-9}$ and $10^{-7}$ M endothelin-1 (ET-1) (a) and by $3 \times 10^{-7}$, $6 \times 10^{-7}$ and $10^{-5}$ M ET-3 (b), constructed from data in Figure 1a and b. The individual points of these figures represent values 0.25, 0.5, 1,2,3,4,5,6,8,10,12,15,17,20,30,40 and 60 min after the application of ET-1 (a) and ET-3 (b). Data are means ± s.e.mean (shown by vertical and horizontal lines $n \geqslant 5$). The curved arrows indicate the direction of time. The dashed line in these figures indicates the 'basic' $[Ca^{2+}]_i$-tension relationship of $Ca^{2+}$-induced contraction which was obtained from data in Figure 4a and b. The straight dashes with arrowheads indicate the 4 min points after the application of ET-1 (a) and ET-3(b).

Figure 5b shows the $[Ca^{2+}]_i$-tension curves during contractions induced by various concentrations of ET-3. In the $[Ca^{2+}]_i$-rising phase, the $[Ca^{2+}]_i$ and tension increased linearly with time until $[Ca^{2+}]_i$ reached its peak, and even in the $[Ca^{2+}]_i$-declining phase, the tension changed almost in parallel with $[Ca^{2+}]_i$. Therefore, in contrast to stimulation with ET-1, the $[Ca^{2+}]_i$-tension relation was not significantly different ($P > 0.05$) from that for $Ca^{2+}$-contractions at any phase. Only at high concentrations of $10^{-6}$–$10^{-5}$ M ET-3, did the $[Ca^{2+}]_i$-tension curve shift a little to the left in the $[Ca^{2+}]_i$-declining phase. However, there was no significant difference in the steady-state of the $[Ca^{2+}]_i$-tension relation between the ET-3- and $Ca^{2+}$-induced contractions.

Four min after the application of $10^{-9}$ M ET-1 or of $6 \times 10^{-7}$ M ET-3, in which the rise of $[Ca^{2+}]_i$ due to ET-1 was almost identical to that induced by ET-3 (47.4 ± 3.7%, $n = 7$, and 48.4 ± 2.6%, $n = 5$, respectively), the extent of rise of tension induced by ET-1 (13.3 ± 4.0% $n = 7$) was significantly smaller than that induced by ET-3 (42.8 ± 4.6%, $n = 5$), and the $[Ca^{2+}]_i$-tension relation of ET-1 was shifted to the right of that for ET-3 (Figure 5).

### *Effects of BQ-123, a selective $ET_A$ receptor antagonist, on ET-1- and ET-3-induced contractions*

Application of a selective $ET_A$-receptor antagonist, BQ-123, in normal PSS had no apparent effect on the resting $[Ca^{2+}]_i$ and tension. Ten minutes pretreatment with $10^{-5}$ M and $10^{-6}$ M, BQ-123 completely inhibited elevations of $[Ca^{2+}]_i$ and tension induced by $10^{-9}$–$10^{-8}$ M ET-1 and by $10^{-6}$ M ET-3, respectively.

## Discussion

ET-1 was a much more potent vasoconstrictor that ET-3 and the maximal elevations of $[Ca^{2+}]_i$ and tension elicited by ET-1 were much greater than those elicited by ET-3 (Figure 1), consistent with the involvement of an $ET_A$ receptor subtype. This quantitative difference in maximal developed tension, but not to $[Ca^{2+}]_i$, has been reported by others (Inoue *et al.*, 1989). As shown in Figure 1a, we found that the ET-1 ($10^{-8}$–$10^{-7}$ M) induced increase in $[Ca^{2+}]_i$, consisted of an initial transient and a subsequent sustained phase in the presence of extracellular $Ca^{2+}$. Following removal of extracellular $Ca^{2+}$, the transient increase in $[Ca^{2+}]_i$ was attenuated and the sustained phase abolished (Figure 3). These results suggest that both intracellular $Ca^{2+}$ release and extracellular $Ca^{2+}$ influx contribute to the initial transient phase, whereas the sustained phase is dependent on extracellular $Ca^{2+}$ influx. In contrast, in the presence of extracellular $Ca^{2+}$, ET-3 caused monophasic ($<10^{-6}$ M) or biphasic ($10^{-6}$–$10^{-5}$ M) sustained increases in $[Ca^{2+}]_i$ (Figure 1b), but in the absence of extracellular $Ca^{2+}$, even $10^{-5}$ M ET-3 caused little, if any, elevation of $[Ca^{2+}]_i$ (Figure 3). These results indicate that the major mechanism whereby ET-3 increases $[Ca^{2+}]_i$ is $Ca^{2+}$ influx from the extracellular space. We have found that the biphasic $[Ca^{2+}]_i$ elevation induced by $10^{-5}$ M ET-3 and the second component of $[Ca^{2+}]_i$ elevation induced by $10^{-7}$ M ET-1 were markedly inhibited by diltiazem, a blocker of voltage-operated $Ca^{2+}$ channels (VOCs) (Figure 2), suggesting that ET-1 and ET-3 induce $Ca^{2+}$ influx mainly through VOCs. ET-1 activation of VOCs is reported to be direct, or indirect via receptor-coupled G-proteins (Kasuya *et al.*, 1992), because membrane depolarization induced by a maximal concentration of ET-1 did not exceed 8.4 mV in the porcine coronary artery (Kasuya *et al.*, 1989). The mechanism for ET-3 activation of VOCs remains to be clarified.

As shown in Figure 5a, for a given steady-state change in $[Ca^{2+}]_i$, ET-1 produced greater tension development than did $Ca^{2+}$, indicating an increase in $Ca^{2+}$ sensitivity of the contractile apparatus. In contrast, the ET-3 $[Ca^{2+}]_i$-tension relationship in the steady-state phase was not significantly different from that for $Ca^{2+}$-contractions (Figure 5b); hence, ET-3 probably did not affect the $Ca^{2+}$ sensitivity of the contractile apparatus. However, in the absence of extracellular $Ca^{2+}$, ET-3 ($10^{-6}$–$10^{-5}$ M) induced a small sustained contraction accompanied with a small transient $[Ca^{2+}]_i$ elevation (Figure 3), so a small $Ca^{2+}$-sensitizing action of high concentrations of ET-3 cannot be excluded. Therefore, the present study suggests that ET-1-induced contractions are mediated not only via stimulation of $Ca^{2+}$ influx and intracellular $Ca^{2+}$ release, but also via an increase in $Ca^{2+}$ sensitivity of the contractile apparatus, while ET-3-induced contractions are mediated mainly by $Ca^{2+}$ influx from the extracellular space.

There was a dissociation between the time courses of changes in $[Ca^{2+}]_i$ and tension during ET-1-induced contractions, but no such dissociation during the ET-3-induced contractions (Figure 1), reflected in differences between ET-1 and ET-3 in temporal changes in the ET-1 and ET-3 $[Ca^{2+}]_i$-tension relationships (Figure 5): (1) During the $[Ca^{2+}]_i$-rising phase of the ET-1-induced contraction, tension increased more slowly, thereby shifting the $[Ca^{2+}]_i$-tension relation to the right of that for $Ca^{2+}$-contractions. After the $[Ca^{2+}]_i$ reached a peak, tension continued to increase to a maximum. During the $[Ca^{2+}]_i$-declining or steady-state phases, the $[Ca^{2+}]_i$-tension relationship was shifted to the left of that for $Ca^{2+}$-contractions, thereby indicating an increase in $Ca^{2+}$ sensitivity of the contractile apparatus (Figure 5a). (2) During the entire time-course ($[Ca^{2+}]_i$-rising and steady-state phase) of ET-3-induced contractions, the change of tension correlated well with that of $[Ca^{2+}]_i$, resulting in a $[Ca^{2+}]_i$-tension relationship that almost overlapped that for $Ca^{2+}$-contractions (Figure 5b). Thus, ET-3-induced contractions may be mostly $[Ca^{2+}]_i$-dependent. (3) When ET-1 and ET-3-induced tension development was compared at concentrations which induced a similar increase in $[Ca^{2+}]_i$ during the $[Ca^{2+}]_i$-rising phase, it appeared that ET-1 increased tension more slowly than did ET-3 (Figure 1a and b), the $[Ca^{2+}]_i$-tension relation of ET-1 being significantly shifted to the right of that for ET-3 (Figure 5a and b). These results suggest that the temporal changes in the $[Ca^{2+}]_i$-tension relation (time-dependent changes in $Ca^{2+}$-sensitivity of the contractile apparatus) during contractions differ between ET-1 and ET-3. In other words, the $Ca^{2+}$-sensitivity of the contractile apparatus varies with time during ET-1-induced contraction, but that it is almost constant, and similar to $Ca^{2+}$-induced contractions during ET-3-induced contractions.

There are several possible mechanisms to explain the rightward shift of the $[Ca^{2+}]_i$-tension relation during the $[Ca^{2+}]_i$-rising phase of ET-1-induced contractions: (1) A delay in mechanical response for given rapid changes in $[Ca^{2+}]_i$, (2) stimulation of synthesis of negative feedback signals, perhaps cyclic AMP or cyclic GMP or both, which decrease $Ca^{2+}$-sensitivity of the contractile apparatus (Abe *et al.*, 1990; Ushio-Fukai *et al.*, 1993), and (3) modulation of the myosin light chain (MLC) kinase (MLCK)/MLC phosphatase (MLCP) activity ratio. (1) Four minutes after the onset of contractions to $10^{-9}$ M ET-1 and $6 \times 10^{-7}$ M ET-3, $[Ca^{2+}]_i$ increased to a similar extent, but ET-3 did not shift the $[Ca^{2+}]_i$-tension relationship to the right of that for $Ca^{2+}$-contractions (Figure 5), thereby indicating that delay in the mechanical response for the rapid $[Ca^{2+}]_i$ increase was not a major reason for the rightward shift of the $[Ca^{2+}]_i$-tension relation of ET-1. (2) ET-1 is reported to stimulate phospholipase $A_2$ to produce prostanoids from endothelium-denuded strips (Suzuki *et al.*, 1992), which might decrease the $Ca^{2+}$ sensitivity of the contractile apparatus by increasing cyclic AMP levels (Halushka *et al.*, 1989). However, in our preparation, pretreatment with indomethacin ($10^{-5}$ M), an inhibitor of cyclo-oxygenase (Vane, 1971), did not affect increases in $[Ca^{2+}]_i$ and tension induced by either ET-1 or ET-3. In intact preparations of the rabbit aorta, ET-1 produced no effects on cyclic AMP or cyclic GMP levels (Ohlstein *et al.*, 1989). Thus, the possibility of involvement of

cyclic nucleotides, which are known to decrease the $Ca^{2+}$-sensitivity of the contractile apparatus in ET-1-stimulated signal transduction pathways (Suzuki *et al.*, 1992) can be excluded. (3) The modulation of the MLCK/MLCP activity ratios may be important, because they can modulate the $[Ca^{2+}]_i$-tension relation during contraction (Somlyo *et al.*, 1989) and are also mediated by G-proteins (Kitazawa *et al.*, 1989; Somlyo *et al.*, 1989). Indeed, it has been reported that the phosphorylation of MLC develops at a slower rate during ET-1-induced contractions than during histamine-induced contractions in porcine carotid arteries (Rembold, 1990) and in porcine coronary arteries (Abe *et al.*, 1991). Thus, the rate-limiting step in ET-1-induced contractions located in the pathway transforming early signals of $[Ca^{2+}]_i$ elevation into a MLC phosphorylation, may be intrinsically slower than that associated with ET-3-induced contractions.

In the $[Ca^{2+}]_i$-tension relationship, the time onset to increase $Ca^{2+}$ sensitivity of the contractile apparatus by agonists corresponds to the point on the relation shifting to the left of that for $Ca^{2+}$-contractions. Thus, in this study, it was found that ET-1 increased $Ca^{2+}$ sensitivity of the contractile apparatus in the later phase of contraction. Consistent with this, we have reported an increase in the $Ca^{2+}$ sensitivity in the later phase of histamine-induced contractions (Hirano *et al.*, 1991). Since it has been suggested that agonist-induced $Ca^{2+}$ sensitization is mediated through several substrates for protein kinase C (PKC) (Rasmussen *et al.*, 1987; Nishimura *et al.*, 1988) or the inhibition of protein phosphatase (Somlyo *et al.*, 1989), which is modulated by several complicated and slow biological steps, the leftward shift of $[Ca^{2+}]_i$-tension relationship might be observed in the later phrase of agonist-induced contractions. There are reports suggesting that ET-1 increases $Ca^{2+}$ sensitivity of the contractile apparatus by activating PKC (Kodama *et al.*, 1989; Nishimura *et al.*, 1992).

Since these results suggest that ET-1 and ET-3 activated vasoconstrictor mechanisms are quantitatively and qualitatively different, we used the $ET_A$-selective receptor antagonist, BQ-123, to determine whether the mechanisms of action of ET-1 and ET-3 are dissociated at the level of the receptor or at post-receptor levels of signal transduction pathways. Both ET-1- and ET-3-induced contractions were completely inhibited by BQ-123, thereby indicating that the receptor subtype mediating ET-1- and ET-3-induced contractions can be classified as $ET_A$ in the porcine coronary artery. This is supported by the fact that the $ET_A$ receptor has a much higher affinity for ET-1 than ET-3 (Arai *et al.*, 1990), and that the elevations of $[Ca^{2+}]_i$ and tension induced by ET-1 were substantially greater than those induced by ET-3, as shown in this study. However, this is inconsistent with reports that ET-1 and ET-3 act via distinct receptors on vascular smooth muscle (Harrison *et al.*, 1992; Fukuroda *et al.*, 1992). The reasons for this discrepancy are not clear. However, since it has been demonstrated that ET-3 stimulates $Ca^{2+}$ mobilization by an $IP_3$-independent mechanism in aortic smooth muscle cells (Little *et al.*, 1992), it may be that ET-1 and ET-3 activate different signal transduction pathways through different second messengers produced after binding to receptors. Thus, it seems that the $ET_A$-receptor-mediated effects of ET-1 and ET-3 can be dissociated at the sub-receptor level of signal transduction pathways, presumably via different coupling to G-proteins, resulting in different vasoconstrictor mechanisms for ET-1 and ET-3.

In summary, the present study demonstrated that: (1) ET-1 elicits vasoconstriction by activation of $Ca^{2+}$ influx from the extracellular space, $Ca^{2+}$ release from intracellular storage sites and $Ca^{2+}$ sensitivity of the contractile apparatus, whereas ET-3 induces vasoconstriction mainly through $Ca^{2+}$ influx from the extracellular space. Thus, ET-3 depends on free extracellular $Ca^{2+}$ . (2) A distinct pattern of temporal changes in the $[Ca^{2+}]_i$-tension relationship during contractions induced by ET-1 and ET-3 was observed, hence, distinct mechanisms of a time-dependent modulation of the $Ca^{2+}$ sensitivity function in the contractile actions of ET-1 and ET-3. (3) Both ET-1 and ET-3-induced contractions semm to be mediated via $ET_A$-receptors in the porcine coronary artery, thereby indicating that the $ET_A$-receptor-mediated effects of ET-1 and ET-3 can be dissociated at sub-receptor levels of signal transduction pathways.

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# Pharmacology of stretch-activated K channels in *Lymnaea* neurones

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**1** Single-channel recording was used to describe the pharmacology of stretch-activated K channels in *Lymnaea* neurones using channel blockers amiloride, tetraethylammonium (TEA), quinidine, gadolinium (Gd) and diltiazem.

**2** Amiloride, TEA and quinidine applied to the outside face of the membrane all produced a fast flickery block of stretch-activated K channels. All of these agents were without effect when applied at the inside face at concentrations as high as 10, 200 and 10 mM respectively. Neither Gd nor diltiazem had any effect on stretch-activated K channels extracellularly (100 μM).

**3** Amiloride, TEA and quinidine block were voltage-independent with $IC_{50}$ values at positive (and negative membrane potentials of 2.3 (and 2.0) mM, 48 (and 54) mM and 0.8 (and 0.7) mM respectively. Woodhull plots for TEA and quinidine block confirmed the voltage independence of stretch-activated K channel block by these agents.

**4** Hill plots of the amilorde, TEA and quinidine block yield Hill coefficients at positive (and negative) membrane potentials of 1.7 (and 1.5), 1.4 (and 1.2) and 1.5 (and 1.6 mM) respectively.

**5** Ethanol (3%) had no apparent effect on stretch-activated K channel kinetics or conductance yet reduced the efficacy of quinidine block.

**6** The above pharmacological fingerprint of the stretch-activated K channel is discussed with reference to other K-selective and stretch-activated channels.

**Keywords:** Stretch-activated channels; mechanotransduction; tetraethylammonium; quinidine; amiloride; ethanol; diltiazem; gadolinium.

## Introduction

A wide variety of mechanosensitive channels have been described in eukaryotic cells (Morris, 1990), of which two major types, based on ion selectivity, are cation- and K-selective stretch-activated channels. The role of most of these channels is unknown (Morris, 1992), but there is growing evidence that some may function as cellular mechanotransducers, with the strongest case being that for mechanosensitive channels in supraoptic neurones functioning as osmomechanical transducers (Oliet & Bourque, 1993).

Since they are most mechanosensitive in mechanically disrupted membrane (Morris & Horn, 1991; Small & Morris, 1994), stretch-activated K channels may act to signal incipient cell trauma but they show little promise of serving as physiological transducers. Moreover, it has been shown that a channel with a well-defined non-mechanical function, the *Aplysia* S-channel (a serotonin and neuropeptide modulated K channel whose activation reduces neuronal excitability), behaves as a stretch-activated K channel in cell-attached and excised patch recordings (Vandorpe *et al.*, 1994). Stretch-activation is facilitated by the actin reagent, cytochalasin D, suggesting that stretch-activated K channel mechanosensitivity is normally held in check by cytoskeletal restraints (Small & Morris, 1994). This observation raises the possibility that mechanosensitivity is transduced via the phospholipid bilayer as suggested for bacterial mechanosensitive channels (Martinac *et al.*, 1990).

In general, the physiological functions of channels are difficult to assess if the channel cannot be selectively disrupted. What is required for mechanosensitive channels, therefore, is a high-affinity blocker or a cloned channel whose function could be manipulated. Although a mechanosensitive channel from *E. coli* has recently been cloned (Sukharev *et al*, 1994), its behaviour is unlike any mechanosensitive channel in eukaryotes and its structure does not resemble any other cloned channel, making it unlikely that it is similar to mechanosensitive channels of eukaryotes. Site-directed mutagenesis studies (Heginbotham *et al.*, 1992) have shown that a two amino acid deletion readily can convert a K-selective channel to a cationic channel, suggesting that stretch-activated K and cationic channels might be closely related. Additionally, permeation studies of stretch-activated cationic channels revealed pore characteristics more like those of a K channel than, for instance, those of ligand-gated non-selective cation channels like the nicotinic acetylcholine receptor (Yang & Sachs, 1990).

Others have been developing a pharmacological profile for stretch-activated cationic channels (Lane *et al.*, 1991; 1993; Hamill *et al.*, 1992). In this study we describe the pharmacology of stretch-activated K channels in *Lymnaea* neurones with the hope that a pharmacological profile will facilitate comparisons of other K-selective and mechanosensitive channels.

## Methods

### *Cultured* Lymnaea stagnalis *neurones*

The circumoesophageal ring of ganglia was dissected out of *Lymnaea stagnalis* and gently agitated for 60 min in normal saline (NS) (in mM: 55 NaCl, 1.6 KCl, 2 $MgCl_2$, 3.5 $CaCl_2$, 5 HEPES-NaOH adjusted to pH 7.6) with $CaCl_2$ removed and 0.25% protease type XIV (Sigma) added. The ganglia were then washed in NS and individual neurones were plated in NS with 5 mM glucose and 0.1 mg ml gentamicin sulphate (Sigma). Cells were 1–6 days in culture before patch-clamp recording (Small & Morris, 1994).

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### *Patch-clamp recording*

Cell-attached recordings were made in a bath solution of NS and excised inside-out patches were made with intracellular solution in the bath (in mM: 52 KCl, 2.2 EGTA, 2 $MgCl_2$, 0.2 $CaCl_2$, 5 HEPES-KOH adjusted to pH 7.2). Pipettes contained high-K solution (in mM: 52 KCl, 2 $MgCl_2$, 1 TEA-Cl, 5 HEPES-KOH adjusted to pH 7.6). External blocking agents were in the pipette during cell-attached recordings and internal agents were tested in the bath during excised patch recordings. Patch pipettes with an outside diameter of ~2.5 μm pre polishing, ~2.0 μm post polishing and a resistance of 8 MΩ with high-K solution in the pipette and NS in the bath were made from borosilicate glass [N51A (i.d. 1.15 mm, o.d. 1.65 mm)] (Garner Glass, Claremont, CA, U.S.A.) using a List L/M-3P-A pipette puller (Darmstadt, Germany). They were coated with Sylgard 184 (Dow Corning, Midland, MI, U.S.A.) and fire polished.

To distinguish stretch-activated K channels from other K channels, namely $Ca^{2+}$-activated K channels, we routinely used 1 mM TEA in the pipette for recordings of neurones. (We show here that the $K_d$ for external TEA block of stretch-activated K channels is about 50 mM. When forming gigaseals, ~+ 10 mmHg was maintained in the pipette until contact was made with the membrane. This served to prevent dilution of any drugs applied in the pipette.

### *Analysis*

Channel currents were recorded using an Axopatch 1D (Axon Instruments, Foster City, CA, U.S.A.) connected to a PC microcomputer via a TL-1 interface (Axon Instruments, Claremont, CA, U.S.A.). pClamp 5.5 (Axon Instruments, Claremont, CA, U.S.A.) was used to digitize and export records to Sigmaplot 4.1 (Jandel Scientific, Corte madeira, CA, U.S.A.). Once in Sigmaplot 4.1 the records were analysed then exported to Coreldraw 3.0 (Corel, Ottawa, Ontario, Canada).

For current/voltage ($I/V$) relations of single-channel events from cell-attached patches the membrane voltage was taken to be $V_m = (V_{rest} - V_p)$, where $V_p$ is the pipette holding potential and $V_{rest}$ is the resting membrane potential, which was assumed to be − 50 mV (Sigurdson & Morris, 1989) for *Lymnaea* neurones. Unless otherwise stated, $I/V$ relations were obtained by measuring single stretch-activated K channel currents during 2 s steps to indicated voltages. Woodhull plots of channel block were obtained by plotting the ratio of the mean currents without ($i_O$) and with ($i_D$) drug over a range of voltages from $I/V$ relations. The first-order linear regressions were fit to data to obtain slopes which were stated with corresponding correlation coefficients of fit. Concentration–response curves for block of stretch-activated K channels were expressed as a percentage of the maximum possible inhibition (i.e. to zero current) of the control single-channel current during 2 s steps to indicated voltages. Hill plots of channel block were obtained by transforming the per cent inhibition data ($y$) from concentration–response curves using the term, $\log[y/(\max - y)]$, where max is 100% inhibition. $IC_{50}$ values and Hill coefficients were obtained from $x$-intercepts and slopes of (best fit) first-order linear regressions to mean data, carried out using fitting algorithms in Sigmaplot (4.1). To provide clear resolution of both inward and outward K currents, high-K solutions were routinely used in the pipette. Currents flowing into the pipette are illustrated as upward deflections. Symbols in figures are means with standard error of mean (s.e.mean) unless otherwise indicated.

## Results

### *Amiloride*

*Cell-attached patches* External amiloride (2 mM) decreased stretch-activated K single channel currents in cell-attached patches of *Lymnaea* neurones without affecting the apparent $P_{open}$ of the channel (Figure 1a–e). A fast-flickery amiloride block of stretch-activated K channels was evident over a wide voltage range. Although some of amiloride's actions on other membrane proteins are steeply voltage dependent (Lane *et al.*, 1991), the extent of stretch-activated K channel block by amiloride was similar at the extremes of positive and negative voltage we tested (Figure 1e). The concentration–response curves (Figure 1f) demonstrate that amiloride's effect on single stretch-activated K channel current at both positive and negative membrane potentials was concentration dependent. Hill plots of amiloride block for both positive and negative membrane potentials (Figure 1g) yield similar $IC_{50}$ values (2.3, 2.0) and Hill coefficients (1.7, 1.5) for positive and negative membrane potentials, consistent with a voltage-independent block. Given that the Hill coefficients are closer to 2 than 1, and that block is not voltage dependent, a mechanism of amiloride block of stretch-activated K channels involving the binding of two amiloride molecules at sites external to the transmembrane voltage field seems likely.

*Excised inside-out patches* Excised inside-out patches were formed with high K in the pipette and single stretch-activated K channel conductance was examined using 2 s voltage ramps from − 100 to 120 ($V_m$, mV). On four patches, channel activity was recorded before and after the addition of amiloride to the inside face of the patch. No effect was detectable on either the conductance or kinetic properties using concentrations as high as 10 mM (not shown). Thus, unlike *Xenopus* oocyte mechanosensitive channels (Lane *et al.*, 1991), amiloride did not block stretch-activated K from the internal side.

### *Gadolinium*

*Cell-attached patches* Gadolinium (Gd), a cation with a complex blocking action on stretch-activated cationic channels (Yang & Sachs, 1989) was tested. With Gd (100 μM) in the recording pipette of five cell-attached patches, 2 s voltage ramps from − 100 to 120 ($V_m$, mV) yielded single stretch-activated K channel $I/V$s which were compared with control patches. The single stretch-activated K channel conductance and apparent kinetics of ramp responses from both groups of patches were indistinguishable (not shown).

### *Dilitazem*

*Cell-attached patches* Using the same protocol, but with 100 μM diltiazem (diltiazem has been shown to block one type of stretch-activated cationic channel; Ruknudin *et al.*, 1993 in the pipette, four cell-attached patches were tested. Single stretch-activated K channel conductance and apparent kinetics of these patch responses were not detectably different from control patches (not shown).

### *Tetraethylammonium (TEA)*

*Cell-attached patches* TEA has been a useful probe for K channels, and though it is a poor blocker of molluscan stretch-activated K channels (Small & Morris, 1994; Vandorpe *et al.*, 1994) it does interact to some extent with these channels. To explore this interaction further, we tested external TEA on cell-attached patches by including it in the recording pipette at concentrations from 5 to 200 mM and ramping $V_m$ from − 100 to 120 mV over 2 s. TEA produced a fast-flickery block of stretch-activated K channels, associated with a decreased single channel amplitude (Figure 2), as measured from 2 s steps to the indicated pipette potentials. The single-channel $IV$s (Figure 2a) and the corresponding Woodhull plots (Figure 2b) both indicate that this block was not voltage dependent. The effects of increasing concentrations of TEA on block of stretch-activated K channels

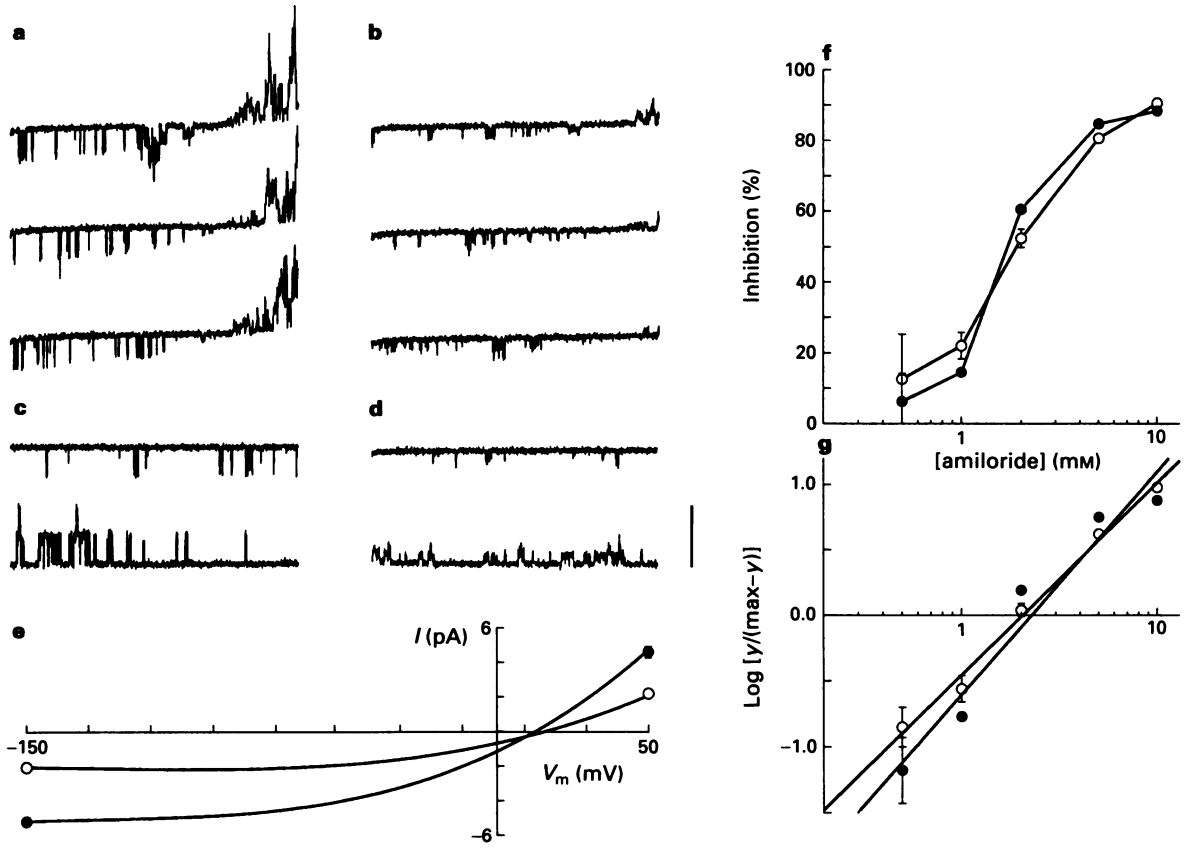


**Figure 1** Effects of extracellular amiloride on stretch-activated K channels in cell-attached patches. Stretch-activated K channel current responses to three 2 s voltage ramps from − 150 to 50 ($V_m$, mV) in the absence (a) and presence (b) of 2 mM amiloride. Stretch-activated K channel responses to a 2 s voltage step to −150 (top) and 50 (bottom) ($V_m$, mV) in the absence (c) and presence (d) of 2 mM amiloride. Scale bar: 10 pA for a–d (e) Superimposed ramp *I/V* data (as in a and b) from four patches were fit by eye to give average single-channel current levels (solid line). At the extremes, single-channel current was measured from step recordings (symbols as in c and d). Standard error bars are mostly within the symbols ($n = 12$). (f) Concentration–response curves for amiloride block of stretch-activated K channels at − 150 (●) and 50 (○) ($V_m$, mV). Between three and twelve patches were used for concentrations of 0.5–10 mM amiloride respectively. (g) Hill plots of data in (f). The *x*-intercepts indicate $IC_{50}$ values (for negative and positive membrane potentials) of 2.0 and 2.3 mM. Slopes give Hill coefficients (for negative and positive potentials) of 1.5 a;nd 1.7 (correlation coefficients 0.99 and 0.97).

were evident at both positive and negative membrane potentials, giving $IC_{50}$ values of 48 and 54 mM at the voltage extremes (Figure 2c). The Hill plots of concentration dependence data at the voltage extremes suggest that a single TEA molecule is sufficient to produce the block, since the Hill coefficients are closer to 1 than 2 (1.2, 1.4) (Figure 2d).

*Excised inside-out patches* On four patches, the effect of internal TEA was tested on single stretch-activated K channels by examining the responses to 2 s voltage ramps before and after bath application of TEA. Concentrations as high as 200 mM had no apparent effect on conductance or kinetics (not shown).

## *Quinidine*

*Cell-attached patches* External quinidine (1 mM) was shown to reduce currents through single *Lymnaea* heart cell stretch-activated K channels (Brezden *et al.*, 1986). Here we show that external quinidine produces a concentration-dependent block of the neuronal stretch-activated K channels (Figure 3a). With quinidine in the pipette, 2 s voltage ramps from − 100 to 120 ($V_m$, mV), and 2 s voltage steps to the indicated potentials were applied to cell-attached patches. The quinidine block resulted in a reduction in single stretch-activated K channel amplitude at all membrane potentials. Woodhull plots of quinidine block *IV* data (Figure 3b) have slopes close to zero, indicating a voltage-independent block.The reduction in current amplitude obtained from voltage steps to − 100 and 120 ($V_m$, mV) (Figure 3c) was identical, consistent with the Woodhull plots. Hill plots constructed from concentration–response data (Figure 3d) revealed $IC_{50}$ values of 0.8 and 0.7 mM at positive and negative membrane potentials respectively. Hill coefficients closer to 2 than 1 (1.5 and 1.6) at these potentials suggest that binding at two sites may be necessary for quinidine block of stretch-activated K channels.

*Excised inside-out patches* Quinidine was also applied to the intracellular face of excised inside-out patches. Voltage ramps were used as usual to examine single stretch-activated K channels' conductance before and after the addition of quinidine. In all four patches tested, internal quinidine was without effect; there was no discernible difference in the single-channel conductance or the apparent kinetic properties of the stretch-activated K channel with internal quinidine at concentration as high as 10 mM (not shown).

## *Ethanol*

*Cell-attached patches* We tested the effects of 3% (v/v) ethanol, a concentration that significantly increases the fluidity of membranes (Goldstein, 1986; Silberman *et al.*, 1990), on the behaviour of stretch-activated K channels. The ethanol was applied in the pipette and patches were exposed

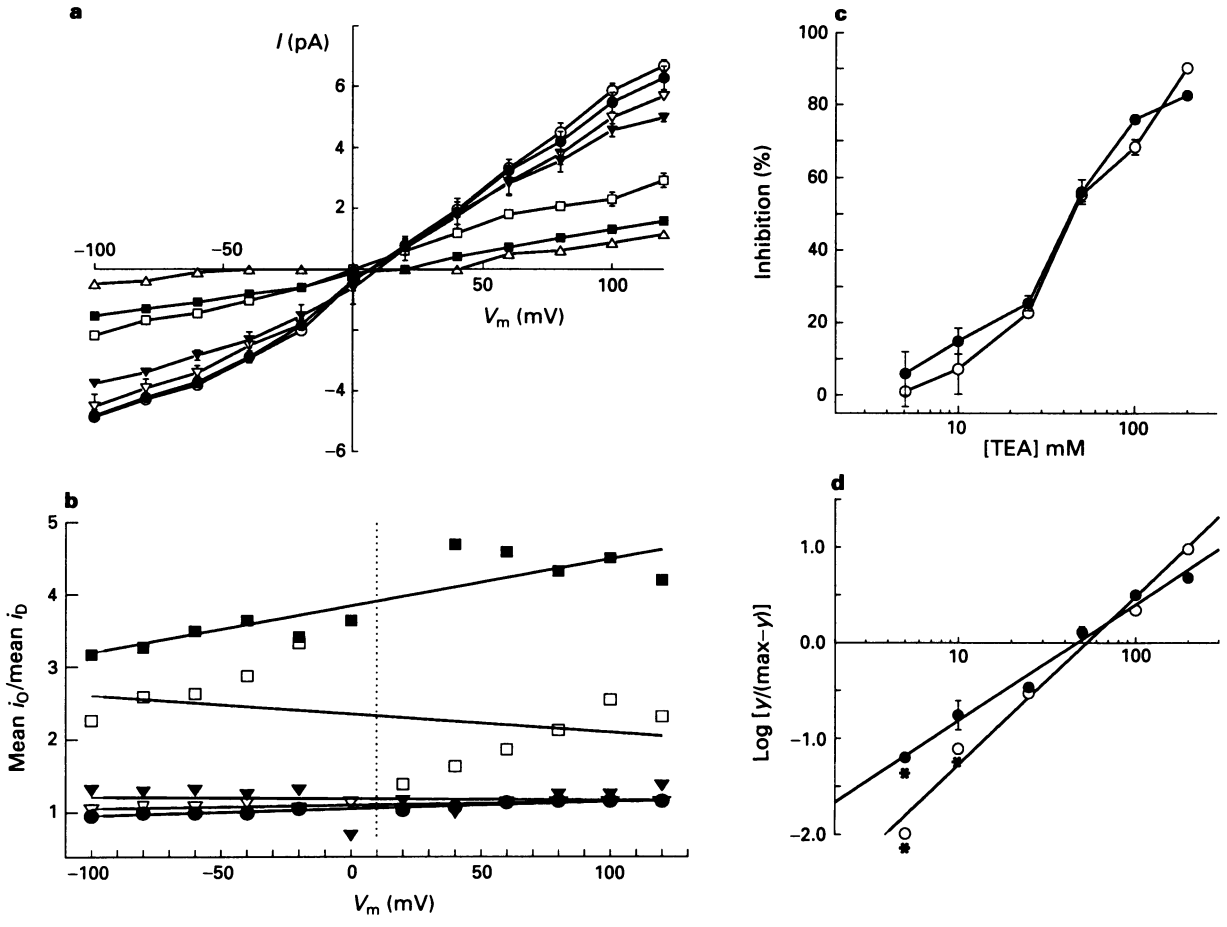


**Figure 2** Effects of extracellular TEA on stretch-activated K channels in cell-attached patches. (a) Single stretch-activated K channel *IV* relations. Symbols are as follows: control (1 mM TEA) (○) $n = 7$, 5 mM TEA (●) $n = 7$, 10 mM TEA (▽) $n = 7$, 25 mM TEA (▼) $n = 3$, 50 mM TEA (□) $n = 3$, 100 mM TEA (■) $n = 4$, 200 mM TEA (△) $n = 3$. (b) Woodhull plots of TEA block of stretch-activated K channel. Symbols are as above. First-order linear regressions fit to data gave slopes (and corresponding correlation coefficients) for 5–100 mM TEA of 0.0010 (0.96), 0.0005 (0.90), −0.0002 (0.84), −0.0025 (0.34) and 0.0065 (0.87). (c) Concentration–response curves for TEA block of stretch-activated K channels at − 100 (●) and 120 (○) ($V_m$, mV). Between three and seven patches were used for concentrations of 5–200 mM TEA. (d) Hill plots of TEA block of stretch-activated K channel. Symbols are as in (c). $IC_{50}$ values for negative (and positive) membrane potentials were 54 (and 48) mM. Hill coefficients for negative (and positive) membrane potentials were 1.4 (and 1.2). Correlation coefficients to fits were 0.99 and 0.99. Asterisks indicate no error bar possible.

for at least 5 min prior to testing. Ethanol did not cause stretch-activated K channels to lose their mechanosensitivity; no difference in the activation of stretch-activated K channels by 20–40 mmHg suction was noted. The responses of ethanol-treated patches to 2 s voltage ramps ($V_m$ = − 100 to 120 mV) were indistinguishable from control patches (not shown). Likewise, the single stretch-activated K channel *I/V* relations obtained using 2 s steps to various voltages (Figure 4), were not significantly different between control and ethanol-treated patches.

### *Quinidine with ethanol*

*Cell-attached patches* The *IV* relationship of single stretch-activated K channels in patches exposed simultaneously to 0.5 mM quinidine and 3% ethanol in the recording pipette was examined. The single stretch-activated K channel current amplitude was measured during 2 s voltage steps (Figure 4). Although ethanol alone did not affect stretch-activated K channel characteristics, it decreased the efficacy of stretch-activated K channel block by external quinidine. In the presence of ethanol, quinidine still produced a flickery block of stretch-activated K channels but the extent of block decreased from 52 ± 1.8 to 25 ± 4.2% ($P < 0.05$) and from 50 ± 5.8 to 33 ± 1.6% ($P < 0.05$) at positive and negative membrane potentials respectively (i.e. ~50% and ~34% reductions in quinidine's efficacy) (see Figure 4).

## Discussion

### *Amiloride*

Although amiloride's highest affinity is for epithelial Na channels, it, along with structurally relate analogues, has been used in constructing a pharmacological profile of mechanosensitive channels in *Xenopus* oocytes (Lane *et al.*, 1992). On these channels, amiloride appears to have two distinct types of binding site: one type (Hill coefficient 2) is accessible from the external side and causes a distinctly voltage-dependent flickery block and has $K_d$ 0.5 mM at − 150mV and another type of lower affinity that is voltage independent and accessible from the inner face (Lane *et al.*,a 1991). Amiloride blocks mechanosensitive currents of hair cells (Jorgensen & Ohmorie, 1988) with even greater potency ($K_d$ 50 μM); the suggested binding reactions are complex. We found no published reference to amiloride blockade of K-selective channels in the mM range, so we cannot say whether a voltage-independent block with a $K_d$ of 2 mM – the result we obtained for *Lymnaea* stretch-activated K channels – is

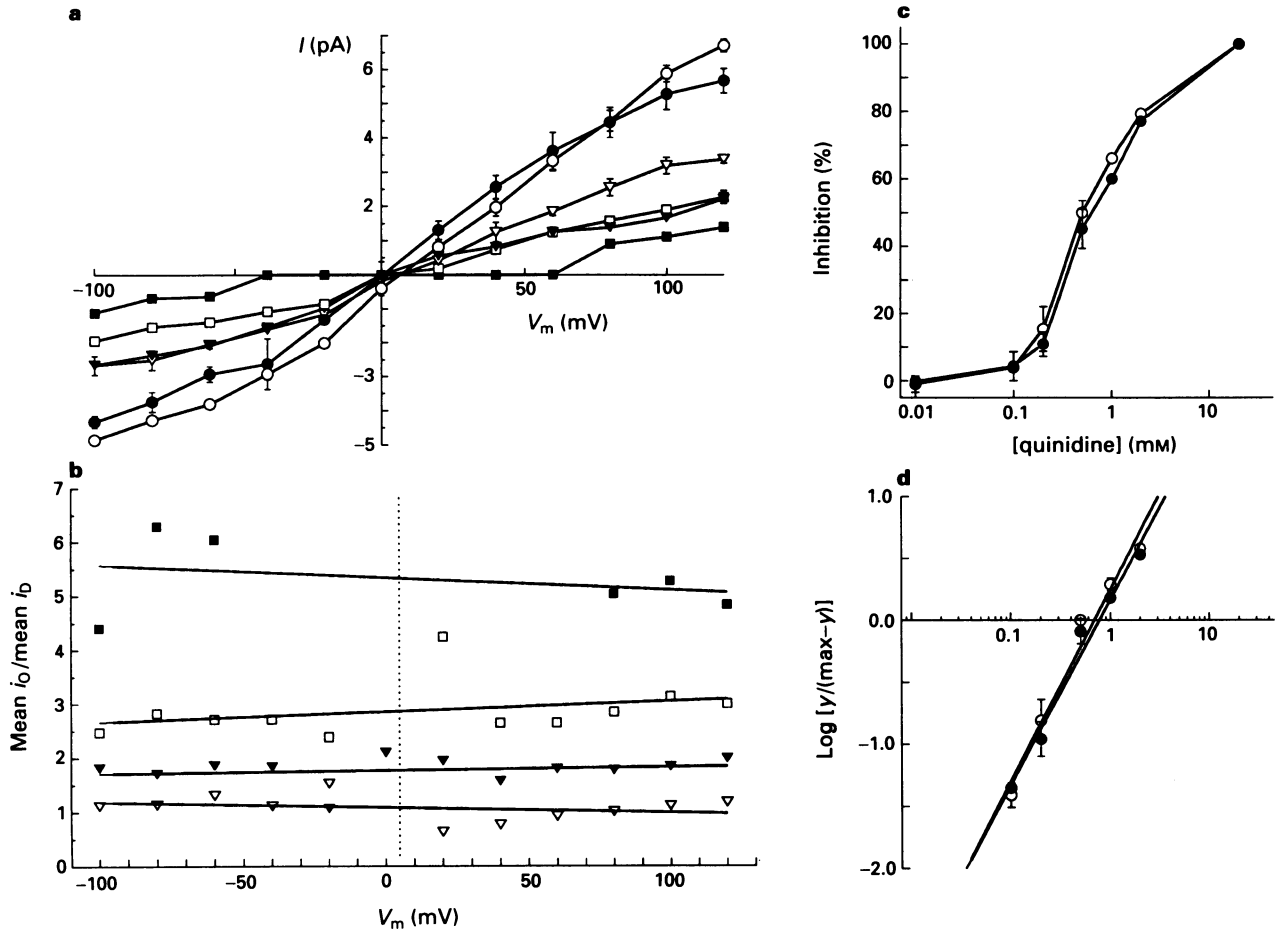


**Figure 3** Effects of extracellular quinidine on stretch-activated K channels in cell-attached patches. (a) Single stretch-activated K channel *IV* relations. Symbols are as follows: control (○) $n = 7$, 0.1 mM quinidine (●) $n = 3$, 0.2 mM quinidine (△) $n = 3$, 0.5 mM quinidine (▲) $n = 5$, 1 mM quinidine (□) $n = 3$, 2 mM quinidine (■) $n = 3$, (b) Woodhull plots of quinidine block of stretch-activated K channel. Symbols are as above. First-order linear regressions fit to data to give slopes (and corresponding correlation coefficients) for 0.2–2 mM quinidine of −0.00094 (0.88), 0.00064 (0.88), 0.00198 (0.79), and −0.0002 (0.31). (c) Concentration–response curves for quinidine block of stretch-activated K channels at −100 (●) and 120 (○) ($V_m$, mV). Three or four patches were used for concentrations of 0.01–12 mM quinidine respectively. (d) Hill plots of quinidine block of stretch-activated K channel. Symbols are as in (c). $IC_{50}$ values for negative (and positive) membrane potentials were 0.7 (and 0.8) mM. Hill coefficients for negative (and positive) membrane potentials were 1.6 (and 1.5). Correlation coefficients to fits were 0.99 and 0.98. No points were possible for 0.01 mM because the mean per cent inhibition was <0 so that the transformed value to be plotted in (d) was undefined.

normal or unusual for K channels. Nevertheless, given the paucity of known blockers for stretch-activated K channels, knowledge of amiloride's action may eventually be helpful, in a biophysical context, as a distinguishing characteristic.

### *Gadolinium and diltiazem*

External gadolinium (Gd) can fully block cation-selective mechanosensitive channels at 10 μM; it is not specific for mechanosensitive channels, however, since it inhibits some Ca channels (Yang & Sachs, 1989). Do stretch-activated K channels share the sensitivity of stretch-activated cationic channels to Gd? It has been reported anecdotally (Yang & Sachs, 1989) that a rat astrocyte stretch-activated K channel is not Gd sensitive at 10 μM, and we have now shown that 100 μM had no discernible effect on molluscan stretch-activated K channels. Evidently, being sensitive to membrane tension does not in itself render channels susceptible to Gd.

The antiarrhythmic diltiazem blocks a wide spectrum of channels including cyclic nucleotide-gated cation channels (Chen *et al.*, 1993), L-type Ca channels (Budavari, 1989), *eag*-type K channels (Nguyen *et al.*, 1994) and an avian cationic channel of marginal mechanosensitivity (Ruknudin *et al.*, 1993) with $K_d$ values in the mocromolar range. Nevertheless, on stretch-activated K channels, 100 μM extracellular diltiazem had no effect. Since it has been reported (Ruknudin, *et al.*, 1993) that 20 μM external diltiazem inhibits a vertebrate stretch-activated cation channel, we tested 100 μM on *Xenopus* oocyte stretch-activated cationic channels but found that it had no effect (unpublished observation).

### *Tetraethylammonium*

The classic K channel blocker, TEA, binds to K channels with a 1:1 stoichiometry, usually producing a voltage-independent block from the outside and, from the inside, a voltage-dependent block at ~20% across the electric field (Pongs, 1992).

For external TEA block, $K_d$ varies from 0.1 mM (Rettig *et al.*, 1992) to >200 mM (Tasaki & Hagiwara, 1957) in many types of K channels [i.e. delayed rectifiers; 0.3 (Dubois, 1981) to >200 mM (Tasaki & Hagiwara, 1957); calcium-activated *K* 0.14 (Bokvist *et al.*, 1990) to 52.2 mM (Wong & Adler, 1986); inward rectifiers, <10 mM (Schachtman *et al.*, 1992)]. Stretch-activated K channels of *Lymnaea* neurones are shown

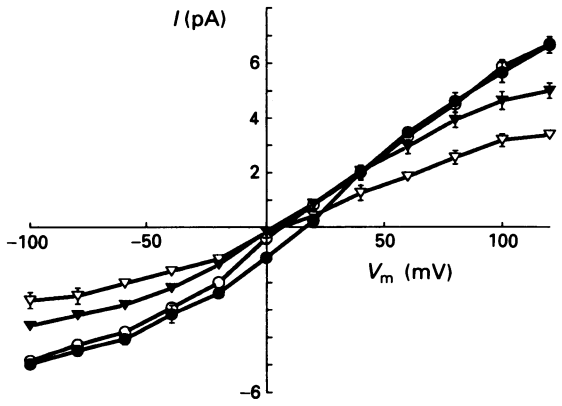


**Figure 4** Effects of 3% ethanol, quinidine and both together on stretch-activated K channels in cell-attached patches. Stretch-activated K channel $I/V$ relations. Symbols are as follows: control (○) $n = 7$, 3% ethanol (●) $n = 4$, 0.5 mM quinidine (△) $n = 3$, 0.5 mM quinidine in 3% ethanol (▲) $n = 10$.

here to have a high $K_d$ for external TEA block (~50 mM). Among the stretch-activated K channels, Hill coefficients for external TEA block are all less than 1.5, consistent with 1:1 stoichiometry for TEA channel binding. For the *Aplysia* (Shuster & Siegelbaum, 1987) and *Drosophila* (Gorczyca & Wu, 1991) channels, Hill coefficients were not originally given, but the data were sufficient to make the calculation, which yielded 0.9 and 1.3. In both cases, the block is voltage independent. For *Lymnaea* channels, our average Hill coefficient was 1.3 and block was voltage independent.

The intracellular TEA pharmacology of the two molluscan stretch-activated K channels differed, however. Intracellular TEA blocks the S-channel with $K_d$ 40 mM (Shuster & Siegelbaum, 1987), whereas 200 mM intracellular TEA was without effect on *Lymnaea* stretch-activated K channels. No information is available for the *Drosophila* channel on this issue.

## *Quinidine*

Quinidine is another agent commonly used to block K channels. In the 10–100 μM range it blocks delayed rectifiers, transient outward A-currents and Ca-activated K channels, as well as voltage-gated sodium channels. Extracellular quinidine produced a voltage-independent block of stretch-activated K channels with $K_d$ 0.8 mM. This is similar to quinidine's efficacy on the newly-cloned *reag* channels, which are blocked by quinidine with $IC_{50}$ 0.4 mM (Ludwig *et al.*, 1994).

Since quinidine is a tertiary amine of pK 8.9 (Rich *et al.*, 1994a), 95% would be cationic in a pH 7.6 aqueous solution. Because quinidine's actions on some channels occur in the 10–100 μM range, it is possible that the micromolar quantities of the neutral form (e.g. the bath concentration of neutral quinidine would be ~40μM at the $K_d$ we determined for stretch-activated K channels) are responsible for stretch-activated K channel block. If the neutral form blocked the channel via the bilayer, quinidine should have been equally effective presented from either side. We found, however, that quinidine blocked exclusively from the outside. This establishes that the site is external and accessed from the aqueous medium, but does not rule out the possibility that the neutral form is the effective species.

At the pH of our solutions (7.6) quinidine was predominantly cationic; blockade by a cation should be voltage dependent unless it occurs outside the voltage field. Because extracellular quinidine blocked stretch-activated K channels independent of voltage, we must conclude either that the neutral form is effective or that the cation acts at an external site. Since a quaternary cationic form of quinidine blocks hKv1.5 by binding to a site in the mouth of the channel (Rich *et al.*, 1994b), the second hypothesis seems more likely.

A two-state channel with open channel block would be consistent with our observations of a dose-dependent reduction of channel amplitude in the presence of quinidine (Figure 4). The single-channel amplitude would be decreased with filtering only when the on rate ($k_{on}$) of the blocking drug is large. This behaviour is typical of fast, open channel blockade (Moczydlowski, 1992), and seems the likeliest explanation for the effect of quinidine on stretch-activated K channels.

## *Ethanol*

It is thought that, by binding to the phospholipid head groups, ethanol interferes with the packing of acyl chains in the bilayer (Mitchell & Litman, 1994), thereby increasing membrane fluidity (Goldstein, 1986). For native membranes exposed to<2% ethanol, fluidity increases of as much as 12% have been measured (Silberman *et al.*,. 1990). Alcohols can affect ligand–channel interactions. On acetylcholine channels, ethanol (1.8%) causes the channel to open more readily and stay open longer (Dilger *et al.*, 1994); the efficacy of the agonist increases, not the agonist's affinity for the channel. In another case, PCP binding to acetylcholine channels is competitively inhibited by hexanol (Lin & Wang, 1994).

On stretch-activated K channels, 3% ethanol had no effect on conductance but decreased the efficacy of 0.5 mM quinidine block. Based on the conclusion that we have arrived at above, namely that quinidine is a fast, open channel blocker, ethanol decreased the $k_{on}$ for quinidine block.

Two possible explanations for our ethanol/quinidine interaction data can be envisaged. One is based on the somewhat exotic possibility that quinidine binds at the lipid/protein/aqueous interface. In that case, the interactions of ethanol with the phospholipid head groups (Mitchell & Litman, 1994) could hinder quinidine's action. A more likely possibility (Rich *et al.*, 1994b) is that there is a quinidine binding site in the mouth of the channel.

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# Effects of macrophage depletion on the induction of histidine decarboxylase by lipopolysaccharide, interleukin 1 and tumour necrosis factor

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1 Our previous work has shown that injection into mice of lipopolysaccharide (LPS) and the cytokines interleukin 1 (IL-1) and tumour necrosis factor (TNF) induces histidine decarboxylase (HDC), the enzyme forming histamine, in various tissues such as liver, lung, spleen and bone marrow, but not in the blood. The induction of HDC also occurs in nude mice and mast cell-deficient mice. On the other hand, haematopoietic cytokines such as IL-3, granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage CSF (GM-CSF) only induce HDC in the haematopoietic organs, i.e. bone marrow and spleen. In the present study, the effect of macrophage depletion on the induction of HDC was examined.

2 On day 1 after a single intravenous injection of a macrophage depletor (liposomes encapsulating dichloromethylene diphosphonate, which is toxic when ingested into macrophages), macrophages were almost completely depleted in the liver and reduced by about 50% in the spleen and bone marrow, but not significantly affected in the lung. On day 3, the degrees of the depletion were similar to those of day 1. In the spleen, macrophages were depleted in the red pulp, and there was a structural destruction.

3 In macrophage-depleted mice, the induction of HDC by LPS, IL-1α or TNF-α was not impaired in the liver, and was potentiated in the lung and bone marrow. The induction of HDC was decreased only in the spleen at day 3.

4 HDC was not induced by LPS in the spleen of the adult rat, which is correspondingly inactive in haematopoiesis.

5 These results indicate that the major cells in which HDC activity is induced in response to LPS, IL-1 and TNF are not circulating granulocytes, circulating monocytes, T cells derived from thymus, mast cells or phagocytic macrophages. Based on these results, we discuss the possibility that the major cells in which HDC was induced in non-haematopoietic and haematopoietic organs were endothelial cells and haematopoietic precursor cells respectively.

**Keywords:** Histidine decarboxylase; interleukin 1; cytokines; lipopolysaccharide; macrophages

## Introduction

Histamine affects the microcirculation by stimulating endothelial cells to increase capillary permeability and by stimulating microvascular smooth muscles to dilate them. The inflammatory response reflects these changes and is characterized by an increased capillary permeability and an increased blood flow in fine blood vessels caused by a capillary dilatation. We have shown that the activity of histidine decarboxylase (HDC), the enzyme forming histamine, is increased in the liver, lung, bone marrow and spleen in response to lipopolysaccharide (LPS), inflammatory mitogenic agents (Endo, 1982; 1983a) and cytokines such as IL-1 and TNF (Endo, 1989; Endo *et al.*, 1986; 1992a). As these tissues have a well-developed microcirculation, it is expected that, in addition to the release of histamine from mast cells or basophils, the newly formed histamine produced by the increased HDC activity may be involved in the activation of the microcirculation. Because the increase in HDC activity is accompanied by a rapid increase in mRNA for HDC (unpublished data), this increase is due to the induction of the HDC enzyme itself.

Histamine has also been shown to stimulate *in vitro* haematopoietic precursor cells such as granulocytic precursor cells or IL-3-responder cells (Byron, 1977; Nakaya & Tasaka, 1988; Shounan & You-Heng, 1988; Schneider *et al.*, 1990). In contrast to the induction of HDC in various tissues by LPS, IL-1 and TNF as described above, the haematopoietic cytokines IL-3, GM-CSF and G-CSF induce HDC preferentially in the haematopoietic organs, i.e. bone marrow and spleen (Lebel *et al.*, 1990; Endo *et al.*, 1992a). These results suggest that histamine is involved in the proliferation or differentiation of some haematopoietic precursor cells.

Apart from IL-3, other cytokines capable of inducing HDC (i.e. IL-1, TNF, GM-CSF and G-CSF) are known to be produced mainly by macrophages and endothelial cells (Oppenheim *et al.*, 1986; Beutler & Cerami, 1987; Dinarello, 1991). Correspondingly, the organs in which HDC is induced are the tissues rich in these cells, and LPS is known to be a potent stimulator of these cells to produce the cytokines described above. Therefore, macrophages or endothelial cells may be important in the induction of HDC.

Van Rooijen *et al.* (1989, 1990) have reported that intravenous injection of dichloromethylene diphosphonate, which was encapsulated in liposomes, can deplete macrophages from the liver and spleen. In the present study, therefore, the effect of the macrophage depletor on the induction of HDC by LPS, IL-1 and TNF was examined.

## Materials and methods

### *Animals and materials*

Male BALB/c mice (6 weeks old) were obtained from the mouse centre of our university. Wistar rats (7 weeks old)

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were obtained from Japan SLC (Shizuoka, Japan). LPS of *Escherichia coli* 055B5, prepared by Boivin's method, was obtained from Difco Labs (Detroit, MI, U.S.A.). Recombinant human IL-1α and recombinant human TNF-α, which were prepared according to Furutani *et al.* (1985) and Yamada *et al.* (1985), were provided by Dainippon Pharmaceutical (Osaka, Japan). Dichloromethylene diphosphonate was provided by Kissei Pharmaceutical (Matsumoto, Japan). Reagents and cytokines were dissolved in sterilized saline and injected intravenously (i.v.) or intraperitoneally (i.p.) (0.1 ml per 10 g body weight).

### *Depletion of macrophages from the liver*

Suspensions of liposomes encapsulating dichloromethylene diphosphonate ($Cl_2$MDP-liposomes) were prepared according to Van Rooijen *et al.* (1989, 1990). Briefly, 75 mg of phosphatidylcholine and 11 mg of cholesterol were dissolved in chloroform in a round-bottom flask. The thin film that formed on the walls of the flask after rotary evaporation at 37°C was dispersed by gentle shaking for 10 min in 10 ml of $Cl_2$MDP solution (200 mg $ml^{-1}$) in 10 mM sodium phosphate buffer (PBS, pH 7.4). This suspension was kept for 2 h at room temperature and sonicated for 3 min (50 Hz) and kept for another 2 h. The resulting liposomes were centrifuged at 100,000 *g* for 30 min. The volume of precipitated liposomes was about 0.5 ml, which contained a maximum of 100 mg of $Cl_2$MDP. The liposomes were finally suspended in 4 ml of PBS, and this preparation was injected intravenously into the tail vein (0.2 ml per mouse) within 3 days.

### *Detection of macrophages*

Macrophages were detected by immunohistochemical staining of F4/80 antigen which is expressed specifically on the surface of these cells (Austyn & Gordon, 1981; Hirsch *et al.*, 1981). Monoclonal antibody to F4/80 antigen was purchased from SEROTEK (Kindlington, U.K.).

Tissues were fixed with 4% paraformaldehyde in PBS. After washing with PBS, specimens were immersed in 20% sucrose in PBS, embedded in Cryoform (International Equipment, Needham, MA, U.S.A.) and quickly frozen in an acetone–dry ice mixture. After the fixation, tibias were decalcified in 15% EDTA in PBS, washed with 20% sucrose in PBS, embedded in Cryoform and quickly frozen in an acetone–dry ice mixture. Frozen sections (8 μm thick) were cut, placed on poly-L-lysine-coated glass slides and air dried. After incubation in 0.3% $H_2O_2$-methanol for 30 min, sections were incubated in 5% normal goat serum in PBS containing 5% bovine serum albumin and 0.025% Triton X-100, then incubated with an F4/80 monoclonal antibody. After several rinses, sections were incubated with a biotinylated goat anti-rat IgG antibody (Cappel, West Chester, PA, U.S.A.), followed by incubation with ABC (avidin–biotin complex) reagent (Vector Lab., Burlingame, CA, U.S.A.). After washing, sections were treated with a mixture of 0.05% 3,3-diaminobenzidine and 0.005% $H_2O_2$ in Tris–HCl buffer (0.05 M, pH 7.6). Counter-staining was produced with methyl green. As controls, sections were incubated with either normal rat serum or PBS instead of the primary antibody.

### *Electron microscopy*

The livers were removed rapidly from decapitated mice and electron microscopic analysis was carried out as described previously (Endo & Nakamura, 1992).

### *Assay of HDC activity*

HDC activity was assayed by a previously described method (Endo, 1983b) with a slight modification (Endo *et al.*, 1992b). HDC activity in the liver, lung and spleen was expressed as nmol of histamine formed during 1 h by the enzyme contained in 1 g of each tissue (nmol $h^{-1}$ $g^{-1}$). HDC activity in the bone marrow was expressed as the activity of 1 g of the tibia plus femur, because these bone tissues themselves were subjected to the assay of HDC activity (Endo *et al.*, 1992b).

### *Data analysis*

Experimental values are given as mean ± standard deviation. Statistical significance of differences between two means of data were evaluated by Student's unpaired *t*-test, and *P*-values less than 0.05 were considered to be significant.

## Results

### *Depletion of macrophages*

On day 1 after a single intravenous injection of $Cl_2$MDP-liposomes, F4/80-positive macrophages were almost completely depleted in the liver (Figure 1a and b). In the spleen and bone marrow, depletion of the macrophages was about 50% (Figure 1c–f). In the spleen, macrophages were depleted in the region of the red pulp. On day 3, the degrees of the depletion in the liver, spleen and bone marrow were similar to those observed on day 1. These results are essentially the same as those observed by Van Rooijen *et al.* (1989, 1990). There was no apparent depletion of macrophages in the lung on either day 1 or day 3.

In spite of the drastic depletion of macrophages in the liver, no other histochemical changes were observed in this organ. Electron microscopic analysis indicated that endothelial cells were not decreased in the liver of mice treated with the liposome (Figure 2). In addition, there was no loss of body weight and no apparent sign of illness or weakness in the mice. There was a structural destruction only in the red pulp in the spleen (Figure 3) and a slight decrease in its weight on day 3 (about 20%).

### *Effect of macrophage depletion on the induction of histidine decarboxylase*

Injection of LPS into mice induced a marked increase in HDC activity in the liver, lung, spleen and bone marrow of control mice treated with saline. On day 1 after injection of the liposomes, the induction of HDC by LPS was not decreased and was, in fact, enhanced in the lung and bone marrow (Figure 4).

Both IL-1 and TNF can also induce HDC, and this induction is potentiated by their combination (Endo, 1989). As shown in Figure 5, a combination of 10 μg $kg^{-1}$ of each of IL-1 and TNF induced HDC activities similar to those evoked by 0.1 mg $kg^{-1}$ LPS. On day 3 after injection of the liposomes, the induction of HDC by LPS or by the cytokines was not decreased in the liver, lung and bone marrow, and was again enhanced in the lung and bone marrow as observed on day 1. A significant decrease in the induction of HDC by LPS or by the cytokines was observed only in the spleen.

### *Comparison of the induction of histidine decarboxylase activity by lipopolysaccharide in mice and rats*

Compared with the experiments described above, a large dose of LPS (1 mg $kg^{-1}$) was injected into rats and mice. As shown in Figure 6, LPS induced HDC in a number of tissues of these animals. However, while there was a large increase in the level of the enzyme in the spleen of mice, there was no increase in HDC activity in the spleen of rats, even by the large dose of LPS used in this experiment.

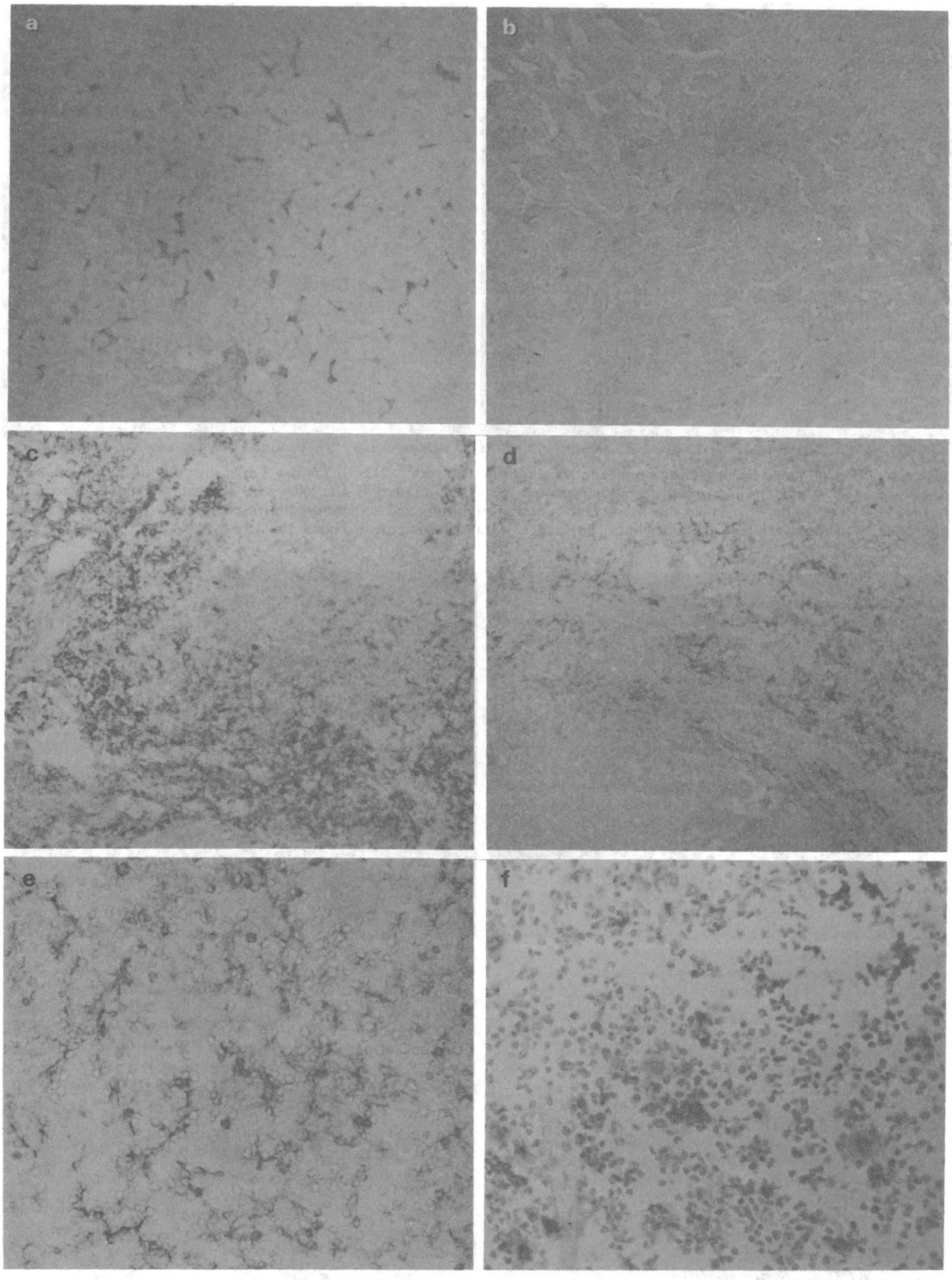


**Figure 1** Effect of a macrophage depletor on mouse tissues on day 1 after its injection ( × 180). On day 1 after intravenous injection of saline or liposomes encapsulating dichloroethylene diphosphonate ($Cl_2$MDP-liposomes), the liver, spleen and tibia were subjected to immunohistochemical staining for F4/80 antibody. There are many F4/80-positive cells in the normal liver (a), spleen (c) and bone marrow of tibia (e). In the mice treated with $Cl_2$MDP-liposomes, these cells are almost completely depleted in the liver (b) and reduced by about 50% in the spleen (d) and bone marrow of tibia (f).

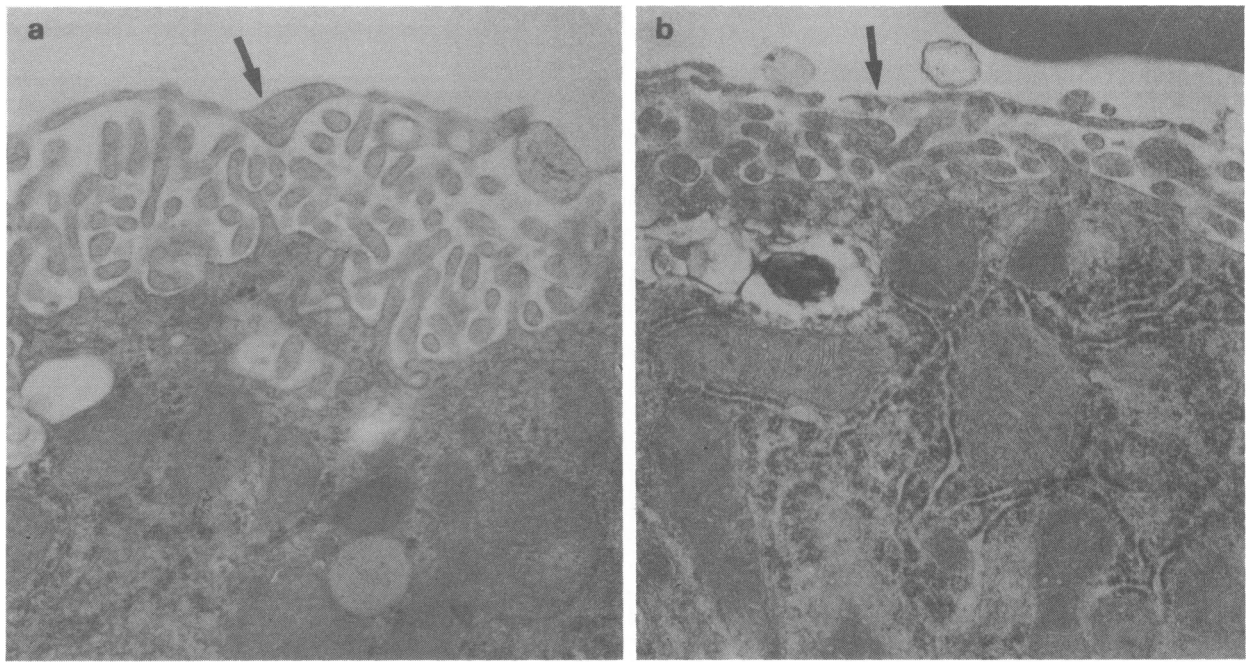


**Figure 2** Effect of a macrophage depletor on endothelial cells in the liver ( × 21,000). On day 3 after intravenous injection of saline or liposomes encapsulating dichloroethylene diphosphonate ($Cl_2$MDP-liposomes), the mice were injected with lipopolysaccharide (0.1 mg $kg^{-1}$, i.p.) and, 4.5 h later, the livers were removed and subjected to electron microscopic analysis. Endothelial cells (indicated by arrows) are seen in both control (a) and $Cl_2$MDP-liposome-treated mice (b).

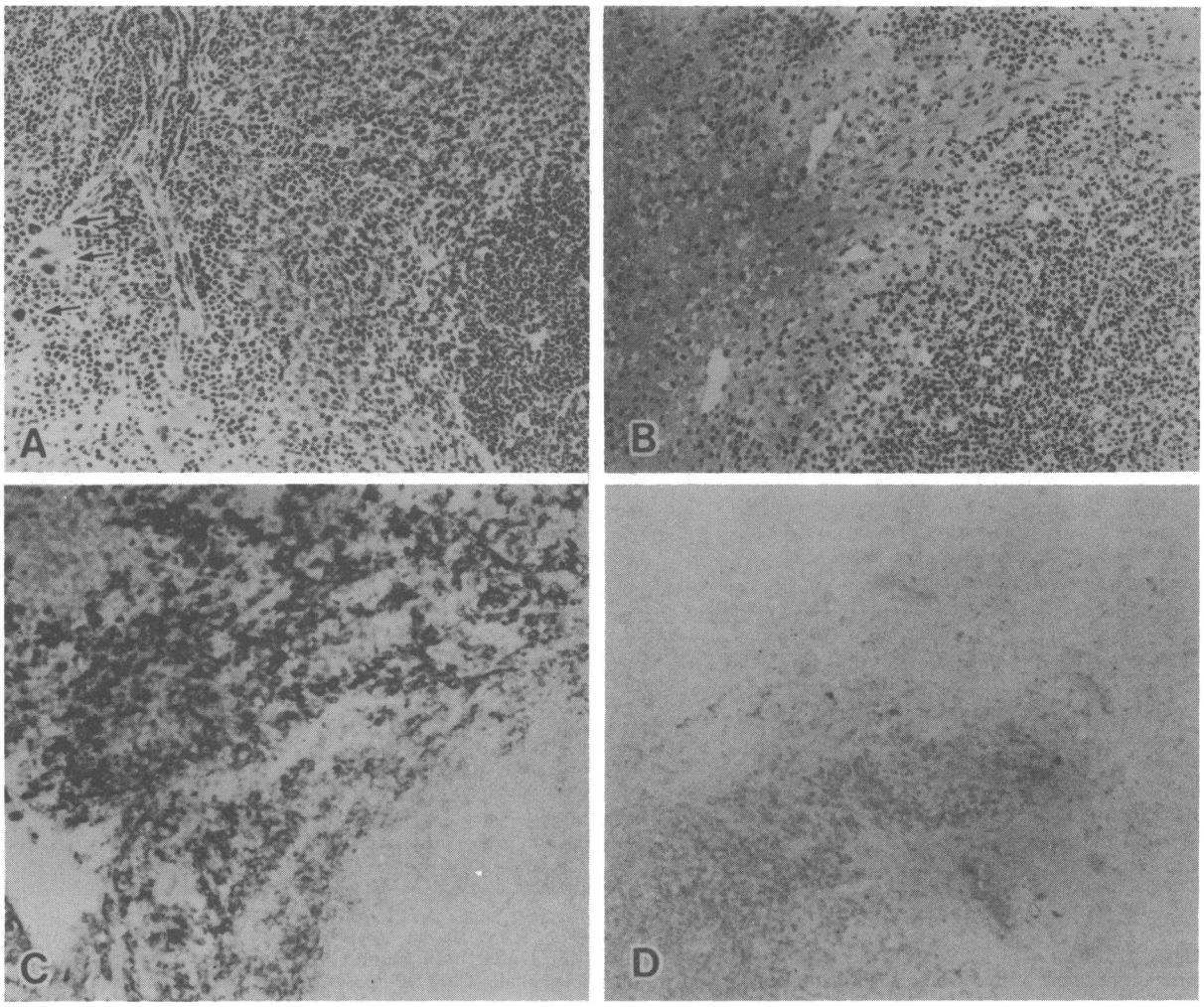


**Figure 3** Effect of a macrophage depletor on the structure of the red pulp in the spleen of mice ( × 180). On day 3 after intravenous injection of saline or liposomes encapsulating dichloroethylene diphosphonate ($Cl_2$MDP-liposomes), the spleens were subjected to haematoxylin–eosin staining (A and B) and immunohistochemical staining for F4/80 (C and D). (A) There are many cells including megakaryocytes (arrows) in the red pulp of the control mice. (B) Cells are distributed sparsely in the red pulp of the mice treated with $Cl_2$MDP-liposomes, and there are no megakaryocytes. (C) There are many F4/80-positive cells in the red pulp of the control mice. (D) There are no F4/80-positive cells in the red pulp of the mice treated with $Cl_2$MDP-liposomes.

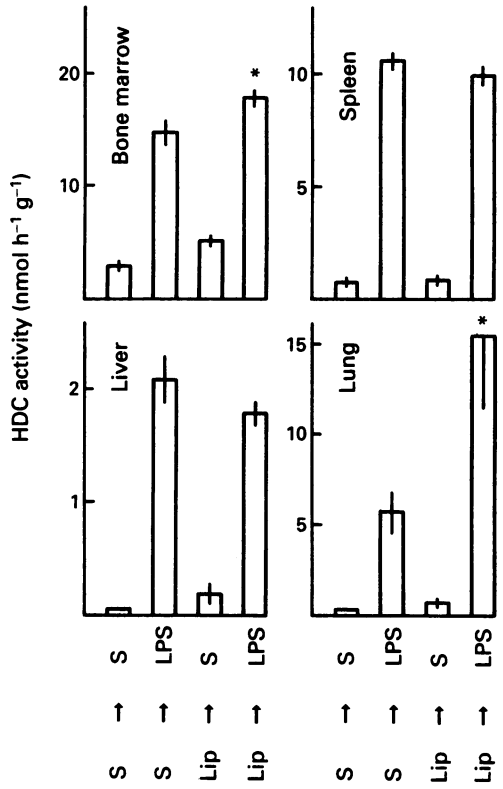


**Figure 4** Induction of histidine decarboxylase (HDC) on day 1 after injection of a macrophage depletor. On day 1 after intravenous injection of saline (S) or liposomes encapsulating dichloroethylene diphosphonate (Lip), the mice were injected with lipopolysaccharide (LPS, 0.1 mg $kg^{-1}$, i.p.) and, 4.5 h later, tissues were removed and HDC activities were measured. Each value is the mean ± s.d. of four mice. *$P<0.05$ vs saline LPS.

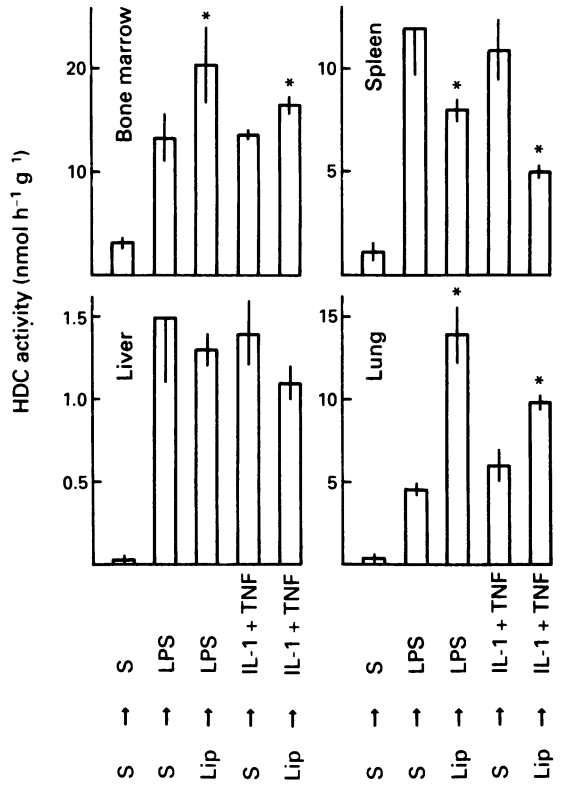


**Figure 5** Induction of histidine decarboxylase (HDC) on day 3 after injection of a macrophage depletor. On day 3 after intravenous injection of saline (S) or liposomes encapsulating dichloroethylene diphosphonate (Lip), the mice were injected with lipopolysaccharide (LPS, 0.1 mg $kg^{-1}$, i.p.) or IL-1α plus TNF-α (10 μg $kg^{-1}$ each, i.p.) and, 4 h later, tissues were removed and HDC activities were measured. Each value is the mean ± s.d. of four mice. *$P<0.05$ vs saline LPS or saline IL-1 + TNF.

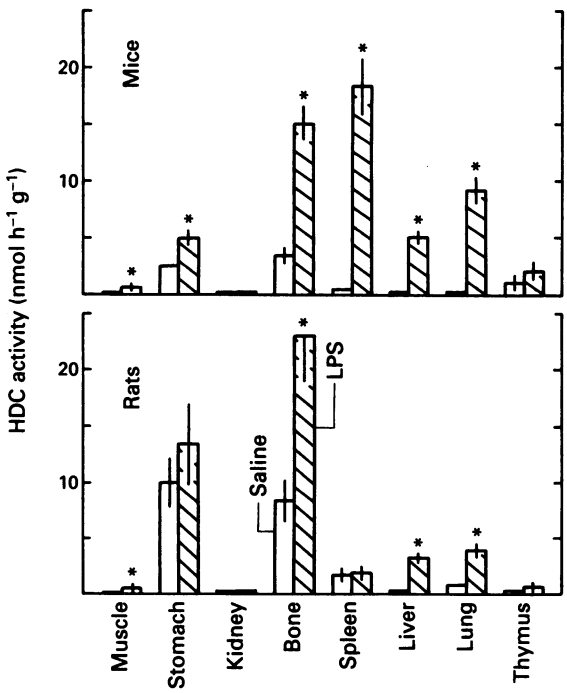


**Figure 6** Comparison of the induction of histidine decarboxylase (HDC) between mice and rats. Mice (6 weeks old) and rats (7 weeks old) were injected with saline (open columns) or lipopolysaccharide (hatched columns, 1 mg $kg^{-1}$, i.p.) and, 4.5 h later, their tissues were removed and HDC activities were measured. Each value is the mean ± s.d. of four mice. *$P<0.05$ vs control (saline injected).

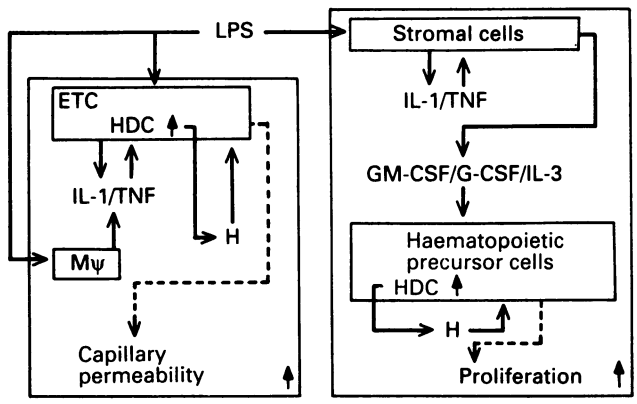


**Figure 7** Hypothetical schema of the induction of histidine decarboxylase (HDC) in response to lipopolysaccharide (LPS). In non-haematopoietic organs such as the liver, lung and skeletal muscle (left), LPS stimulates endothelial cells (ETC) and/or macrophages (Mψ), resulting in a production of IL-1 and/or TNF. These cytokines stimulate the ETC and induce HDC within them. The newly formed histamine also stimulates the ETC, resulting in an enhanced capillary permeability. In haematopoietic organs (right), LPS stimulates stromal cells to produce IL-1 and/or TNF. These cytokines stimulate the stromal cells and result in production of GM-CSF, G-CSF and IL-3. These cytokines stimulate haematopoietic precursor cells and induce HDC in these cells. The newly formed histamine stimulates the haematopoietic precursor cells themselves to stimulate their proliferation.

## Discussion

We have shown that injection into mice of various inflammatory mitogenic substances such as LPS and concanavalin A (Endo, 1983a) or cytokines such as IL-1, TNF, G-CSF and GM-CSF (Endo, 1989; Endo *et al.*, 1986, 1992a) induces HDC in various tissues. HDC activities are also increased by LPS in nude mice to similar extents as in normal controls (Endo, 1983a). $W/W^V$ mice are known to be deficient in mast cells, but LPS also induces HDC activity in these animals to a rather greater extent than in controls (Endo & Nakamura, 1993). These results indicate that neither lymphocytes derived from the thymus (i.e. T-cells) nor mast cells are the major cells involved in the induction of HDC. Hepatocytes are similarly not the major cells involved in the induction of HDC in the liver, because inhibition of RNA synthesis in these cells does not decrease the induction of HDC in this organ, although the induction of ornithine decarboxylase is strongly inhibited (Endo *et al.*, 1992c). In addition, the experiment on the isolation of liver cells indicated that the increase in histamine after injection of LPS occurred largely in cells other than hepatocytes (Endo & Nakumura, 1993).

The increase in HDC activity is most marked in the tissues rich in macrophages and endothelial cells, such as liver, lung, spleen and bone marrow. Macrophages and endothelial cells are known to be the major cells which produce the cytokines capable of inducing HDC, i.e. IL-1, TNF, G-CSF and GM-CSF. It is also possible that HDC is induced within these cells.

F4/80 antigen has been shown to be present specifically on the surface of macrophages, but not on neutrophils, mast cells, eosinophils, lymphocytes, dendritic cells, fibroblasts, erythrocytes, platelets and immature precursor cells of macrophages and neutrophils (Austyn & Gordon, 1981; Hirsch *et al.*, 1981). Within 24 h of a single intravenous injection of $Cl_2$MDP-liposomes, F4/80-positive macrophages were almost completely depleted in the liver, reduced by about 50% in the spleen and bone marrow, but not affected in the lung. When $Cl_2$MDP-liposomes are administered intratracheally, alveolar macrophages have been shown to be eliminated (Thepen *et al.*, 1989). On day 3, the degrees of the depletion in these tissues were similar to those observed on day 1. There were no other noticeable changes in these tissues of the mice given $Cl_2$MDP-liposomes except for the spleen. In the spleen on day 3, there was a slight decrease in its weight. Macrophages in the spleen decreased in the red pulp as observed by Van Rooijen *et al.* (1989). Histochemical staining of the spleen indicated a structural destruction of the red pulp. An electron microscopical examination indicated that endothelial cells in the liver are normal after injection of $Cl_2$MDP-liposomes.

In spite of complete depletion of macrophages or Kupffer's cells in the liver, the induction of HDC by LPS or IL-1 plus TNF was not impaired on either day 1 or day 3. In the lung and bone marrow, the HDC induction by LPS or IL-1 plus TNF was actually enhanced in the mice treated with $Cl_2$MDP-liposomes on both day 1 and day 3. There was a decrease in the induction of HDC only in the spleen on day 3. However, there was no reduction of HDC induction in the spleen on day 1 when about 50% of its macrophages had been depleted. These results suggest that the reduction of HDC induction in the spleen on day 3 is not due to the decrease in macrophages. Therefore, phagocytic macrophages located at the sites where they are exposed to $Cl_2$MDP-liposomes and eliminated from tissues are not the major cells in which HDC is induced. In addition, these results also indicate that the phagocytic macrophages are not the major cells which produce the cytokines capable of inducing HDC in response to LPS.

As HDC activity is not detected in the blood even after injection of LPS (Endo, 1982), circulating or mature granulocytes and circulating macrophages (i.e. monocytes) are not the cells in which HDC is induced. The present results, however, do not rule out a possible involvement of non-phagocytic or F4/80-negative macrophages.

The tissues in which HDC is induced have well-developed microvascular systems. Histamine activates endothelial cells to enhance capillary permeability and induces capillary dilatation to increase blood flow. These are essential mechanisms in inflammation for supplying or exchanging various substances and enabling immune cells to move outside the blood vessels. We have observed that forced walking for long hours or *in situ* electric stimulation of skeletal muscles of mice induces a marked HDC activity in the skeletal muscles (unpublished data). In order to keep the mobility of the skeletal muscles or to recover from fatigue, enhanced microcirculation may be required in the skeletal muscles. Schayer (1962), who found that HDC was an inducible or adaptive enzyme, has suggested that the induced histamine that is produced by the induction of HDC is an intrinsic regulator of the microcirculatory system. Although it is speculative at present, our results suggest that endothelial cells may be the major cells involved in the induction of HDC in tissues such as the liver, lung and skeletal muscles. IL-1 and/or TNF derived from endothelial cells (or non-phagocytic macrophages) may induce HDC in the endothelial cells. In addition, endothelial cells may stimulate themselves by producing histamine through the induction of HDC, implying an autocrine or paracrine mechanism.

It is also possible that the newly formed histamine may be utilized in the regulation of various immune responses (Beer *et al.*, 1984), including haematopoiesis. It is known that haematopoiesis in the spleen is inactive in adult rats. In correspondence with this, HDC induction by LPS did not occur in the spleen of adult rats. The red pulp in the spleen of mice is the site of haematopoiesis. The reduction in HDC induction observed in the spleen on day 3 after injection of $Cl_2$MDP-liposomes may be due to a structural destruction of the red pulp, i.e. an impaired haematopoiesis. In addition, haematopoietic cytokines, IL-3, G-CSF and GM-CSF, induce HDC only in the haematopoietic organs of mice (Lebel *et al.*, 1990; Endo *et al.*, 1992a). Therefore, these results suggest that haematopoietic cells or stromal cells are involved in the induction of HDC in haematopoietic organs. The term 'stromal cells' is the generic name for various cells which support haematopoiesis and includes endothelial cells, macrophages and fibroblasts. Stromal cells are known to produce IL-3, G-CSF and GM-CSF in response to LPS, IL-1 and TNF (Dorshkind, 1990). Because spleens of adult rats contain endothelial cells, macrophages and fibroblasts, it seems unlikely that stromal cells are the cells in which HDC is induced. Therefore, the most likely cells in which HDC is induced in the spleen and bone marrow seem to be haematopoietic precursor cells.

Histamine stimulates proliferation of haematopoietic precursor cells, such as granulocytic precursor cells or IL-3 responder cells (Byron, 1977; Shounan & You-Heng, 1988; Nakaya & Tasaka, 1988; Schneider *et al.*, 1990). In addition, we have shown that a prolonged enhancement of HDC activity accompanies an increase in granulocytes, macrophages and osteoclasts (Endo *et al.*, 1993), whose precursor cells are known to be identical. Therefore, we expect that HDC is induced in these haematopoietic precursor cells and that newly formed histamine may stimulate the cells themselves, again implying an autocrine or paracrine mechanism.

In conclusion, the major cells in which HDC is induced in response to LPS, IL-1 and TNF are cells other than T-cells, mast cells, phagocytic macrophages, mature granulocytes, circulating monocytes and hepatocytes. The most likely candidate in the liver, lung and skeletal muscles appears to be endothelial cells. On the other hand, in the haematopoietic organs, the candidate seems to be haematopoietic precursor cells. These hypotheses are summarized in Figure 7. We are currently attempting to visualize the cellular distribution of

HDC mRNA by *in situ* hybridization techniques but have encountered difficulties owing to the low content of the RNA.

Finally, it is necessary to consider the significance of the induction of HDC. As pharmacological administration of histamine produces shock, histamine has been believed to be an important mediator of shock syndromes. Therefore, the induction of HDC appears to contribute in the development of endotoxin shock or septic shock, because LPS is a potent inducer of HDC. Galactosamine is known to sensitize experimental animals to the lethal effect of LPS, and their combined administration produces severe congestion in the liver, resulting in a rapid death. We have shown that galactosamine enhances the LPS-induced elevation of hepatic HDC activity in mice (Endo *et al.*, 1992c). However, even a complete inhibition of the HDC activity by an irreversible inhibitor of the enzyme prevented neither the hepatic congestion nor the death, indicating that the induction of HDC is not involved in the hepatic congestion. Neugebauer *et al.* (1987) have also shown that histamine is not a predominant factor in the lethal outcome of endotoxin shock in rats induced by a large amount of LPS alone.

Major actions of histamine, such as dilatation and increased permeability of capillary vessels, stimulation of sensory nerve endings producing itch and pain and stimulation of gastric acid secretion, are responses to exogenous stimuli or materials. IL-1, IL-3, GM-CSF and G-CSF, i.e. HDC-inducing cytokines, are important factors triggering non-specific immune responses and stimulating proliferation of various immune cells. Therefore, it seems likely that the induction of HDC is an event in self-defence mechanisms.

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# Vascular pharmacology of methylene blue *in vitro* and *in vivo*: a comparison with $N^G$-nitro-L-arginine and diphenyleneiodonium

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**1** The vascular effects of the soluble guanylyl cyclase inhibitor, methylene blue as well as the nitric oxide (NO) synthase inhibitors, $N^G$-nitro-L-arginine (L-NOARG) and diphenyleneiodonium (DPI) were studied in rat isolated aortic rings and conscious, unrestrained rats.

**2** Acetylcholine (ACh) and sodium nitroprusside (SNP) caused concentration-dependent relaxation of preconstricted aortic rings. Both methylene blue ($1 \times 10^{-5}$ M) and L-NOARG ($3 \times 10^{-5}$ M) abolished ACh-induced relaxation; however, methylene blue but not L-NOARG shifted the concentration-response curve of SNP to the right.

**3** In conscious rats, i.v. infusion of methylene blue ($1.1 \times 10^{-5}$ mol kg$^{-1}$ min$^{-1}$), at a concentration which reduced the aortic tissue level of cyclic GMP by 50%, did not significantly alter mean arterial pressure (MAP) and heart rate (HR). In contrast, i.v. bolus injection of L-NOARG ($1.5 \times 10^{-4}$ mol kg$^{-1}$) markedly increased MAP and decreased HR.

**4** Both ACh and SNP dose-dependently decreased MAP in conscious rats. Methylene blue did not alter the magnitude or duration of ACh- or SNP-induced depressor responses. L-NOARG, on the other hand, significantly though incompletely, reduced the magnitude and duration of the depressor response to ACh but not SNP. The depressor response to ACh or SNP was not altered by pretreatment with indomethacin ($1.4 \times 10^{-5}$ mol kg$^{-1}$) or capsaicin ($3.3 \times 10^{-4}$ mol kg$^{-1}$).

**5** $N^G$-nitro-L-arginine methyl ester (L-NAME) also caused dose-dependent increases in MAP in conscious rats. Both methylene blue and DPI ($1 \times 10^{-5}$ mol kg$^{-1}$) selectively shifted the dose-pressor response curve of L-NAME to the right.

**6** These results suggest that: (1) the inhibition of endogenous NO biosynthesis does not necessarily lead to pressor response *in vivo*, (2) L-NOARG may not produce pressor response solely via the inhibition of endogenous endothelial NO biosynthesis, and (3) the depressor responses to ACh and SNP may not involve the release of NO or prostanoids or afferent nerve transmitters.

**Keywords:** Methylene blue; $N^G$-nitro-L-arginine (L-NOARG); nitric oxide (NO); acetylcholine (ACh); sodium nitroprusside (SNP); diphenyleneiodonium (DPI); zaprinast; guanosine 3':5'-cyclic monophosphate (cyclic GMP); indomethacin; capsaicin; blood pressure

## Introduction

Nitric oxide (NO) is known to activate soluble guanylyl cyclase, causing the formation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) and vascular relaxation (Schultz *et al.*, 1977; Katsuki *et al.*, 1977; Gruetter *et al.*, 1979; Martin *et al.*, 1985; Ignarro *et al.*, 1986). $N^G$-substituted arginine (Arg) derivatives, which include $N^G$-nitro-L-Arg (L-NOARG) and its methyl ester L-NAME, are well-known to inhibit endothelium-dependent relaxation and produce pressor response in whole animals. It has been postulated that the Arg analogues vasoconstrict *in vivo via* the inhibition of endothelial NO biosynthesis (Rees *et al.*, 1989; Aisaka *et al.*, 1989b; see review by Moncada *et al.*, 1991).

This hypothesis is supported by several observations: Arg analogues inhibit NO biosynthesis and endothelium-dependent relaxation *in vitro* (Palmer *et al.*, 1988; Rees *et al.*, 1990; Wang *et al.*, 1993a,b) and *ex vivo* (Rees *et al.*, 1990; Pucci *et al.*, 1992; Wang *et al.*, 1993a), the inhibition of endothelium-dependent relaxation *in vitro* and the vasoconstriction response to the Arg analogues *in vivo* are antagonized by L-Arg but not D-Arg (Rees *et al.*, 1989, 1990; Wang *et al.*, 1991, 1993b), and the inability of conventional pharmacological antagonists (e.g., reserpine, mecamylamine, phentolamine, captopril) to block the vasoconstrictor responses to Arg analogues (Rees *et al.*, 1989; Wang & Pang, 1991, 1993a). However, there is evidence to challenge this hypothesis. Firstly, $N^G$-substituted Arg analogues caused greater inhibition of endothelium-dependent relaxation *in vitro* than vasodilatation *in vivo* (Rees *et al.*, 1990; Wang *et al.*, 1993b; see review by Moncada *et al.*, 1991). Secondly, diphenyleneiodonium (DPI), which is chemically unrelated to Arg (Stuehr *et al.*, 1991; Kwon *et al.*, 1991; Keller *et al.*, 1992), suppressed endothelium-dependent vasodilatation *in vitro* and *in vivo* (Stuehr *et al.*, 1991; Rand & Li, 1993; Wang *et al.*, 1993c), but did not cause pressor response related to the inhibition of NO biosynthesis (Wang & Pang, 1993a,b; Wang *et al.*, 1993c). Therefore, further investigations using other inhibitors of the NO/cyclic GMP pathway are needed to resolve the issue of whether or not *in vivo* inhibition of the NO/cyclic GMP pathway is causally related to the elevation of blood pressure and suppression of NO-dependent vasodilatation.

Methylene blue is a suitable drug to examine this hypothesis. It interferes directly with soluble guanylyl cyclase activated by NO, but not by arachidonic acid, protoporphyrin or phenylhydrazine (Gruetter *et al.*, 1981; see review by Ignarro & Kadowitz, 1985; see Gryglewski *et al.*, 1992), via the oxidation of its active haemo centre (Martin *et al.*, 1985) or inactivation of its haemo-deficient apoenzyme (Tsai *et al.*, 1983), thereby reducing the level of cyclic GMP. Like oxyhaemoglobin, methylene blue binds to and inactivates NO (Marshall *et al.*, 1988; Wolin *et al.*, 1990). Furthermore, the release of NO by organic nitrates is suppressed by methylene

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blue (Feelisch & Noack, 1987). Methylene blue should therefore inhibit the vasodilatation response to NO, irrespective of its release by endothelium-dependent or endothelium-independent vasodilators, and elicit a pressor response via inhibition of the action of endogenous NO.

This study examined the *in vitro* as well as *in vivo* inhibitory effects of methylene blue on vasodilatation responses to both acetylcholine (ACh) and sodium nitroprusside (SNP) in preconstricted rat aortic rings and conscious, unrestrained rats, respectively. The effects of methylene blue were compared with those of L-NOARG on blood pressure and inhibitions of ACh- and SNP-induced relaxation *in vivo* as well as *in vitro*. To evaluate the effectiveness of methylene blue in inhibiting guanylyl cyclase activity *in vivo*, the effect of methylene blue on aortic tissue cyclic GMP content and depressor response to zaprinast, the cyclic GMP phosphodiesterase inhibitor (Kukovetz *et al.*, 1979; Lugnier *et al.*, 1983, 1986), were also investigated. The possible selective inhibitory effect of methylene blue on pressor response induced by L-NAME *in vivo* was compared to that of DPI.

It was recently reported that vasodilator response to ACh in the rat skin microvasculature (Ralevic *et al.*, 1992) and SNP in feline cerebral arterioles (Wei *et al.*, 1992) also involved the release of transmitters from sensory nerve endings, as these responses were inhibited by capsaicin. Although ACh-induced vasodilatation is generally believed not to involve prostanoid biosynthesis (Furchgott & Zawadzki, 1980; Wang *et al.*, 1993a), there are reports of the attenuation by indomethacin of ACh-induced vasodilatation *in vivo* (Cairns *et al.*, 1991; Rosenblum *et al.*, 1992). Therefore, the effects of capsaicin and indomethacin on depressor responses to ACh and SNP *in vivo* were also investigated.

## Methods

Sprague-Dawley rats (320–420 g) were used for all studies. Each group consisted of 5–6 rats *in vivo*, or 6–7 aortic rings *in vitro*, with each ring derived from a different rat.

### *Isolated aortic preparations*

The rats were killed by a blow on the head followed by exsanguination. The thoracic aorta was removed and cleared of connective tissue. Four ring segments of 0.5 cm length were prepared from one aorta and suspended randomly in separate organ baths. Each ring was connected to a Grass FT-03-C force-displacement transducer for isometric recording with a preload of 1 g and equilibrated for 1 h (with 3 washouts) in normal Krebs solution (pH 7.4, 37°C) bubbled with 95% $O_2$ and 5% $CO_2$. The Krebs solution had the following composition (mM): NaCl 118, glucose 11, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, $MgCl_26H_2O$ 1.2.

The rings were incubated with the vehicle or drugs (see later). Afterwards, $10^{-6}$ M ($EC_{90}$) phenylephrine was added to the baths and 20–25 min later, at the steady state phase of the contractile response to phenylephrine, a cumulative concentration-response curve to ACh ($10^{-8}$ to $3 \times 10^{-8}$ M) or SNP ($10^{-8}$ to $10^{-6}$ M) was constructed. Each concentration of ACh or SNP was left in the bath for 1–3 min to reach a plateau response. The time taken to complete each curve was approximately 20 min.

In studies involving the measurement of cyclic GMP, the thoracic aorta was rapidly removed, placed in Krebs solution at 4°C and trimmed of connective tissue. Each aorta was divided into two segments and bathed for 3 min at 37°C in Krebs solution (with or without added drug) prior to being clamp-frozen and stored at − 70°C. The frozen tissues were processed within 1 week as described by Diamond & Hartle (1974) and Diamond & Blisard (1976). Briefly, the tissues (15–40 mg) were homogenized in 1 ml of cold (4°C) 6% trichloroacetic acid (TCA) and the homogenate was centrifuged for 20 min at 2000 *g*. TCA was later removed from the supernatant by extraction (5 ×) with 5 ml water-saturated ether. Any remaining ether was removed by warming the tubes for 5 min in a 80°C water bath. The cyclic GMP levels in the extracts were measured by a radioimmunoassay kit (Amersham International) and expressed as pmol $g^{-1}$ wet weight of tissue.

### *Surgery*

The rats were anaesthetized with halothane (4% in air for induction and 1.5% in air for surgery). A polyethylene cannula (PE50) was inserted into the left iliac artery for the measurements of mean arterial pressure (MAP) by a pressure transducer (P23DB, Gould Statham, CA, U.S.A.), and heart rate (HR), which was determined electronically from the pulse pressure using a tachograph (Grass, model 7P4G). PE50 cannulae were also inserted into both iliac veins for the administration of the vehicle or drugs. The rats were given $>6$ h to recover from the effects of halothane and surgery before use as conscious and unrestrained rats.

### *Experimental protocol*

*(1) Effects of methylene blue and L-NOARG on relaxation responses to ACh and SNP* Six groups of aortic rings were incubated with the vehicle (normal saline, 50 µl), methylene blue ($10^{-5}$ M) or L-NOARG ($3 \times 10^{-5}$ M), followed 10 min later by the addition of phenylephrine ($10^{-6}$ M) and the construction of a concentration-response curve to ACh ($10^{-8}$ to $3 \times 10^{-5}$ M) or SNP ($10^{-8}$ to $10^{-6}$ M).

*(2) Effects of methylene blue and L-NOARG on MAP and HR in conscious rats* Two groups of rats were i.v. infused with methylene blue ($1.1 \times 10^{-5}$ mol $kg^{-1}$ $min^{-1}$) or given a bolus injection of L-NOARG ($1.5 \times 10^{-4}$ mol $kg^{-1}$). MAP and HR were monitored for 2 h.

*(3) Effects of methylene blue and L-NOARG on depressor response to zaprinast in conscious rats* Three groups of rats were i.v. infused with the vehicle (normal saline, 0.09 ml $kg^{-1}$ $min^{-1}$), methylene blue ($1.1 \times 10^{-5}$ mol $kg^{-1}$ $min^{-1}$) or i.v. bolus injected with L-NOARG ($1.5 \times 10^{-4}$ mol $kg^{-1}$), followed 20 min later by i.v. bolus injections of zaprinast ($7.4 \times 10^{-6}$ to $2.4 \times 10^{-4}$ mol $kg^{-1}$), at dose-intervals of 5 min.

*(4) Effects of methylene blue and L-NOARG on depressor responses to ACh and SNP in conscious rats* Four groups of rats were given the vehicle (normal saline, 0.09 ml $kg^{-1}$ $min^{-1}$ by i.v. infusion or 8 ml $kg^{-1}$ by i.v. bolus), methylene blue ($1.1 \times 10^{-5}$ mol $kg^{-1}$ $min^{-1}$, i.v. infusion) or L-NOARG ($1.5 \times 10^{-4}$ mol $kg^{-1}$, i.v. bolus), followed 20 min later by i.v. bolus injections of ACh ($3.4 \times 10^{-10}$ to $8.8 \times 10^{-8}$ mol $kg^{-1}$) and SNP ($1.7 \times 10^{-9}$ to $4.2 \times 10^{-7}$ mol $kg^{-1}$), at dose-intervals of 3–7 min, to allow the recovery of depressor responses. During the recovery phase, the chart speed was increased to facilitate the measurement of half-recovery time. The sequence of ACh and SNP administered was reversed in half of the rats in each group with a recovery interval of 20 min.

*(5)* Ex vivo *effects of methylene blue on cyclic GMP level in aorta in the absence and presence of sodium nitroprusside* Two groups of rats were given i.v. infusion of either the vehicle (normal saline, 0.09 ml $kg^{-1}$ $min^{-1}$) or methylene blue ($1.1 \times 10^{-5}$ mol $kg^{-1}$ $min^{-1}$) for 30 min. The thoracic aortae were divided into two segments and incubated for 3 min in Krebs solution with or without added SNP ($10^{-6}$ M). Afterwards, the segments were frozen in liquid nitrogen and stored at − 70°C for less than 1 week until assayed for cyclic GMP levels. All assays were run in blocks

of four aortic segments (with or without added SNP) from a control rat and a methylene blue treated-rat.

*(6) Effects of methylene blue on pressor responses to L-NAME and noradrenaline in conscious rats* Two groups of rats were i.v. infused with the vehicle (normal saline, 0.09 ml $kg^{-1}$ $min^{-1}$) or methylene blue ($1.1 \times 10^{-5}$ mol $kg^{-1}$ $min^{-1}$). Twenty min later, cumulative dose-MAP response curves to L-NAME ($10^{-6}$ to $1.2 \times 10^{-4}$ mol $kg^{-1}$) were constructed, at dose-intervals of 10 min, in order to obtain plateau responses. Another two groups were treated similarly, except that noradrenaline ($3 \times 10^{-10}$ to $4 \times 10^{-8}$ mol $kg^{-1}$) was given instead of L-NAME.

*(7) Effect of indomethacin on pressor response to L-NAME in conscious rats* Two groups of rats were i.v. bolus injected with the vehicle (100% DMSO, 1 ml $kg^{-1}$) or indomethacin ($1.4 \times 10^{-5}$ mol $kg^{-1}$). Twenty min later, cumulative dose-MAP response curves of L-NAME ($10^{-6}$ to $1.2 \times 10^{-4}$ mol $kg^{-1}$) were constructed, at dose-intervals of 10 min.

*(8) Effects of DPI on pressor responses to L-NAME, phenylephrine and angiotensin II* The first two groups of rats were i.v. bolus injected with the vehicle of DPI ($10^{-5}$ mol $kg^{-1}$) followed 20 min later by the construction of a cumulative dose-response curve of L-NAME (i.v. bolus, $10^{-6}$ to $1.2 \times 10^{-4}$ mol $kg^{-1}$ for the control group and $10^{-5}$ to $1.2 \times 10^{-3}$ mol $kg^{-1}$ for the DPI-pretreated group). Another two groups of vehicle- or DPI-pretreated rats were given i.v. bolus injections of noradrenaline ($3 \times 10^{-10}$ to $4 \times 10^{-8}$ mol $kg^{-1}$) and angiotensin II ($1 \times 10^{-11}$ to $6.4 \times 10^{-10}$ mol $kg^{-1}$) with 2–6 min recovery between doses. The sequence of noradrenaline and angiotensin II administered was reversed in half of the experiments.

*(9) Effects of capsaicin and indomethacin on depressor responses to ACh and SNP* Two groups of rats were s.c. injected twice daily with the vehicle (100% DMSO, 0.5 ml $kg^{-1}$, each dose) or capsaicin ($8.2 \times 10^{-5}$ mol $kg^{-1}$ each dose) for 2 days. Dose-depressor response curves of ACh and SNP were constructed as described in Protocol (4). The other two groups of rats were i.v. bolus injected with the vehicle (100% DMSO, 1 ml $kg^{-1}$) or indomethacin ($1.4 \times 10^{-5}$ mol $kg^{-1}$). The construction of dose-depressor response curves of ACh and SNP was also as described in Protocol (4). The dose of capsaicin selected blocked the 'nociceptive response' induced by i.v. bolus injections of DPI (unpublished observations).

### *Drugs*

$N^G$-nitro-L-arginine (L-NOARG), $N^G$-nitro-L-arginine methyl ester hydrochloride (L-NAME), acetylcholine chloride (ACh), phenylephrine hydrochloride, methylene blue, indomethacin, noradrenaline hydrochloride, angiotensin II acetate and capsaicin were obtained from Sigma Chemical Co. (MO, U.S.A.). Sodium nitroprusside (SNP) and diphenyleneiodonium sulphate (DPI) were obtained from Fisher Scientific Co. (NJ, U.S.A.) and Colour Your Enzyme Co. (Ontario, Canada), respectively. Zaprinast (M&B 22948) was kindly provided by Rhone-Poulenc Rorer Central Research. All drugs were dissolved in normal saline (0.9% NaCl solution) except for capsaicin and indomethacin which were dissolved in 100% dimethylsulphoxide (DMSO) and DPI which was dissolved in 5% glucose solution.

### *Statistical analyses*

The magnitude of the depressor response was expressed as % MAP while duration was expressed as 'modified half-recovery time', calculated as the ratio of the time taken for 50% recovery of the depressor responses to baseline MAP (s $mmHg^{-1}$, ratio of half-recovery time to baseline MAP). $ED_{50}$ and $E_{max}$ were calculated from individual dose-response curves (Wang & Pang, 1993a). All results were analysed by the analysis of variance (ANOVA) followed by Duncan's multiple range test, with $P<0.05$ selected as the criterion for statistical significance. To obtain homogeneity of variances, data of cyclic GMP levels were logarithm-transformed prior to statistical analysis. The cyclic GMP results are expressed as mean + s.e.mean (results transformed back from log values). All other results are expressed as mean ± s.e.mean.

## Results

### *Effects of methylene blue and L-NOARG on ACh- and SNP-induced relaxations in preconstricted aortic rings*

Phenylephrine caused sustained contractions in vehicle-, methylene blue- and L-NOARG treated rings, which reached plateau values (pooled data) of 1.35 ± 0.11 g ($n = 14$), 1.62 ± 0.15 g ($n = 13$, $P>0.05$ compared to the vehicle group) and 2.02 ± 0.13 g ($n = 12$, $P<0.05$ compared to the vehicle group), respectively. ACh and SNP caused concentration-dependent relaxations of phenylephrine-induced contraction, with maximal relaxations of ≈70% and 100%, respectively (Figure 1a, 1b). Methylene blue and L-NNA abolished ACh-induced relaxation (Figure 1a). Methylene blue shifted the relaxation curve of SNP to the right without affecting the maximum while L-NOARG did not affect the SNP curve (Figure 1b).

### *Effects of methylene blue and L-NOARG on MAP in conscious rats*

Baseline MAPs and HRs of rats to be given methylene blue and L-NOARG were 105 ± 2 and 109 ± 5 mmHg, and

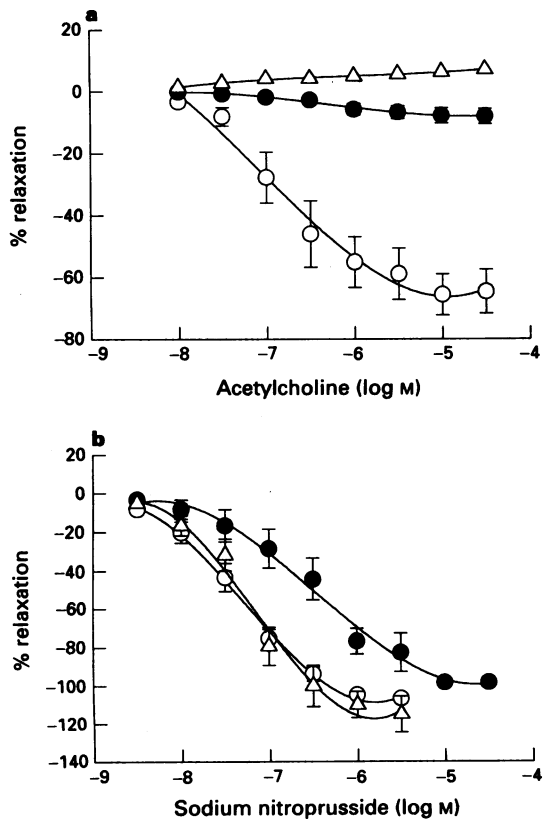


**Figure 1** Effects (mean ± s.e.mean) of methylene blue ($10^{-5}$ M, ●) and $N^G$-nitro-L-arginine ($3 \times 10^{-5}$ M, Δ) on acetylcholine (a) and sodium nitroprusside (b) induced relaxations in phenylephrine ($10^{-6}$ M)-preconstricted aortic rings ($n = 6–7$ each group), (○) vehicle.

353 ± 5 and 352 ± 9 beats $min^{-1}$, respectively. Methylene blue did not significantly alter MAP or HR during the 2 h observation (Figure 2a,b), although the skin of the rats as well as the urine and plasma turned blue during and after the infusion of methylene blue. In contrast, L-NOARG caused sustained increases in MAP (Figure 2a) and bradycardia (Figure 2b).

### *Effects of methylene blue and L-NOARG on depressor response to zaprinast in conscious rats*

Baseline MAPs are summarized in Table 1. L-NOARG raised MAP whereas methylene blue did not alter MAP. In vehicle-treated rats, i.v. bolus injection of zaprinast caused immediate and dose-dependent depressor responses (Figure 3) with modified half-recovery time of 1.63 ± 0.27 s $mmHg^{-1}$ at $2.4 \times 10^{-4}$ mol $kg^{-1}$, which was longer than those for ACh and SNP (see later). L-NOARG inhibited, whereas methylene blue did not alter, the magnitude of the depressor response to zaprinast (Figure 3). On the other hand, the modified half-recovery time of the depressor response to the high dose ($2.4 \times 10^{-4}$ mol $kg^{-1}$) of zaprinast was reduced ($P<0.05$) by methylene blue to 0.88 ± 0.09 s $mmHg^{-1}$. Methylene blue did not alter the tachycardic effect of zaprinast (results not shown). The duration of the depressor response to zaprinast in the presence of L-NOARG was unavailable as MAP in three rats did not return to control.

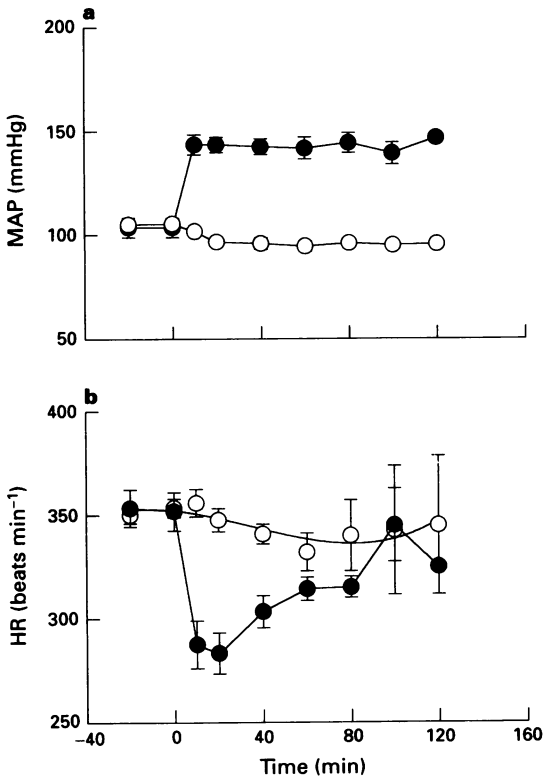


**Figure 2** Effects of (mean ± s.e.mean) of methylene blue ($1.1 \times 10^{-5}$ mol $kg^{-1}$ $min^{-1}$, i.v. infusion, ○) and $N^G$-nitro-L-arginine ($1.5 \times 10^{-4}$ mol $kg^{-1}$, i.v. bolus injection, ●) on mean arterial pressure (MAP, a) and heat rate (HR, b) in conscious rats ($n = 6$ each group).

**Table 1** Values (mean ± s.e.mean) of mean arterial pressure (MAP) before (a) and 20 min (b) or 120 min (c) after the i.v. administrations of the vehicle (infusion or bolus injection), methylene blue ($1.1 \times 10^{-5}$ mol $kg^{-1}$ $min^{-1}$, infusion) and $N^G$-nitro-L-arginine (L-NOARG, $1.5 \times 10^{-4}$ mol $kg^{-1}$, bolus injection) in conscious rats

| | *MAP* (mmHg) | | |
|---|---|---|---|
| *Treatment* | *a* | *b* | *c* |
| Vehicle (saline) | 109 ± 5 | 108 ± 6 | 116 ± 3 |
| Methylene blue | 112 ± 2 | 110 ± 2 | 110 ± 3 |
| Vehicle (saline) | 122 ± 2 | 122 ± 2 | 126 ± 4 |
| L-NOARG | 118 ± 5 | 158 ± 4* | 150 ± 8* |
| Vehicle (saline) | 105 ± 5 | 104 ± 5 | – |
| L-NOARG | 104 ± 5 | 146 ± 7* | – |
| Methylene blue | 103 ± 3 | 94 ± 3 | – |

$n = 6$ in each group.
*Significant difference from baseline (a) MAP ($P<0.05$).

### *Effects of methylene blue and L-NOARG on depressor responses to ACh and SNP*

Baseline MAPs of the rats are summarized in Table 1. As with previous observations, L-NOARG raised MAP while methylene blue did not alter MAP. Intravenous bolus injections of both ACh and SNP caused dose-dependent and transient reductions in MAP in the two groups of vehicle-treated rats (Figures 4,5). Methylene blue affected neither the magnitude nor duration of ACh or SNP-induced depressor responses (Figure 4a,b). L-NOARG, on the other hand, significantly reduced the magnitude and shortened the duration of the depressor responses to ACh but did not alter responses to SNP (Figure 5a,b).

### Ex vivo *effects of methylene blue on cyclic GMP level in aorta in the absence and presence of sodium nitroprusside*

Cyclic GMP level in aortae from methylene blue-treated rats (369 + 71 pmol $g^{-1}$ wet wt., data transformed back from log value) was less ($P<0.05$) than that from vehicle-treated rats (780 + 69 pmol $g^{-1}$ wet wt.). Three min exposure to SNP significantly elevated aortic cyclic GMP levels of both control and methylene blue-treated rats to 2,541 + 737 and 817 + 59 pmol $g^{-1}$ wet wt., respectively. However, the level of cyclic GMP in aorta of methylene blue-treated rats remained lower ($P<0.05$) than that of control rats.

### *Effects of methylene blue on pressor responses to L-NAME and noradrenaline*

In another 2 groups of rats, baseline MAPs (107 ± 3 vs 104 ± 2 mmHg) were similar to MAPs at 20 min after i.v.

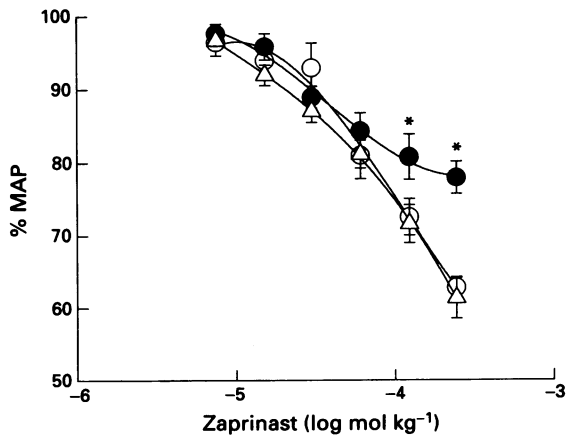


**Figure 3** Effects (mean ± s.e.mean) of methylene blue ($1.1 \times 10^{-5}$ mol $kg^{-1}$ $min^{-1}$, i.v. infusion, Δ) and $N^G$-nitro-L-arginine ($1.5 \times 10^{-4}$ mol $kg^{-1}$, i.v. bolus injection, ●) on mean arterial pressure (MAP, expressed as % baseline MAP) response to i.v. bolus injections of zaprinast in conscious rats ($n = 6$ each group); (○) vehicle. *Significant difference from the corresponding values in the vehicle-treated group.

infusion of the vehicle or methylene blue (106 ± 2 vs 100 ± 2 mmHg). L-NAME caused dose-dependent increases in MAP in the control group, with $ED_{50}$ of 9.2 ± 1.5 × $10^{-6}$ mol $kg^{-1}$ and $E_{max}$ of 46 ± 3 mmHg, respectively. Methylene blue shifted the dose-MAP response curve of L-NAME to the right, with a 4 fold increase of $ED_{50}$ (3.8 ± 0.8 × $10^{-5}$ mol $kg^{-1}$) and insignificant reduction of $E_{max}$ (36 ± 5 mmHg) (Figure 6a). Methylene blue did not significantly alter the bradycardic effect of L-NAME (results not shown).

In another 2 groups of rats, baseline MAPs (108 ± 4 vs 109 ± 2 mmHg) were also similar to MAPs 20 min after i.v. infusion of the vehicle or methylene blue (108 ± 4 vs

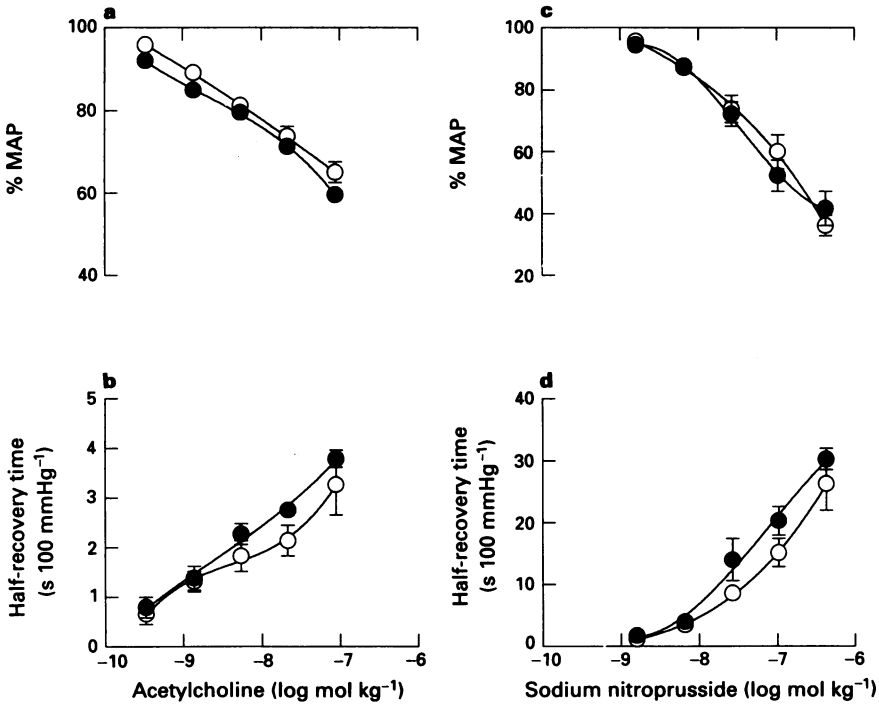


**Figure 4** Effects (mean ± s.e.mean) of methylene blue (1.1 × $10^{-5}$ mol $kg^{-1}$ $min^{-1}$, i.v. infusion, ●) on mean arterial pressure (MAP, expressed as % baseline MAP, a) and duration (expressed as modified half-recovery time, calculated as half-recovery time/baseline MAP, s 100 $mmHg^{-1}$, b) of responses to i.v. bolus injections of acetylcholine and sodium nitroprusside in conscious rats ($n = 6$ each group); (○) vehicle.

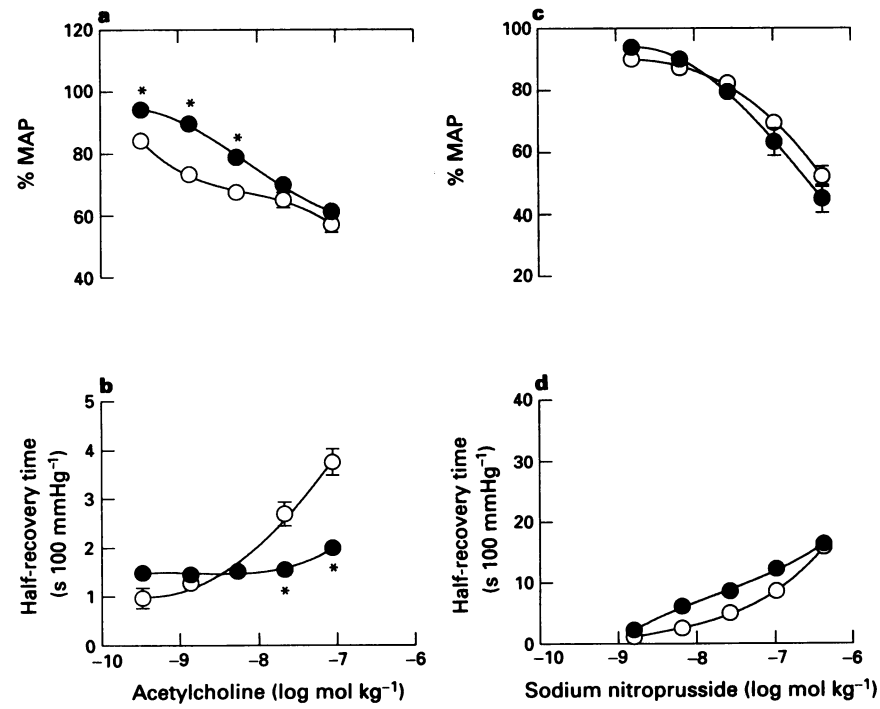


**Figure 5** Effects (mean ± s.e.mean) of $N^G$-nitro-L-arginine (1.5 × $10^{-4}$ mol $kg^{-1}$, ●) on mean arterial pressure (MAP, expressed as % baseline MAP, a) and duration (expressed as modified half-recovery time, calculated as half-recovery time/baseline MAP, s 100 $mmHg^{-1}$, b) of responses to i.v. bolus injections of acetylcholine and sodium nitroprusside in conscious rats ($n = 6$ each group); (○) vehicle. *Significant difference from the corresponding values in the vehicle-treated group.

104 ± 2 mmHg). Intravenous bolus injections of noradrenaline also dose-dependently increased MAP in the vehicle group, with $ED_{50}$ of $5.1 \pm 1.6 \times 10^{-9}$ mol kg$^{-1}$ and $E_{max}$ of 71 ± 5 mmHg. Methylene blue did not alter the $ED_{50}$ ($5.2 \pm 1.4 \times 10^{-9}$ mol kg$^{-1}$) or $E_{max}$ (69 ± 5 mmHg) of responses to noradrenaline (Figure 6b).

### *Effect of indomethacin on pressor responses to L-NAME*

In another 2 groups of rats, baseline MAPs (112 ± 6 vs 109 ± 3 mmHg) were similar to MAPs at 20 min after the injection of the vehicle or indomethacin (112 ± 6 vs 110 ± 3 mmHg). L-NAME dose-dependently raised MAP, with $ED_{50}$ of $1.0 \pm 0.2 \times 10^{-5}$ mol kg$^{-1}$ and $E_{max}$ of 56 ± 6 mmHg. Intravenous bolus injection of indomethacin did not alter the $ED_{50}$ ($8.8 \pm 0.9 \times 10^{-6}$ mol kg$^{-1}$) or $E_{max}$ (53 ± 5 mmHg) of L-NAME.

### *Effects of DPI on pressor responses to L-NAME, noradrenaline and angiotensin II*

In another 2 groups of rats, baseline MAPs (108 ± 2 vs 110 ± 4 mmHg) were similar to MAPs at 20 min after treatment (1084 ± 2 vs 104 ± 6 mmHg) with the vehicle or DPI. L-NAME caused dose-dependent increases in MAP, with $ED_{50}$ of $6.7 \pm 4 \times 10^{-6}$ mol kg$^{-1}$ and $E_{max}$ of 61 ± 4 mmHg (Figure 7a). Pretreatment with DPI shifted the dose-MAP response curve of L-NAME to the right, with 12 fold increase in the $ED_{50}$ ($8.3 \pm 2.5 \times 10^{-5}$ mol kg$^{-1}$), but no change in the $E_{max}$ (53 ± 4 mmHg).

Baseline MAPs (115 ± 3 vs 104 ± 3 mmHg) were also similar to MAPs 20 min after treatment (113 ± 3 vs 94 ± 2 mmHg) with the vehicle or DPI in another two groups of rats. Intravenous bolus injections of noradrenaline caused dose-dependent increases in MAP in the vehicle-treated rats, with $ED_{50}$ of $1.5 \pm 0.5 \times 10^{-9}$ mol kg$^{-1}$ and $E_{max}$ of 69 ± 3 mmHg (Figure 7b). Pretreatments with DPI shifted the dose-MAP curve of noradrenaline to the right, with $ED_{50}$ ($4.2 \pm 1.1 \times 10^{-9}$ mol kg$^{-1}$) increased almost 3 fold but $E_{max}$ (73 ± 4 mmHg) unaffected.

Intravenous bolus injections of angiotensin II also dose-dependently increased MAP in the vehicle-treated rats, with $ED_{50}$ of $3.2 \pm 0.8 \times 10^{-11}$ mol kg$^{-1}$ and $E_{max}$ of 57 ± 4 mmHg (Figure 7c). Pretreatments with DPI also shifted the dose-MAP curve of angiotensin II to the right, with $ED_{50}$ ($8.6 \pm 1.4 \times 10^{-11}$ mol kg$^{-1}$) increased 3 fold and $E_{max}$ (60 ± 4 mmHg) unchanged.

### *Effects of indomethacin and capsaicin on depressor responses to ACh and SNP*

Intravenous bolus injections of ACh and SNP caused similar depressor responses in the two groups of rats pretreated with different doses of DMSO. Pretreatment with indomethacin or capsaicin did not alter the magnitude or duration of the depressor responses to ACh or SNP, compared to the corresponding values in vehicle-treated rats (results not shown).

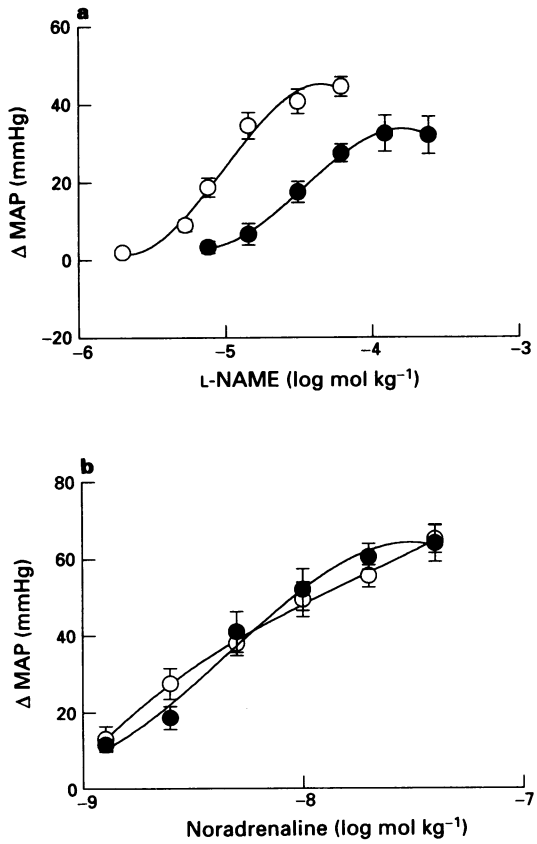


**Figure 6** Effects (mean ± s.e.mean) of methylene blue ($1.1 \times 10^{-5}$ mol kg$^{-1}$ min$^{-1}$, i.v. infusion, ●) on the mean arterial pressure (MAP) responses to i.v. bolus injections of $N^G$-nitro-L-arginine methyl ester (L-NAME, a) and noradrenaline (b) in conscious rats ($n = 5$–6 each group); (○) vehicle.

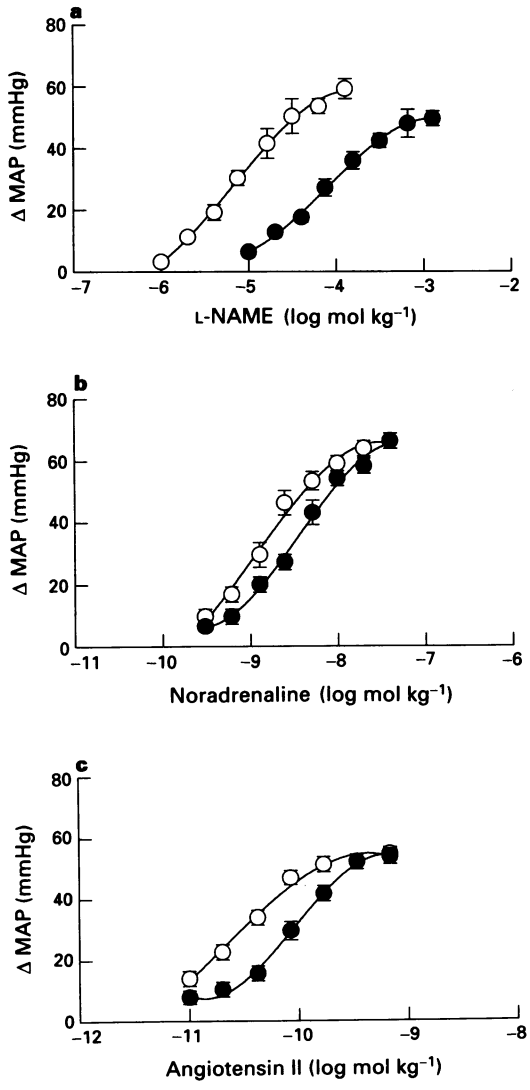


**Figure 7** Effects (mean ± s.e.mean) of diphenyleneiodonium (DPI, $10^{-5}$ mol kg$^{-1}$, i.v. bolus injection, ○) on mean arterial pressure (MAP) responses to i.v. bolus injections of $N^G$-nitro-L-arginine methyl ester (L-NAME, a), noradrenaline (b) and angiotensin II (c) in conscious rats ($n = 6$ each group); (●) vehicle.

## Discussion

It is known that ACh evokes endothelial NO biosynthesis and release *via* the activation of muscarinic receptors (probably the $M_3$ subtype). The released NO activates soluble guanylyl cyclase in smooth muscle cells, causing the formation of cyclic GMP and subsequent vascular relaxation (Martin *et al.*, 1985; Ignarro *et al.*, 1986). Our present results of the ability of L-NOARG and methylene blue to block completely the *in vitro* relaxation response to ACh are supportive of this hypothesis. SNP and other nitrovasodilators are known to liberate NO in vascular smooth muscles *via* biotransformation of the compounds by a reducing agent (Bates *et al.*, 1991). As expected, the relaxation effect of SNP was unaffected by L-NOARG. However, at a concentration which completely blocked the ACh response, methylene blue caused only a right-displacement of the SNP curve. Less inhibition by methylene blue of the responses to nitrovasodilators than ACh has been reported (Martin *et al.*, 1985; Ignarro *et al.*, 1985). These results suggest that either an additional mechanism independent of the guanylyl cyclase/cyclic GMP pathway accounts for the relaxation response to nitrovasodilators (Martin *et al.*, 1985), or that methylene blue blocks ACh-response *via* additional pathways. It has been suggested that methylene blue blocks the vasodilator response to ACh *via* extracellular generation of reactive $O_2$ species in the rat cremaster muscle (Wolin *et al.*, 1990) or cat cerebral microcirculation (Marshall *et al.*, 1988).

Our *in vivo* results show that methylene blue does not alter depressor responses to ACh and SNP. There are conflicting reports on the effects of methylene blue on responses to ACh and nitrovasodilators in anaesthetized animals or perfused beds. Methylene blue potentiated depressor responses to ACh but not nitroglycerin in dogs (Sobey *et al.*, 1988) and potentiated vasodilatation responses to nitrovasodilators and bradykinin in blood perfused dog lungs (Hofman *et al.*, 1992). On the other hand, methylene blue inhibited depressor responses to ACh in rabbits (Cairns *et al.*, 1991), to ACh, adenosine and ATP but not SNP in rats (Loeb & Longnecker, 1992); and to ACh, ATP and SNP but not 8-bromocyclic GMP in lambs (Fineman *et al.*, 1991). Methylene blue did not inhibit the *in vivo* responses to ACh and SNP in our conscious rats. Lack of inhibition was not due to insufficient dose, since the dose used was the highest reported in the published studies. Methylene blue, a tissue dye, readily penetrates cells and this was exemplified by the bluish colour of the rats, the urine and the plasma during and after the administration of the drug. The dose of methylene blue used reduced baseline as well as SNP-stimulated cyclic GMP levels in the aorta by 53 and 68%, respectively, compared to the corresponding values in the aorta of vehicle-treated rats. A doubling of the infusion dose in conscious rats occasionally impaired respiration and increased blood pressure and heart rate (data not shown). Furthermore, a bolus injection of $10^{-3}$ mol $kg^{-1}$ of methylene blue also did not modify depressor response to ACh or to SNP (results not shown). Further support of the effectiveness of methylene blue in inhibiting the activity of guanylyl cyclase was provided by studies using zaprinast, a selective cyclic GMP phosphodiesterase inhibitor. Zaprinast was reported to increase vascular smooth muscle cyclic GMP level and produced vasodilatation (Kukovetz *et al.*, 1979; Lugnier *et al.*, 1983; 1986) and depressor response (Dundore *et al.*, 1991). Our results show that methylene blue shortened the duration while L-NOARG reduced the magnitude of depressor responses to zaprinast, suggesting that methylene blue inhibited guanylyl cyclase *in vivo*. Our result of the attenuation of the depressor response of zaprinast by L-NOARG was similar to those reported by Dundore *et al.* (1991).

L-NOARG at a supramaximal pressor dose (Wang *et al.*, 1991), partially inhibited the depressor response to ACh but not to SNP. Partial inhibition by L-NOARG or L-NAME of depressor responses to ACh is in accordance with published observations (Aisaka *et al.*, 1989a; Rees *et al.*, 1990; Wang *et al.*, 1993b) and has been suggested to be due to the partial involvement of NO biosynthesis for the vasodilatation response to ACh (Aisaka *et al.*, 1989a; Rees *et al.*, 1990; see review by Moncada *et al.*, 1991). However, our results of the lack of inhibition by methylene blue of the depressor response to ACh may suggest that the MAP effect of ACh is entirely unrelated to NO biosynthesis and release. Therefore, the partial inhibition of depressor response to ACh by $N^G$-substituted Arg analogues may not involve the inhibition of NO biosynthesis.

$N^G$-substituted Arg analogues are known to cause pressor response by elevating peripheral vascular resistance and not cardiac output (Gardiner *et al.*, 1990; Wang *et al.*, 1993d). Gardiner *et al.* (1990) showed that the renal, mesenteric and hindquarter beds of conscious rats have varying time course and intensity of vasoconstrictor responses to L-NAME. In accordance to Gardiner's results, we have also found that although L-NAME reduced conductances in all vascular beds of conscious rats, the degree of vasoconstriction varied in different beds (Wang *et al.*, 1993d). These results suggest that blood pressure measurement alone can lead to underestimation or overestimation of the regional haemodynamic effects of L-NAME or L-NOARG.

It is widely believed that pressor responses to $N^G$-substituted Arg analogues are due to the suppressions of NO biosynthesis and guanylyl cyclase activation. Therefore, methylene blue, an inhibitor of guanylyl cyclase, should also increase blood pressure. However, unlike L-NOARG, methylene blue did not raise blood pressure. Methylene blue also did not alter blood pressure in dogs (Archer *et al.*, 1986) or cats (Hyman *et al.*, 1989). However, methylene blue lowered blood pressure in rats (Loeb & Longnecker, 1992) and dogs (Takai *et al.*, 1992) and raised blood pressure in lambs (Fineman *et al.*, 1991). In the latter study, methylene blue also caused tachycardia and pulmonary edema (Fineman *et al.*, 1991). With the exception of the present study, all the above *in vivo* studies were performed in anaesthetized animals. The influence of anaesthetics on responses to methylene blue is not known. Our results clearly show that methylene blue, at a dose which inhibited guanylyl cyclase activity *in vivo* did not raise blood pressure.

It is unclear why L-NOARG, but not methylene blue, causes pressor response. One possibility is that pressor effects of $N^G$-substituted Arg analogues are not related to the modulation of NO biosynthesis. It has been shown that the irreversible NO synthase inhibitor DPI (Stuehr *et al.*, 1991; Kwon *et al.*, 1991; Keller *et al.*, 1992) causes long-lasting inhibition of endothelium-dependent vasodilatation *in vitro* and *in vivo* (Stuehr *et al.*, 1991; Rand & Li, 1993; Wang *et al.*, 1993c) but only transient (1–2 min duration) pressor responses which are entirely mediated *via* sympathetic activation (Wang & Pang, 1993a,c). In contrast, L-NOARG and L-NAME caused prolonged (>2 h in duration) pressor responses that were antagonized by L-Arg (Wang & Pang, 1990; Wang *et al.*, 1991, 1993b) but not by blockers of the sympathetic nervous system (Wang & Pang, 1991). The inability of DPI to evoke NO-related pressor responses suggests that the inhibition of NO biosynthesis may not necessarily cause vasoconstriction *in vivo* and that pressor effects of $N^G$-substituted L-Arg analogues are unrelated to the inhibition of NO biosynthesis.

Another possible reason for the lack of pressor effect of methylene blue is that the inhibition of endothelial NO biosynthesis is a necessary but an insufficient requirement to elicit vasoconstriction *in vivo*. One of the requirements for the NO synthase inhibitors to evoke prolonged pressor response may be the presence of substituted-Arg structures. If this were the case, the inhibition of NO biosynthesis by drugs without $N^G$-substituted Arg structures would not cause pressor responses, but would be expected to antagonize the pressor effects of $N^G$-substituted Arg analogues, the actions of which also rely on the inhibition of NO biosynthesis. In the

present study, methylene blue did not cause pressor responses but selectively displaced the pressor response curve of L-NAME and not noradrenaline to the right, with 4 fold increase in the $ED_{50}$ value of L-NAME. In addition, DPI shifted the pressor response curves of L-NAME, noradrenaline and angiotensin II to the right, with increases in $ED_{50}$ of 12, 3 and 3 fold, respectively, showing that there is a major, specific component of inhibition by DPI of pressor responses to L-NAME and a minor, non-specific component of inhibition of the pressor effect of vasoconstrictors. The mechanism by which DPI caused non-specific inhibition of vasoconstriction is unclear. Our results show that the specific inhibition of NO biosynthesis by DPI or the inhibition of guanylyl cyclase activation by methylene blue selectively antagonized pressor responses caused by $N^G$-substituted Arg analogues. Since neither methylene blue nor DPI caused NO-related pressor response, the inhibition of endogenous NO biosynthesis or the guanylyl cyclase/cyclic GMP pathway alone does not necessarily elicit vasoconstriction *in vivo*. Therefore, the inhibitory effect of $N^G$-substituted Arg analogues on *in vitro* but not *in vivo* vascular relaxation involves the inhibition of NO biosynthesis. Although highly speculative, it is possible that $N^G$-substituted Arg analogues may elicit vasoconstriction *in vivo* by interacting with 'Arg-sensitive receptors' which are not coupled to NO biosynthesis. $N^G$-substituted Arg analogues such as $N^G$,$N^G$-dimethyl-L-Arg and $N^G$-monomethyl-L-arginine are known to be present in the human plasma and urine (Vallance *et al.*, 1992). Whether or not these endogenous compounds have direct pressor action unrelated to the inhibition of NO/cyclic GMP pathway needs to be investigated. The hypothesis that $N^G$-substituted Arg analogues cause pressor responses by inhibiting endogenous NO biosynthesis should be questioned.

In contrast to the results of Rosenblum *et al.* (1992) and Klabunde *et al.* (1991), indomethacin did not alter pressor responses to L-NAME, suggesting that pressor effects of the $N^G$-substituted Arg analogues are not due to activation of the prostaglandin system. Moreover, in contrast to the results of Rosenblum *et al.* (1992) and Cairns *et al.* (1991), indomethacin did not alter the depressor response to ACh or SNP suggesting the lack of involvement of prostanoids for the vasodilatation responses to these drugs. Our results are in accordance to the *in vitro* findings of Furchgott & Zawadzki (1980) and Wang *et al.* (1993a). We also find that capsaicin did not alter the depressor responses to ACh and SNP, suggesting that afferent nerve transmitters (e.g., substance P, calcitonin gene-related peptide) do not contribute to the vasodilatation responses to ACh or SNP.

In summary, methylene blue inhibited relaxation responses to ACh and SNP in preconstricted aortic rings and reduced aortic cyclic GMP level *ex vivo* but did not alter baseline blood pressure or inhibit depressor responses to ACh or SNP in conscious rats. Both methylene blue and DPI selectively shifted the dose-pressor response curve of L-NAME to the right. Neither indomethacin nor capsaicin affected depressor responses to ACh and SNP. These results suggest that: (1) the *in vivo* depressor responses to ACh and SNP may not involve the biosynthesis or release of NO, prostaglandins or afferent nerve transmitters, (2) the inhibition of either NO biosynthesis or the action of NO does not necessarily cause prolonged pressor response, and (3) pressor response to L-NOARG is unlikely to be due to the inhibition of NO biosynthesis alone.

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# Mechanisms of pulmonary vasoconstriction and bronchoconstriction produced by PAF in the guinea-pig: role of platelets and cyclo-oxygenase metabolites

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1 The mechanisms of action of platelet activating factor (PAF) in the bronchial and cardiovascular systems have not yet been fully elucidated. In order to characterize better and to ascertain whether the effects of PAF in both these systems may be ascribed to the same mechanisms, we examined the actions of PAF in the heart-lung preparation of guinea-pig (HLP). The role of platelets and of cyclo-oxygenase metabolites was investigated.

2 In HLPs perfused with autologous blood, bolus injections of PAF (4–32 ng) produced major effects at the pulmonary vascular and bronchial levels. Both dose-related pulmonary vascular hypertension and bronchoconstriction produced by PAF were diminished to the same extent (46% and, respectively, 47%) when HLPs were perfused with a medium consisting of homologous red blood cells suspended in physiological solution containing 3.5% dextran (RBC). This suggests that the effects of PAF partially depend on the presence of formed elements.

3 When indomethacin (30 μM) was added to the perfusing blood, the dose-response curve for the pulmonary hypertensive responses produced by PAF was strongly reduced (90%) in comparison to control preparations, whereas the bronchoconstrictor effects of PAF were only partially diminished (23%). These data constitute direct evidence that products of the cyclo-oxygenase pathway exert a major role in the vascular, rather than in the bronchial actions of PAF.

4 In HLPs perfused with RBC containing indomethacin (30 μM), the pulmonary vascular hypertensive responses produced by PAF were almost completely abolished, thus indicating that cyclo-oxygenase products from tissues are involved in these effects. Conversely, PAF administration continued to cause dose-related bronchoconstrictor responses that were reduced only partially in comparison with HLPs perfused with RBC in the absence of the cyclo-oxygenase inhibitor. This implies that PAF also has direct action on the bronchoconstriction evoked.

5 At the cardiac level, administration of PAF in HLPs perfused with blood caused a dose-related increase in right atrial pressure accompanied by a decrease in left atrial pressure and cardiac output, which were completely suppressed or attenuated by the absence of formed elements and the addition of indomethacin. This suggests that the progressive heart impairment is secondary to the severe pulmonary hypertension induced by PAF.

6 The results of this study performed in the heart-lung preparation of the guinea-pig, which made it possible to simultaneously record cardiovascular and bronchial parameters, indicate that various components are involved in the responses produced by PAF. It is suggested that different mechanisms depending on the relative contribution of these components may account for the PAF-induced effects at the pulmonary vascular and airway levels.

**Keywords:** Platelet activating factor; pulmonary hypertension; bronchoconstriction; platelets; cyclo-oxygenase metabolites; heart-lung preparation

## Introduction

Platelet activating factor (PAF), the phospholipid mediator released during inflammatory and hypersensitivity reactions, is a potent activator of platelet aggregation and possesses a variety of biological activities (Snyder, 1990). Among its actions, it has been supposed that the stimulated release of PAF constitutes an important factor altering circulatory and bronchial performance during inflammatory and allergic processes and contributing to the lethality of anaphylactic shock (Bessin *et al.*, 1983). However, until now, the mechanisms by which both the cardiovascular and bronchial effects are mediated remain uncertain and appear to depend on species and experimental models.

As far as the cardiovascular effects of PAF are concerned, when given intravenously to several animal species, PAF causes systemic hypotension (Lefer *et al.*, 1984; Siren & Feuerstein, 1989), which has been ascribed to pulmonary vascular hypertension or to a decrease in cardiac output (Laurindo *et al.*, 1989). In pigs, a severe increase of pulmonary vascular resistance, depending only partially on thromboxane $A_2$ ($TXA_2$) synthesis, was indicated as the primary mechanism early in the course of PAF-induced shock (Goldstein *et al.*, 1991). Pulmonary vasoconstriction almost completely due to $TXA_2$ synthesis was recently also observed in dogs (Yamanaka *et al.*, 1992), even though it was considered to be of minor importance in the hypotension caused by PAF. In intact guinea-pigs, PAF produced systemic hypotension which was not inhibited after platelet depletion or after the administration of aspirin (Vargaftig *et al.*, 1980). In the same species, a moderate rise in pulmonary arterial pressure was observed after PAF administration in isolated lungs perfused with cell-free solution (Hamasaki *et al.*, 1984), but no data are available on the influence of this hypertensive effect on cardiac performance.

The ability of PAF to produce bronchoconstriction has been extensively demonstrated (Braquet *et al.*, 1987) and species-dependent mechanisms have been described (Chung *et al.*, 1986; Abraham *et al.*, 1989). However, discrepant results

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on the role of cells and mediators have been reported even in the same animal species, such as the guinea-pig, depending on the experimental model utilized. PAF has been shown to induce a platelet-dependent bronchoconstriction *in vivo*, which is not mediated by cyclo-oxygenase metabolites (Vargaftig *et al.*, 1980). Conversely, in *in vitro* experiments the administration of PAF induced a bronchoconstriction that was platelet-independent and blocked by cyclo-oxygenase inhibitors (Hamasaki *et al.*, 1984; Lefort *et al.*, 1984).

The main objectives of the present work were to determine the mechanisms of action of PAF at cardiovascular and bronchial levels and to ascertain whether the PAF-induced effects at both levels may be ascribed to the same mechanisms. The investigation was performed on the heart-lung preparation of guinea-pig (HLP), which can be considered as intermediate between the *in vitro* isolated whole heart and lung models and the *in vivo* situation. In addition the preparation makes it possible to record simultaneously both the cardiovascular and pulmonary parameters. The role of formed elements was verified by comparing PAF-induced effects in preparations perfused with homologous blood or with a medium devoid of platelets, consisting of red blood cells suspended in a modified Tyrode solution. The involvement of arachidonic acid metabolites via the cyclo-oxygenase pathway was also investigated.

A preliminary account of this work was presented at the Vth Joint Meeting between the British and Italian Societies of Pharmacology, in Rome, in 1993 (Argiolas & Del Basso, 1994).

## Methods

As previously described (Bedetti *et al.*, 1990), HLPs were prepared in male albino Hartley Duncan guinea-pigs weighing 350–400 g, anaesthetized with ethyl urethane (1.5 g $kg^{-1}$, i.p.), under artificial respiration (miniature Starling pump, frequency 50 $min^{-1}$; stroke 6 ml). The extracorporeal system consisted of a Starling peripheral resistance (60 mmHg), an electromagnetic flowmeter probe and a collecting thermoregulated reservoir containing 50 ml of perfusion fluid. The peripheral resistance was connected with the aortic cannula and the reservoir with the venous cannula. Right and left atrial pressures (RAP and LAP), pulmonary arterial (PAP), left ventricular (LVP) and aortic (AP) pressures were recorded by means of Statham electromanometers on a Grass multichannel polygraph, together with the cardiac output (CO; measured with a Skalar MDL 1401 electromagnetic flowmeter), heart rate (HR) and ventilation overflow. Signals of haemodynamic variables were fed into an AD/DA card connected to an MS DOS Personal Computer, and then processed. Pulmonary vascular resistance (PVR) was calculated as the ratio between the artero-venous pressure gradient (PAP minus LAP) and the CO by the computer, then re-introduced into the polygraph through the DA channel of the card. Ventilation overflow was recorded by means of a Basile bronchospasm transducer (mod 7020). The gas mixture used for ventilation was 5% $CO_2$ in air. Blood gases and pH determinations were made with a Radiometer blood gas analyser (ABL 30).

### *Perfusion fluids*

The experiments were performed on HLPs perfused with both whole blood and with a suspension of red blood cells in physiological solutions. Blood was obtained from donor guinea-pigs (700–800 g) anaesthetized with ethyl urethane (1.5 g $kg^{-1}$, i.p.). It was collected by a carotid cannula in propylene tubes containing heparin (7.5 iu $ml^{-1}$) and used fresh in the experiments performed with whole blood. In the groups perfused with the suspension of erythrocytes, once collected, the blood was immediately centrifuged at 2600 r.p.m. for 10 min at 4°C. Platelet rich plasma (and a superficial layer containing the majority of the white cells) was discarded. The red blood cell pellet was gently resuspended in an equal volume of a modified Tyrode solution reproducing guinea-pig blood electrolyte composition, as determined in preliminary experiments. The composition (mM) was: NaCl 122.9, KCl 4.63, $CaCl_2$ 2.50, $MgCl_2$ 0.85, $NaH_2PO_4$ 0.54. Centrifugation as described above and washing in physiological solution were repeated three additional times. Finally, the resulting erythrocyte pellet was resuspended in 1.5 volume of the physiological solution, to which $NaHCO_3$ (final concentration mM: 22), D-glucose (final concentration mM: 5.55) and 3.5% dextran (molecular weight 60–90,000) were added. The absence of platelets (and white blood cells) was routinely confirmed by microscopic counts. The haematocrit was measured by a microcentrifuge (ALC 4223). Oncotic pressure was measured with an oncometer (Weil 186) and osmotic pressure with an osmometer (Fiske).

### *Experimental protocol*

Stable values of cardiovascular and pulmonary functions were reached 10–15 min after the surgical procedure was over. Solutions containing PAF (8–96 ng) were administered by bolus injections into the venous cannula at least 10 min after stable values of all parameters were recorded. Each dose increment was initiated on return of parameters to pre-injection or to stable values and, in any case, a period of at least 15 min was allowed to elapse between each dose increment. Only one PAF dose-response curve was performed in each animal for each different treatment.

### *Statistical evaluation*

Data are expressed as means ± standard error of the means. Multiple comparisons between means were peformed by use of Student's *t* test with Bonferroni's correction. Regression analysis calculated by the least squares method was used to study the relationship between the dose of PAF and the responses induced. Statistical differences were calculated by covariance analysis, also taking into account the non homogeneity of variance (Diem & Seldrup, 1982). The potency ratios were estimated according to parallel line or slope ratio assays (Finney, 1952). Results were considered statistically significant when $P < 0.05$; confidence limits were calculated at 0.05 probability.

### *Drugs*

All chemicals used were of analytical grade. PAF (L-α-phosphatidylcholine,β-acetyl-γ-O-hexadecyl) and indomethacin wewre obtained from Sigma Chemical Co. PAF was dissolved to a concentration of 1 mg $ml^{-1}$ in 0.9% saline and stored frozen. Working solutions were prepared from the stock solution and were diluted on a daily basis with the modified Tyrode solution.

## Results

### *Effects of PAF on HLPs perfused with blood*

As shown by a typical tracing (Figure 1), administration of PAF (4–32 ng) in HLPs perfused with homologous blood caused major effects on both pulmonary vascular and bronchial parameters. Dose-dependent increases were obtained in PAP and PVR with little associated changes in CO. At the highest dose, right ventricular overload, manifested by increasing filling pressure of the right atria, was accompanied by a slight decrease in left atrial filling pressure. The rise in ventilation overflow was attributed mainly to bronchoconstriction, since administration of isoprenaline (1–5 μg) into the venous reservoir at the end of some experiments (data not shown) resulted in more or less complete return to the initial level of the PAF-induced effect.

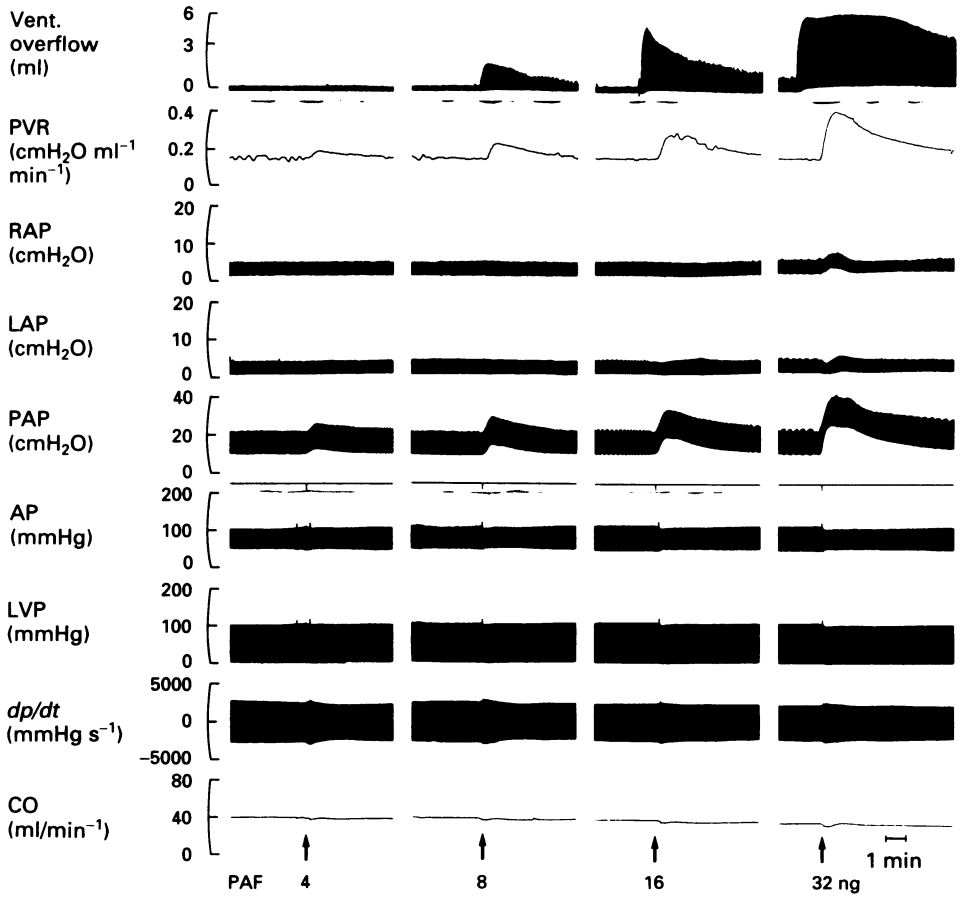


**Figure 1** Typical polygraph tracings showing the effects of PAF on the heart-lung preparation of guinea-pig perfused with homologous blood. PAF (4–32 ng) was administered by bolus injection into the venous cannula. Vent. overflow = resistance to air inflation; PVR = pulmonary vascular resistance calculated as (PAP − LAP) × $CO^{-1}$; RAP = right atrial pressure; LAP = left atrial pressure; PAP = pulmonary arterial pressure; AP = aortic pressure; LVP = left ventricular pressure; *dp/dt* = first derivative of the LVP; CO = cardiac output.

### *Pulmonary vascular responses to PAF in HLPs perfused with blood and with a suspension of red blood cells: effects of indomethacin*

Administration of PAF to HLPs perfused with homologous blood caused an increase in both PVR and PAP (Figure 2). The increases of the pulmonary vascular parameters were both dose-dependent, as shown by the significant log dose-response regressions obtained for ΔPVR and ΔPAP ($P < 0.01$ and, respectively, $P < 0.001$).

To determine whether the pulmonary hypertensive response to PAF is dependent in part on an interaction with formed elements, particularly platelets, responses were investigated in HLPs perfused with the suspension of red blood cells in physiological solution containing dextran (RBC). No significant differences were found between the basal cardiovascular and respiratory parameters of HLPs perfused with blood and with RBC (Table 1). In HLPs perfused with RBC, injection of PAF was also able to induce a dose-dependent rise in PVR and PAP ($P < 0.05$ and $P < 0.01$, respectively). However, the pulmonary vascular responses to all doses of PAF were significantly reduced by perfusion with RBC, when compared with HLPs perfused with blood. Indeed, a significant rightward shift in both ΔPVR ($P < 0.025$) and ΔPAP ($P < 0.001$) dose-response curves was found when compared to the control group. In order to quantify the effects of perfusion with the medium devoid of platelets on the pulmonary vascular effects of PAF, a calculation was made of the relative potency ratio to control preparations. This resulted in a ratio of 0.53 (confidence limits 0.33–0.85) for equiactive rise in PVR and 0.53 (confidence limits 0.45–0.72) for the equiactive rise in PAP, thus indicating that both pulmonary vascular parameters were equally affected by the absence of platelets. This also suggests that, in our experimental conditions, PAP could be considered a reliable parameter as the calculated PVR in order to evaluate the vasoconstrictor activity of PAF, which exert major effects directly on the pulmonary vasculature.

By adding indomethacin (30 μM) to the perfusing blood, the pulmonary vascular responses elicited by PAF decreased considerably, even though significant log dose relationships for ΔPVR and ΔPAP were still found ($P < 0.001$ and $P < 0.05$, respectively). Such dose-response curves were significantly reduced in comparison to control preparations ($P < 0.001$ for ΔPVR and $P < 0.05$ for ΔPAP). The relative potency-ratios, calculated versus control preparations, were, respectively, 0.16 (confidence limits − 0.14–0.47) and 0.10 (confidence limits − 0.13–0.32).

Addition of the cyclo-oxygenase inhibitor to HLPs perfused with RBC substantially blocked the hypertensive pulmonary effects induced by PAF. The responses of both PVR and PAP were almost completely suppressed and no significant dose-response relationships were found between the PAF administered and the increase in PVR and PAP ($P > 0.05$ for both parameters).

### *Bronchial effects produced by PAF*

The results described above refer to the same experimental groups as those just mentioned for pulmonary vascular responses to PAF. As shown in Figure 3, administration of PAF to HLPs perfused with blood and RBC elicited a dose-

related increase in ventilation overflow ($P < 0.001$ for both groups). The bronchial responses to all doses of the phospholipid mediator were reduced ($P < 0.001$) in the HLPs perfused with the medium devoid of platelets, as compared with those obtained in the control group. The potency ratio calculated between equiactive doses of PAF in HLPs perfused with blood and RBC was 0.54 (confidence limits 0.43–0.67). The presence of indomethacin in the perfusing blood caused a slight but significant shift to the right of the ventilatory response-curve ($P < 0.005$). The potency ratio calculated between equiactive doses of PAF in HLPs perfused with blood without and with indomethacin was 0.77 (confidence limits 0.65–0.92). When the cyclo-oxygenase inhibitor was added to the red blood cell suspension, a dose-response relationship between the PAF administered and the bronchoconstrictor responses was still found ($P < 0.001$). This dose-response curve appeared to be only slightly reduced by the presence of indomethacin, although it caused a significant difference in the slope ($P < 0.025$).

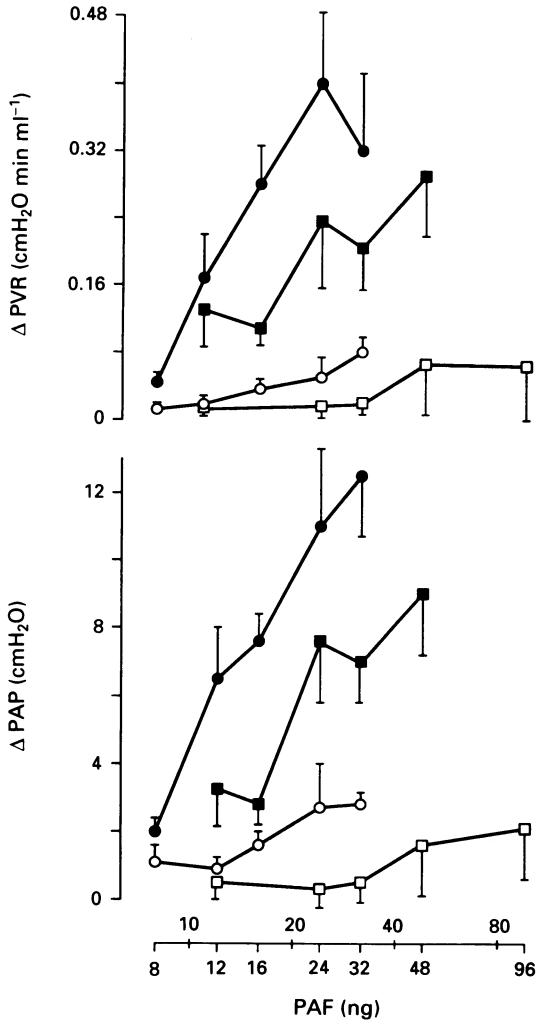


**Figure 2** Effects of increasing doses of PAF on pulmonary vascular pressure (ΔPAP) and on pulmonary vascular resistance (ΔPVR) in heart-lung preparations of guinea-pig perfused with homologous blood (●, $n = 8$) and homologous red blood cells suspended in physiological solution containing 3.5% dextran (■, $n = 8$) in the absence and presence (○, $n = 7$; □, $n = 8$) of indomethacin, 30 μM. Each point indicates the mean with s.e.mean. For statistical differences, see text.

### *Cardiac actions of PAF*

Figure 4 shows the effects of indomethacin and of RBC perfusion on the cardiac action of PAF. Administration of the phospholipid mediator caused a dose-related increase of RAP in HLPs perfused with both blood ($P < 0.001$) and the suspension of RBC ($P < 0.05$). As in the case of the pulmonary vascular effects of PAF, the responses were lower in HLPs perfused with RBC ($P < 0.005$). Moreover, addition of indomethacin to the perfusing medium substantially reduced the rise of RAP ($P < 0.01$) and virtually eliminated the responses of HLPs perfused with RBC. These data, which overlap with those obtained on PAP, suggest that the increase in RAP is probably secondary to the pulmonary hypertension following PAF administration. A progressive heart impairment resulting from the dose-related decrease of LAP ($P < 0.01$) and of CO ($P < 0.01$) was observed in HLPs perfused with blood. The absence of formed elements or the addition of indomethacin attenuated the decrease in CO ($P < 0.025$ for both parameters) and eliminated the fall of LAP. Dose-related increases in HR were observed only after administration of PAF in HLPs perfused with blood ($P < 0.001$); these were unaffected by indomethacin, thus suggesting that in this phenomenon there is a role played by platelet-derived mediators which is different from that of cyclo-oxygenase metabolites.

## Discussion

Even though many studies on the cardiovascular and pulmonary actions of PAF have been performed in guinea-pigs, these effects have not been fully elucidated and appear to be accounted for by different mechanisms, depending on the experimental model. This probably reflects the stimulation of different cell types, as suggested by the fact that lung strips collected by animals desensitized by repeated i.v. infusions of

**Table 1** Basal values of respiratory and cardiovascular parameters in heart-lung preparations of guinea-pig perfused with blood and with the suspension of erythrocytes in physiological solution containing dextran

| *Perfusion with* | *Blood* | *Red blood cells suspension* |
|---|---|---|
| Number of experiments | 8 | 8 |
| Ventilation overflow (ml) | 0.22 ± 0.01 | 0.23 ± 0.02 |
| Cardiac output (ml min$^{-1}$) | 42.4 ± 1.7 | 41.2 ± 1.8 |
| Heart rate (beats min$^{-1}$) | 290 ± 13 | 312 ± 8 |
| Aortic pressure (mmHg) | 77.3 ± 1.2 | 80.1 ± 1.6 |
| Pulmonary arterial pressure ($cmH_2O$) | 15.6 ± 0.5 | 14.1 ± 0.6 |
| Right auricolar pressure ($cmH_2O$) | 2.84 ± 0.30 | 2.02 ± 0.21 |
| Pulmonary resistance ($cmH_2O$ min ml$^{-1}$) | 0.31 ± 0.03 | 0.28 ± 0.01 |
| Left auricolar pressure ($cmH_2O$) | 2.74 ± 0.43 | 2.31 ± 0.36 |
| Intraventricular pressure ($cmH_2O$) | 82.3 ± 2.7 | 81.9 ± 2.7 |

Values represent mean ± s.e. of means of the cardiovascular and respiratory parameters recorded at the beginning of the experiments. Statistical comparisons were performed by Student's $t$ test with Bonferroni's correction.

PAF *in vivo*, still contract when exposed to PAF *in vitro* (Braquet & Vargaftig, 1986).

Thus, our investigation was performed with a preparation that is intermediate between the *in vivo* and *in vitro* situations, making it possible to evaluate simultaneously both the cardiovascular and bronchial parameters. The results obtained show that, in guinea-pig heart-lung preparations perfused with autologous whole blood, intravenous injection of PAF caused major effects at the pulmonary level. Actually, dose-related increases in both PVR and PAP and in bronchial responses to PAF were found. A progressive heart impairment, with an increase in RAP and a decrease of LAP and CO, was also obtained. Perfusion of HLPs with a medium devoid of platelets reduced both vascular and bronchial responses to PAF to the same extent. Conversely, the effects of PAF at the airway and at pulmonary vascular levels were affected in a very different way by the presence of indomethacin. The hypertensive responses were strongly reduced when indomethacin was added to the whole blood and they were completely eliminated when the cyclo-oxygenase inhibitor was added to the suspension of red blood cells. In contrast, the bronchoconstrictor responses were slightly affected by indomethacin and a dose-related bronchoconstriction was still observed after PAF administration in the absence of platelets. These results indicate that the mechanisms of action of the phospholipid mediator may be ascribed to various components which are substantially different at bronchial and cardiovascular levels.

The influence that formed elements, particularly platelets, may exert on PAF-induced cardiovascular and bronchial effects has been studied in various species (Munoz *et al.*, 1991) including guinea-pigs (Benveniste *et al.*, 1983; Hamasaki *et al.*, 1984), mostly by utilizing isolated organs perfused with physiological solution. We assessed the possibility that interaction with formed elements (except red blood cells) may contribute to the response of PAF. This was

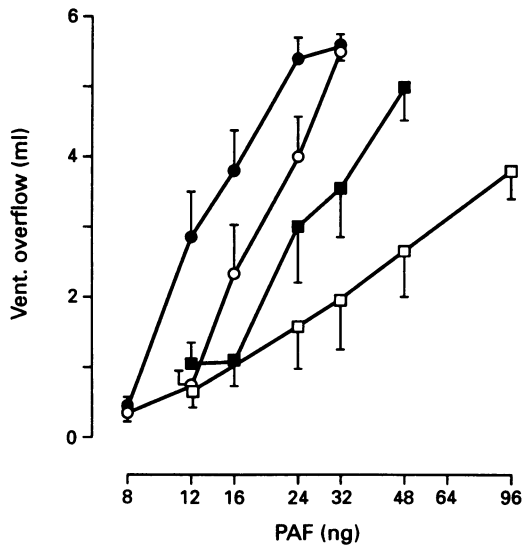


**Figure 3** Dose-response curves for the bronchoconstrictor effects (Vent, overflow) induced by PAF in heart-lung preparations of guinea-pig perfused with blood (●) and homologous red blood cells suspended in physiological solution containing 3.5% dextran (■) in the absence and presence (○,□) of indomethacin, 30 μM. Data refer to the same experimental groups as in Figure 2. Each point indicates the mean with s.e.mean. Number of experiments as in Figure 2. For statistical comparisons, see text.

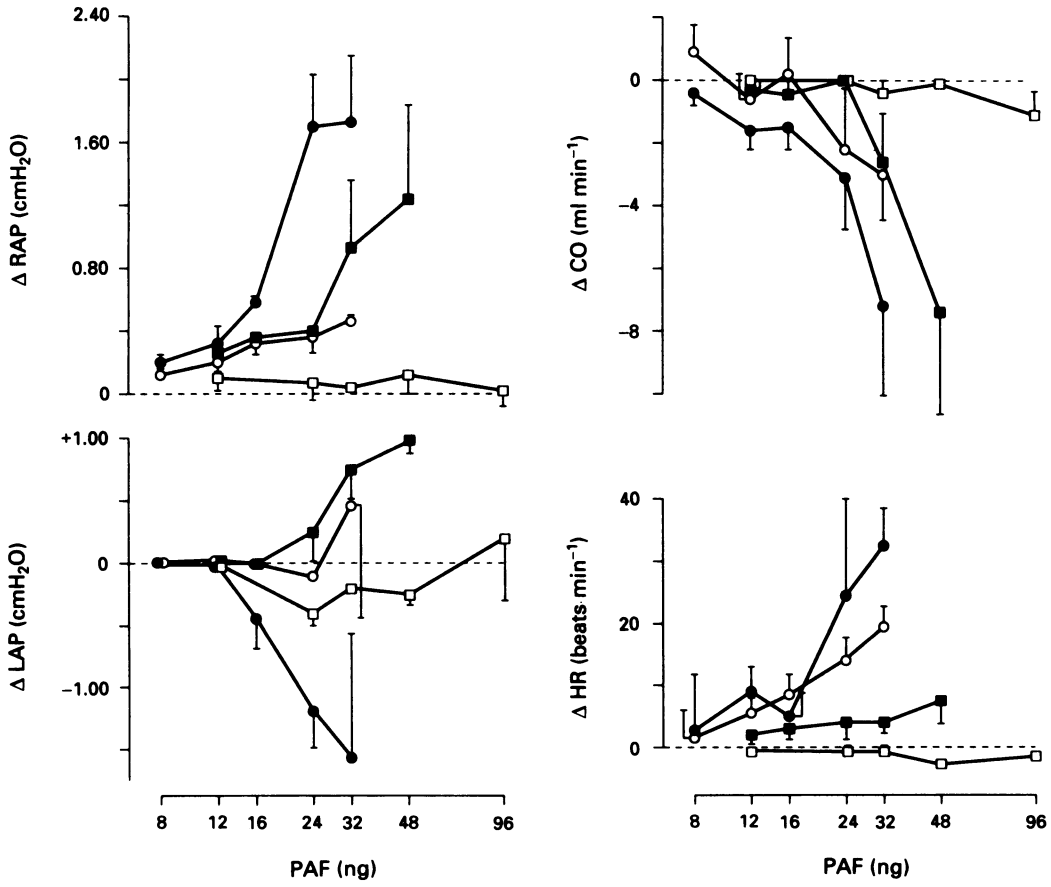


**Figure 4** Cardiac changes in response to increasing doses of PAF in heart-lung preparations of guinea-pig perfused with blood (●) and a suspension of red blood cells in physiological solution containing 3.5% dextran (■) in the absence and presence of indomethacin, 30 μM (○,□). The effects of PAF on right (RAP) and left (LAP) atrial pressures, cardiac output (CO) and heart rate (HR) are plotted. Each point indicates the mean with the s.e.mean. For statistical analysis, see text.

done by perfusing the preparations with a suspension of erythrocytes in physiological solution with the same electrolytic composition as that of guinea-pig plasma. Added to the physiological solution was low molecular weight dextran, the concentration of which was chosen in order to eliminate the differences in physical properties which may be induced by this medium. Actually, the osmotic and oncotic pressures were substantially the same in the groups perfused with blood ($296 \pm 3.5$ mM and $12.8 \pm 0.4$ mmHg, respectively; $n = 20$) and with the suspension of erythrocytes ($289 \pm 2.7$ mM and $12.1 \pm 0.3$ mmHg, respectively; $n = 20$). Moreover, no differences were found in haemodynamic basal values in the two experimental groups.

Hence, our data clearly demonstrated that the responses induced by PAF at both pulmonary vascular and bronchial levels were depressed by perfusion with the suspension of erythrocytes. This suggests that, in our experimental model, a component of both PAF-induced responses may be attributed to the activation and the release of secondary mediators by formed elements, particularly platelets. PAF-initiated stimulation of platelets can cause a release of a variety of substances which may be responsible for an important component of the vasoconstrictor and bronchoconstrictor PAF-induced actions, mainly histamine, 5-hydroxytryptamine, $TXA_2$ (Chignard *et al.*, 1979; Lefer *et al.*, 1984; Popovich *et al.*, 1988). Our results may provide some insight into the source and the type of mediators responsible for PAF-induced effects. When compared with control preparations, the PAF-induced hypertensive responses in HLPs perfused with blood containing indomethacin were more reduced (90% for $\Delta$PAP) than those of preparations perfused with the suspension of erythrocytes (47%). This may support the hypothesis that PAF-induced release of cyclo-oxygenase metabolites from tissues (lung parenchyma or vascular wall) contributes to the pulmonary vascular hypertension due to mediators released by platelets. The slight, but dose-dependent, vasoconstrictor responses induced by PAF still observed in the presence of indomethacin may then be ascribed to a release of mediators such as 5-hydroxytryptamine or histamine from platelets. We have already described how both these amines were able to induce slight hypertensive and severe bronchoconstrictor effects in the heart-lung preparation of guinea-pig (Bedetti *et al.*, 1987). On the other hand, platelet-derived mediators which are different from cyclo-oxygenase metabolites, also appear to contribute to the bronchoconstrictor responses induced by PAF, which were more affected by the absence of platelets (46%) than by the presence of indomethacin in the blood (23%).

Regarding the hypertensive and bronchoconstrictor responses induced by PAF in the medium devoid of platelets, the fact that both pulmonary vascular and bronchial effects of PAF were depressed to a similar degree (46% and 47% respectively, in comparison to control preparations), may suggest that the mechanism of action of the phospholipid mediator at both levels is the same. However, the data obtained with indomethacin do not support this hypothesis. Indeed, when the cyclo-oxygenase inhibitor was added to the suspension of erythrocytes, the hypertensive responses were completely eliminated, thus suggesting that the mediators released by tissues which are responsible for such effects are a product of the cyclo-oxygenase pathway, probably $TXA_2$. Pulmonary hypertensive responses to PAF due to the release of $TXA_2$ (Laurindo *et al.*, 1989) have been reported in pigs. Moreover, we have already found that, in the heart-lung preparation of guinea-pig (Bedetti *et al.*, 1987), the $TXA_2$ agonist, U-46619, induced pulmonary arterial constriction similar to that induced now by PAF. In spite of the major role that the cyclo-oxygenase metabolites exert in the hypertensive effects of PAF, these products may only partially account for the bronchial responses to the phospolipid mediator. The dose-dependent bronchoconstrictor effects of PAF which were still observed even when indomethacin was added to the suspension of erythrocytes may suggest either a direct action of PAF on specific bronchoconstrictor receptors or the ability of PAF to induce the release of mediators with spasmogenic activity (Jancar *et al.*, 1989) different from leukotrienes (Saito *et al.*, 1993) and $TXA_2$.

Thus, our results provide direct evidence that various components may contribute to the responses induced by PAF, also suggesting that the vasoactive and airway responses induced by PAF are mediated by different mechanisms. This concept has already been postulated for PAF-induced effects in the cat (Underwood & Kadowitz, 1989), even though the mechanisms of action of PAF in this species are different from those in guinea-pig (Dyson *et al.*, 1990; Bellan *et al.*, 1992). However, these more closely resemble the mechanisms observed from the results on various experimental models in the dog (Chung *et al.*, 1986; Popovich *et al.*, 1988; Yamanaka *et al.*, 1992). It remains to be seen whether the difference between the mechanisms of action of PAF at bronchial and vascular levels in the guinea-pig may be explained by the existence of two PAF receptor subtypes, as suggested in the case of the rat heart (Hu *et al.*, 1991).

As far as the cardiovascular effects of PAF are concerned, our results may suggest that pulmonary vasoconstriction may play a role in the PAF-induced hypotension observed in the guinea-pig *in vivo*. The severe pulmonary vasoconstriction caused by PAF leads to right heart failure, manifested as increased right atrial pressure. The decrease of cardiac output is induced by inadequate flow to the left side of the circulation, manifested by a decrease of the left atrial pressure. These circulatory effects of PAF appear to be dependent on cyclo-oxygenase metabolites; thus they can only partially account for the hypotensive effects of PAF, which *in vivo* are not affected by aspirin (Vargaftig *et al.*, 1980).

In conclusion, the present results obtained in HLPs perfused both with whole blood and with a suspension of erythrocytes provide some insight into the role that formed elements may play on the pulmonary vascular and bronchial effects of PAF, suggesting their involvement in the responses at airway and pulmonary vascular levels. Moreover, the experiments performed in the presence of the cyclo-oxygenase inhibitor suggest that the pulmonary hypertensive responses to PAF are almost completely dependent on products of the cyclo-oxygenase pathway, part of which may be produced by the lung parenchyma. These products account only partially for the bronchoconstrictor responses induced PAF, suggesting that the PAF-induced bronchial contractile responses may be ascribed either to a direct contractile effect of the phospholipid or to the release of mediators which are different from cyclo-oxygenase metabolites and act only on the bronchial and not the vascular, smooth muscle cells.

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# Pharmacology of the octopamine receptor from locust central nervous tissue (OAR3)

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**1** The present study characterized highly effective agonists from different classes of compounds for the neuronal octopamine receptor ($OAR_3$) of the migratory locust (*Locusta migratoria* L.). Biogenic amines and phenyliminoimidazolidines (PIIs) were employed for the study of structure-activity relationships.

**2** The highest affinity PIIs were predominantly those with substitutions at the positions 2 and 4 of the phenolic ring (e.g. NC 7, $K_I = 0.3$ nM, NC 8, $K_I = 0.81$ nM). Substitutions at these positions always had positive effects on the affinity of the respective agonists.

**3** Substitutions at the positions 3, 5 and 6, however, always had negative effects on the affinity. At the position one of the phenolic ring, heterocyclic substituents are preferred.

**4** Some PIIs had a more than 30 times higher affinity for OARs than for α-adrenoceptors which are the vertebrate homologues of the insect octopamine receptors.

**5** The only non-PII with subnanomolar affinity was the aminooxazoline derivative AC 6 ($K_I = 0.92$ nM).

**6** A variety of substances with known insecticidal activity such as chlordimeform, demethylchlordimeform, amitraz or AC 6 had high affinity for the locust neuronal octopamine receptor.

**Keywords:** Insect; nervous system; *Locusta*; phenyliminoimidazolidines; receptor; octopamine

## Introduction

Since its discovery in the salivary glands of *Octopus* (Erspamer & Boretti, 1951), the biogenic monoamine octopamine (*p*-hydroxyethanolamine) has been well established as a neurotransmitter, neurohormone, and neuromodulator in various invertebrate species (Axelrod & Saavedra, 1977; Orchard, 1982; Evans, 1985; Saavedra, 1989). Especially in insects, the modulatory role of octopamine in different peripheral organs has been studied in great detail (Evans, 1985) and also in the central nervous system some physiological effects of octopamine have been described (Nathanson & Greengard, 1973; Orchard, 1982). It is essential for the coordination of locomotor behaviour, such as flying and running (Sombati & Hoyle, 1984) and even complex functions of the nervous system, such as learning and memory (Bicker & Menzel, 1989; Dudai *et al.*, 1987) are under the control of this neuroactive substance.

Receptors for octopamine were studied in neuronal (Nathanson & Greengard, 1973; Roeder & Gewecke, 1990) as well as in non-neuronal tissues (Evans, 1981; Nathanson, 1985a,b). In the latter, three different receptor classes ($OAR_1$, $OAR_{2A}$ and $OAR_{2B}$) could be distinguished (Evans, 1981). Most octopamine receptors of non-neuronal tissues studied so far, have pharmacological features comparable to the class 2A receptor (Evans, 1985). In the nervous tissue a peculiar receptor class is present (Roeder, 1992). Its pharmacology is (in contrast to octopamine receptors from non-neuronal tissues such as the locust ext.-tibiae-muscle or the firefly lantern), less well studied (Nathanson, 1985a,b). In non-neuronal tissues, compounds such as the formamidines, the phenyliminoimidazolidines (Nathanson, 1985a,b; Evans, 1987) or the aminooxazolines (Jennings *et al.*, 1988) have high affinity properties.

Locust neuronal octopamine receptors have been studied using different methods. In addition to the study of octopamine-sensitive adenylate cyclases, radioligand-receptor assays with either [$^3$H]-octopamine (Roeder & Gewecke, 1990) or the PII-derivative, [$^3$H]-NC-5Z (Nathanson & Kaugars, 1989; Roeder & Nathanson, 1993) were successfully used. The identity of these binding sites with octopamine receptors located on isolated neuronal somata was recently confirmed by electrophysiological methods (Kaufmann & Benson, 1991).

As octopamine receptors are the only non-peptide receptors whose occurrence is restricted to invertebrates, they represent ideal targets for specific insecticides. Therefore, the detailed pharmacological characterization of octopamine receptors, especially of those from neuronal tissues, could result in the development of new leads for more specific insecticides.

## Methods

Migratory locusts (*Locusta migratoria* L.) of either sex were reared in a light/dark cycle of 12h/12h (35°C) under crowded conditions. They were fed with a diet of fresh wheat and bran. Two to 3 weeks after imaginal moult, nervous tissue (supraoesophageal-, suboesophageal-, and thoracic ganglia) was dissected out and immediately stored on ice. The nervous tissue was homogenized in ice cold incubation buffer (HEPES/NaOH 10 mM, 5 mM $MgSO_4$, 100 μM PMSF, pH 7.4) in a glass/teflon homogenizer followed by a glass/glass homogenizer (10 strokes each). This homogenate was centrifuged (20,000 *g*, 30 min), the supernatant discarded, and the pellet resuspended in the original volume. This procedure was repeated twice to obtain washed pellets that were stored frozen until use (−70°C).

The incubation was performed in a total volume of 100 μl with 0.3–0.5 mg ml$^{-1}$ protein, and 10 nM [$^3$H]-octopamine at room temperature. The incubation proceeded for approximately 1 h and was stopped by filtration through glass fibre filters (Whatmann GF/C) preincubated in 0.3% polyethyleneimine. After addition of the sample, the filters were washed with ice cold buffer. All pharmacological experiments were performed in the presence of indicated

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concentrations of tested substances. Each substance was tested in 6–7 different concentrations at least 4–6 times in triplicate. Standard errors in the pharmacological studies were usually below 10% of the respective values. In order to increase clarity, error bars have been omitted. To determine non-specific binding, 10 μM octopamine was added to the incubation medium. Radioactivity on the filters was quantified with a scintillation counter (app. 40% efficiency). $K_I$-values were determined using the LIGAND programme (Munson & Rodbard, 1980). A more detailed description of the procedures was given previously (Roeder & Gewecke, 1990).

Most of the PIIs were from Boehringer Ingelheim (Germany) or Shell Agriculture (Ingelheim), St 92 and NC 5Z were gifts of Dr J.A. Nathanson. Demethylchlordimeform was from CIBA-Geigy (Basel, Switzerland). AC 6 was a gift from Dr Jennings. Amitraz and BTS 23396 were from Cyanamid. Most of the other substances were obtained from SIGMA (Deisenhofen, Germany).

## Results

### *Biogenic amines*

Although the biogenic amines are substances with a structure related to that of octopamine, they vary substantially in their affinity for the octopamine receptor, and minor structural changes lead to different affinities. The natural occurring *p*-isomer of octopamine has an affinity that is approximately 500 times higher if compared with that of the *m*-isomer (Figure 1, Table 1). Comparison of the affinities of these two isomers with the non-hydroxylated analogue, phenylethanolamine, enabled quantification of the effects of hydroxylation at either position 4 or 3 of the phenolic ring. Whereas introduction of an hydroxyl-group at position 4 resulted in an approximately 15 fold increase in affinity, this substitution at position 3 led to an approximately 50 times decreased affinity (Figure 1). This negative effect of hydroxylation at position 3 of the phenolic ring is one of the major structural features of the octopamine receptor that demarcates it from catecholamine and especially from vertebrate adrenoceptors, to which octopamine receptors are presumably homologous.

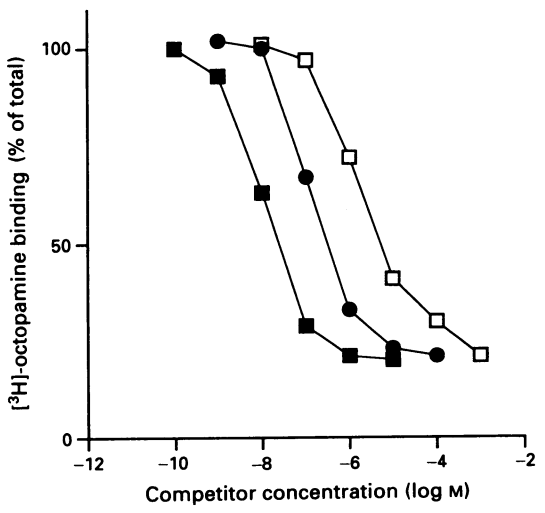


**Figure 1** Displacement of specific [$^3$H]-octopamine binding from locust (*Locusta migratoria* L.) nervous tissue membranes by two octopamine isomers (*meta*- (■) and *para*- (□) octopamine) and phenylethanolamine (●). Each point represents the mean of at least 3–4 separate experiments performed in triplicate. Standard errors are not indicated, but they represent usually less than 10% of the respective values.

The biogenic amine with highest affinity for the locust neuronal octopamine receptor is the N-methylated analogue of octopamine, synephrine ($K_I$ = 3.38 nM). Its affinity is approximately twice as high as that of octopamine ($K_I$ = 7.9 nM). This two fold increase in affinity caused by N-methylation was also observed for noradrenaline and its N-methylated analogue, adrenaline ($K_I$ = 416 and 908 nM respectively; Table 1). Tyramine, the biological precursor of octopamine and itself a neurotransmitter candidate in the insect nervous system (Saudou *et al.*, 1990), has an affinity that is approximately 8 times lower than that of octopamine. The stereospecificity of the binding was shown by the two fold increase in affinity of the (−)-isomer of noradrenaline compared with the (±)-racemate. Ahlquist (1948) used in his pioneering work (which classified adrenoceptors into α- and β-receptors) the three biogenic amines adrenaline, noradrenaline and isoprenaline). Interestingly, the locust neuronal octopamine receptor has the same rank order of affinities for these agonists as the vertebrate α-adrenoceptors.

### *Phenyliminoimidazolidines*

Currently, phenyliminoimidazolidines (PIIs) are the most interesting agonists for octopamine receptors. Among them are various compounds with subnanomolar affinities. To quantify the effects of a given substitution, the affinities of those PIIs were compared that are different at only one position of the phenolic ring. As a simple example, the positive effect of bromination at position 4 of the phenolic ring was demonstrated by comparing the affinities of the 4-bromo-substituted analogue NC 12 ($K_I$ = 14.9 nM) with the nonsubstituted PII, NC 10 ($K_I$ = 23.4 nM, Table 2). Sets of other PIIs could be used to quantify the effects of other substitutions. The chlorine substituted PII derivatives, clonidine (2, 6-dichlor), NC 11 (2,4,6-trichlor), NC 3 (2,4,5-trichlor) and NC 8 (2,4-dichlor) vary substantially in their affinities. The 2,4-chor derivative (NC 8, $K_I$ = 0.812 nM) has higher affinity than either the 2, 4, 5-trichlor (NC 3, $K_I$ = 2.27 nM) or the 2, 4, 6-trichlor derivative (NC 11, $K_I$ = 18.7 nM). The negative effect of chlorination at position 6 is even greater than the negative effect of this substitution at position 5 (Table 2). The positive effect of a chlorine-group at position 4 is shown by the higher affinity of the 2, 4, 6-trichlor derivative NC 13 compared with the 2, 6-dichlor PII (clonidine, $K_I$ = 47.4 nM).

Regarding three different PIIs it was possible to evaluate the effect of methylation at different positions of the phenolic ring. Whereas the increase in affinity of the 2, 4, 6-trimethyl PII (NC 13, $K_I$ = 4.38 nM) compared with the 2, 6-dimethyl derivative (NC 4, $K_I$ = 19.8 nM) indicates the positive effect of *para*-methylation, methylation at position 6 results in a decreased affinity (the 2, 4-dimethyl PII, NC 9, $K_I$ = 1.02 nM compared with NC 13, $K_I$ = 4.38 nM; Figure 2). The positive

**Table 1** Affinity of various biogenic amines for the locust neuronal octopamine receptor that share structural similarities with octopamine

| *Substance* | $K_I$ (nM) | $K_{Ioct}/K_{Ix}$ |
|---|---|---|
| Synephrine | 3.38 ± 0.64 | 2.33 |
| *p*-Octopamine | 7.9 ± 0.9 | 1 |
| Tyramine | 51.6 ± 17.5 | 0.15 |
| Phenylethanolamine | 115 ± 39.1 | 0.07 |
| Adrenaline | 416 ± 74.8 | 0.02 |
| (−)-Noradrenaline | 475 ± 42 | 0.016 |
| (±)-Noradrenaline | 908 ± 245 | 0.009 |
| *m*-Octopamine | 5050 ± 1860 | 0.002 |
| Isoprenaline | 18600 ± 4840 | 0.001 |

$K_I$-values ± s.d. are given. Each substance is tested in at least 5–7 different concentrations 3–4 times in triplicate. $K_{Ioct}$ means $K_I$-value of octopamine, $K_{Ix}$ means $K_I$-value of the respective substance.

effects of methylation or chlorination at position 4 are obvious. If the two derivatives NC 7 (4-chlor, 2-methyl-PII, $K_I = 0.3$ nM) and NC 9 (2, 4-dimethyl derivative, $K_I = 1.02$) are compared a ranking of these two substitutions is possible. The chlorine substitution is approximately 3 times more efficient than the methyl-substitution at the *para* position. The opposite is true for position 2, where methylation is more effective than chloride substitution (NC 7 compared with NC 8, the 2, 4-dichlor derivative, $K_I = 0.81$ nM).

In the firefly lantern, 2, 6 di-alkyl substituted substances have especially high affinities (Nathanson, 1985b). For the locust neuronal octopamine receptor, the affinity rises about 20 times from the 2, 6-dimethyl PII, NC 4 ($K_I = 19.8$ nM) to the 2, 6-diethyl derivative, NC 5 ($K_I = 0.87$ nM). A further enlargement of the substituents at this position results in an approximately 150 fold decreased affinity as shown for the 2, 6-diisopropyl PII, NC 20 ($K_I = 132$ nM) compared with NC 5. The *para*-position of the phenolic ring appears to be the most interesting one. Even bulky substituents such as the azido-group or the amino-group are tolerated without loss of affinity (*p*-azido- and *p*-amino-clonidine, $K_I$ values 44.5 and 58 nM respectively compared with clonidine $K_I = 47.4$ nM). All other substituents tested, although of different chemical structure (halogen substituents such as bromide and chloride as well as alkyl substituents such as methyl and ethyl) have positive effects on the affinity (Table 2).

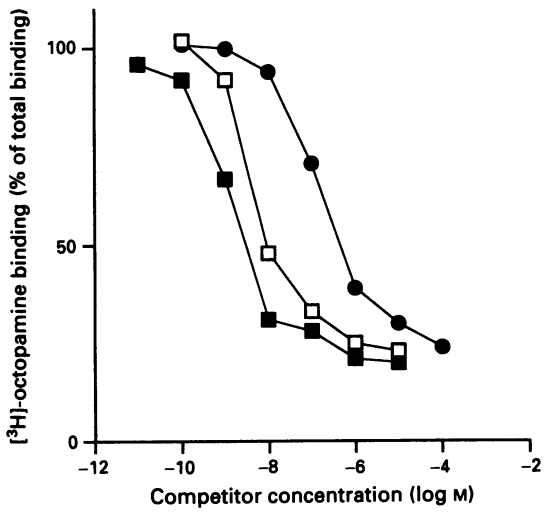


**Figure 2** Comparison of the relative potencies of different methylated phenyliminoimidazolidines (PIIs) in competing with [$^3$H]-octopamine for its specific binding site. Each point represents the mean of 3–4 experiments performed in triplicate: (■) 2, 4-dimethyl-PII; (□) 2, 4, 6-trimethyl-PII; (●) 2, 6-dimethyl-PII.

### *Insecticides*

A variety of substances from different classes of compounds such as the formamidines or the aminooxazolines combine high affinity for the locust neuronal octopamine receptor with insecticidal activity. The two formamidine derivatives, chlordimeform and demethylchlordimeform, are especially potent insecticides (Table 3). Although chlordimeform has a lower affinity ($K_I = 137$ nM), it is thought to be metabolized in the insect to the more potent demethylchlordimeform ($K_I = 1.97$ nM). The substance with highest affinity in this group, and the only non-PII with a subnanomolar affinity, is the aminooxazoline derivative, AC-6 ($K_I = 0.95$ nM). Even substances such as BTS 23376 ($K_I = 8.9$ nM) and amitraz ($K_I = 21.6$ nM) which are known to have a certain insecticidal activity, have relatively high affinity for the locust neuronal octopamine receptor. It is suggested that at least some of these substances exert their insecticidal activity through interaction with the neuronal octopamine receptor.

If the affinities of these substances are compared with each other it is possible to quantify the effects of substitutions at position 1 of the phenolic ring. This is possible because all, except amitraz, have a 4-chlor, 2-methyl substitution at the phenolic ring (Figure 3). Substances with heterocyclic substituents such as the 2-aminooxazolines (AC-6) and phenyliminoimidazolidines (NC 7) have highest affinities (Table 3, Figure 3). Only slight structural changes such as the N-methylation of demethylchlordimeform result in a strongly decreased affinity. Interestingly, the introduction of the

**Table 3** Affinity of selected insecticides derived from different classes of compounds for the locust neuronal octopamine receptor

| *Substance* | $K_I$ (nM) | $K_{Ioct}/K_{Ix}$ |
|---|---|---|
| *Formamidines* | | |
| Demethylchlordimeform | 1.97 ± 0.76 | 4.01 |
| Chlordimeform | 137 ± 70 | 0.06 |
| *Aminooxazolines* | | |
| AC-6 (4-chlor, 2-methyl-) | 0.95 ± 0.24 | 8.3 |
| *Miscellaneous* | | |
| BTS 23376 | 8.9 ± 0.62 | 0.89 |
| Amitraz | 21.6 ± 5 | 0.37 |

$K_I$-values ± s.d. of substances from three different classes of compounds are given (see Table 1).

**Table 2** Affinity of various phenyliminoimidazolidines for the locust neuronal octopamine receptor

| *Substance* | *Substitution* | $K_I$ (nM) | $K_{Ioct}/K_{Ix}$ |
|---|---|---|---|
| NC 7 | 4-chlor, 2-methyl- | 0.3 ± 0.04 | 26.4 |
| St 92 | 2, 4, 6-triethyl | 0.56 ± 0.14 | 14.2 |
| NC 8 | 2, 4-dichlor | 0.81 ± 0.18 | 9.73 |
| NC 5 | 2, 6-diethyl | 0.87 ± 0.32 | 9.04 |
| NC 9 | 2, 4-dimethyl- | 1.02 ± 0.42 | 7.75 |
| NC-5Z | 4-azido, 2, 6-dimethyl- | 1.05 ± 0.47 | 7.52 |
| NC 3 | 2, 4, 5-trichlor- | 2.27 ± 0.89 | 3.48 |
| NC 13 | 2, 4, 6-trimethyl- | 4.38 ± 1.3 | 1.8 |
| NC 12 | 4-bromo- | 14.9 ± 3.3 | 0.53 |
| NC 11 | 2, 4, 6-trichlor- | 18.7 ± 3 | 0.42 |
| NC 4 | 2, 6-dimethyl- | 19.8 ± 6.5 | 0.4 |
| NC 10 | – | 23.4 ± 4.7 | 0.34 |
| *p*-Azidoclonidine | 4-azido, 2, 6-dichlor- | 44.5 ± 7.1 | 0.18 |
| Clonidine | 2, 6-dichlor- | 47.4 ± 17.5 | 0.17 |
| *p*-Aminoclonidine | 4-amino, 2, 6-dichlor- | 58 ± 16.2 | 0.14 |
| NC 20 | 2, 6-diisopropyl- | 132 ± 35.6 | 0.06 |

The $K_I$-values ± s.d. of the different substances are given (see Table 1).

methyl-group at this position has a more negative effect than the extremely bulky side chain of BTS 23376 (Figure 3).

### *Miscellaneous*

As shown for some of the insecticides, this heterogeneous group of compounds enables further quantification of the effect of a given substitution at position 1 of the phenolic ring (Figure 4). 2-Imidazoline substituents are again the most effective substituents (tolazoline, $K_I$ = 18.5 nM; phenyliminoimidazoline, $K_I$ = 23.4 nM). These two agonists together with 2-phenyl-2-imidazolidine enabled us to study the effects of different bridging groups between the phenolic ring and the imidazolidine group. Whereas the methyl and imino group are approximately equipotent, the affinity drops drastically about three orders of magnitude if a spacing group is omitted (2-phenyl-2-iminoimidazolidine, $K_I$ = 16200 nM; Figure 4).

Most of the high affinity agonists have substitutions at the position 2 and 4 of the phenolic ring. An exception is the imidazoline derivative, naphazoline (Table 4; Figure 5; $K_I$ = 3.03 nM). The five fold increase in affinity between naphazoline and tolazoline could be attributed to the structural difference between both substances. An additional benzol ring is added, attached to positions 2 and 3 of the phenolic ring of tolazoline.

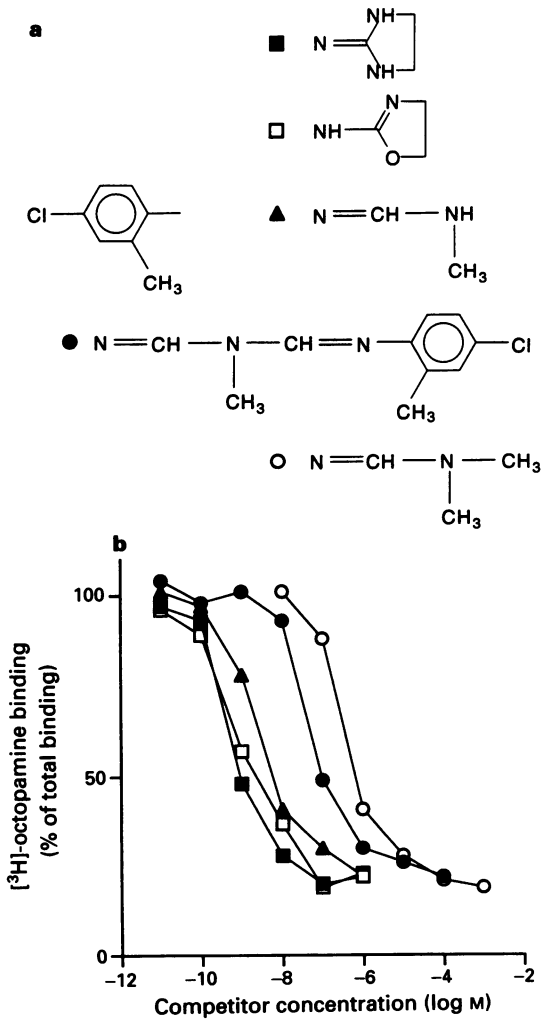


**Figure 3** Effect of different substitutions at position one of the phenolic ring of agonists that share otherwise the same structural motif (4-chlor, 2-methyl-). In (a) the different substitutions at position one of the phenolic ring are shown. In (b) each point represents the mean of 3–4 experiments performed in triplicate: (■) NC7; (□) AC-6; (▲) demethylchlordimeform; (●) BTS 23376; (○) chlordimeform.

### *Receptor specificity*

Some of the tested agonists have a high degree of specificity for the locust neuronal octopamine receptor. The affinity obtained in the current study is compared with the one obtained from [$^3$H]-clonidine binding ($\alpha_2$-adrenoceptor) and [$^3$H]-WB-4101 ($\alpha_1$-adrenoceptor) binding to vertebrate tissues (DeJong & Soudijn, 1981; U'Prichard *et al.*, 1977; Jarrott *et al.*, 1980; Rouot & Snyder, 1979). As seen in Table 5, the biogenic amines octopamine and tyramine are the substances with maximal specificity for the locust neuronal octopamine receptor. Their affinities for the locust receptor are up to 200 times greater than their affinities for vertebrate $\alpha_2$- or $\alpha_1$-adrenoceptors, to which octopamine receptors are presumable homologous. In contrast, the vertebrate adrenergic transmitter, noradrenaline has an affinity that is approximately 30 times lower for the locust neuronal octopamine receptor than for $\alpha_2$-adrenoceptors. Among the other substances tested, the phenyliminoimidazolidines NC 5, NC 9 and NC 10 have the highest degree of specificity although

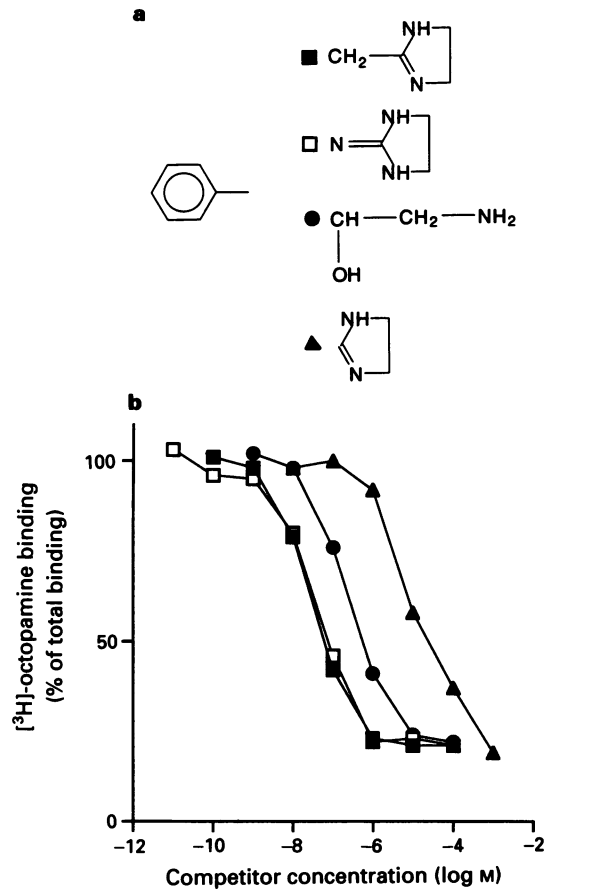


**Figure 4** Differential affinity of otherwise nonsubstituted substances for the locust neuronal octopamine receptor. The structure of the different substances is given in (a). In (b): (■) tolazoline; (□) phenyliminoimidazolidine; (●) phenylethanolamine; (▲) 2-phenyl-2-imidazolidine.

**Table 4** Affinity for the locust neuronal octopamine receptor of miscellaneous compounds

| *Substance* | $K_I$ (nM) | $K_{Ioct}/K_{Ix}$ |
|---|---|---|
| Naphazoline | 3.03 ± 2.61 | 2.61 |
| Tolazoline | 18.5 ± 16 | 0.43 |
| Imidazol-yl-phenol | 813 ± 561 | 0.01 |
| 2-Phenyl-2-imidazolidine | 16200 ± 4700 | 0.0005 |

$K_I$ values ± s.d. are given (see Table 1).

they never reach the specificity of octopamine (Table 5). On the other hand, PIIs such as the well known adrenoceptor ligands, clonidine and *p*-aminoclonidine, have a high degree of specificity for vertebrate adrenoceptors.

## Discussion

Octopamine receptors are thought to represent ideal targets for highly specific insecticides because they are the only known non-peptide ligand receptors whose occurrence is restricted to invertebrates (Evans, 1985). Much effort has been made to characterize the pharmacology of octopamine receptors from non-neuronal tissues (Axelrod & Saavedra, 1977; Harmar & Horn, 1977; Evans, 1981; 1985; Nathanson, 1985a,b). Although, a class of highly specific antagonists was characterized for locust neuronal octopamine receptors (Roeder, 1990), these receptors have been less well studied. A variety of highly specific agonists for octopamine receptors such as the formamidines (Harmar & Horn, 1977; Nathanson & Hunnicutt, 1981), the phenyliminoimidazolidines (Nathanson, 1985a,b) or the 2-aminooxazolines (Jennings *et al.*, 1988) are available. In the current study the affinities of these compounds for a different type of octopamine receptor, the neuronal class 3 receptor (Roeder, 1992) are evaluated, and structure-affinity evaluations have been made.

### *Biogenic amines*

The most interesting pharmacological feature obtained from the study of biogenic amines is their rank order of affinities that makes a simple type of classification possible. With only three amines, octopamine, tyramine and noradrenaline is it possible to distinguish octopamine receptors from their closest relatives, the vertebrate α-adrenoceptors and the insect tyramine receptors (Saudou *et al.*, 1992; Hiripi *et al.*, 1994). Octopamine has the highest affinity, followed by tyramine and noradrenaline for the octopamine receptor, but octopamine and tyramine change places in their affinity for the insect tyramine receptor. Noradrenaline is the substance with the highest affnity for adrenoceptors. Interestingly, octopamine and tyramine are the substances with maximal specificity for insect neuronal octopamine receptors (Table 5). Octopamine receptors and tyramine receptors of insects are both thought to be homologues of vertebrate adrenoceptors receptors. While octopamine receptors appear to be homologous with vertebrate α-adrenoceptors, tyramine receptors are more closely related to vertebrate β-adrenoceptors. Clas-

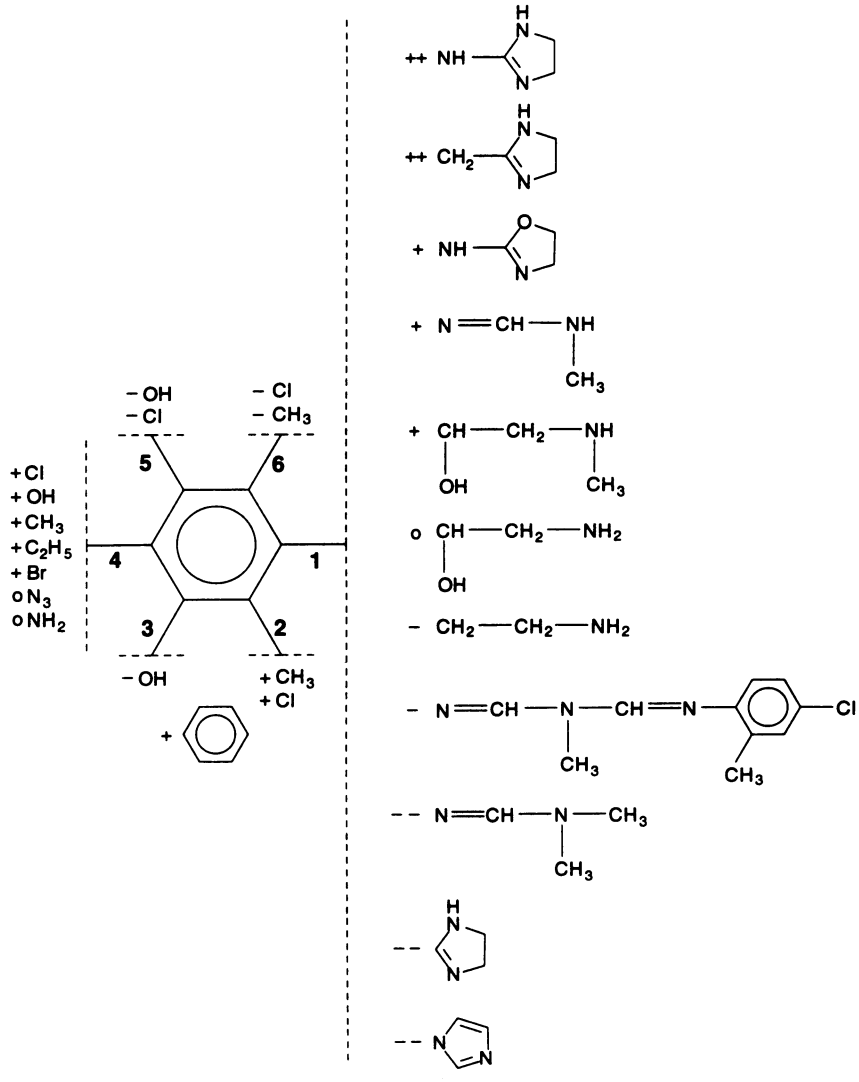


**Figure 5** Structure affinity relationship of agonists for the locust neuronal octopamine receptor. The substitutions of each position of the phenolic ring are listed, in the order of their efficacy, at the respective position. + + indicates an increase in the affinity caused by the substitution of more than 10 times when compared with the nonsubstituted situation (positions 2–6). For position one, comparison is made with the affinity of the natural agonist octopamine; + indicates an increase in affinity between 2 and 10 times; o indicates no change in affinity (0.5–2 times the affinity); – indicates a decrease in affinity (0.5–0.1), and -- indicates a marked decrease in affinity (more than 10 times less affinity).

**Table 5** Relative potencies of octopamine agonists in comparison with mammalian α-adrenoceptors

| *Substance* | $K_I(\alpha_2)/K_I(OA_3$ locust) | $K_I(\alpha_{1A})/K_I(OA_3$ locust) |
|---|---|---|
| *Biogenic amines* | | |
| Octopamine | 197 | 1392 |
| Tyramine | 114 | 445 |
| Phenylethanolamine | 9.6 | 200 |
| Adrenaline | 0.014 | 1.42 |
| Noradrenaline | 0.036 | 2.1 |
| Isoprenaline | 0.3 | – |
| *Phenyliminoimidazolidines* | | |
| NC 5 | 33.5 | – |
| NC 8 | 2.78 | – |
| NC 9 | 5.5 | – |
| NC 10 | 5.4 | – |
| Clonidine | 0.12 | 9.07 |
| *p*-Aminoclonidine | 0.015 | – |
| *2-Benzylimidazolines* | | |
| Tolazoline | 9.7 | 113.5 |
| Naphazoline | 1.88 | 363 |

$K_I$-values for $\alpha_2$-binding ([$^3$H]-clonidine) were obtained from DeJong & Soudijn (1981), from U'Prichard *et al.* (1977), Jarrott *et al.* (1980) and from Rouot & Snyder (1979). For $\alpha_1$-binding ([$^3$H]-WB-4101), the data were obtained from U'Prichard *et al.* (1977).

sical octopamine receptor agonists, such as octopamine itself, demethylchlordimeform, naphazoline and synephrine are relatively ineffective at the locust tyramine receptor (Hiripi *et al.*, 1994). To distinguish the octopamine and tyramine receptor in the locust nervous tissue, even better antagonists are available. Yohimbine, the antagonist with subnanomolar affinity for the insect tyramine receptor (Saudou *et al.*, 1992; Hiripi *et al.*, 1994) has very low affinity for the neuronal octopamine receptor of locusts ($K_I = 82\,\mu M$). On the other hand, high affinity antagonists of the locust neuronal octopamine receptor such as mianserin and phentolamine (Roeder & Gewecke, 1990) have only low affinity for the locust tyramine receptor (Hiripi *et al.*, 1994).

### *Phenyliminoimidazolidines*

The other classes of compounds tested allowed more sophisticated pharmacological questions to be asked. The large number of high affinity phenyliminoimidazolidines used was necessary to obtain quantitative data about structure-activity relationships. PIIs such as NC 5 have, as do octopamine and tyramine, high specificity for octopamine receptors if compared with vertebrate α-adrenoceptors (NC 5 has a more than 30 times higher affinity for neuronal octopamine receptors than for vertebrate α-adrenoceptors, Table 5). Substitutions at only two positions of the phenolic ring have a positive effect on the affinity. These are positions 2 and 4 of the phenolic ring (Figure 5). This is in contrast to the octopamine receptor of the firefly lantern, where PIIs substituted at positions 2 and 6 are most effective (Nathanson, 1985a,b) The neuronal octopamine-sensitive adenylate cyclase of the tobacco hawkmoth *Manduca sexta* has a pharmacological profile that appears to be more similar to that of the locust nervous tissue (Hollingworth & Johnstone, 1983). In both preparations, substances that are substituted at positions 2 and 4 of the phenolic ring are most effective. In addition, the ability to stimulate the octopamine-sensitive tissue, adenylate cyclase is correlated with the insecticidal activity of these compounds (Hollingworth & Johnstone, 1983; Hollingworth *et al.*, 1984). Another class of compounds, the phenylaminomethylimidazolidines were found to have very high affinity for octopamine receptors from cockroaches, whereas their affinity for *Manduca* octopamine receptors is much lower (Orr *et al.*, 1991). Interestingly, the phenylaminomethylimidazolines have a different substitution preference at the phenolic ring. Substitutions at positions 2 and 3 seems to be optimal for this class of compounds (Hollingworth *et al.*, 1984; Orr *et al.*, 1991).

### *Insecticides and miscellaneous*

It is likely that the insecticidal activity of substances such as the formamidines, amitraz, BTS 23376, or AC-6 result from interaction with octopamine receptors. The coincidence of insecticidal activity with high affinity for the octopamine receptor supports the assumption that the neuronal octopamine receptor is the target of these insecticides. Application at sublethal concentrations of the well-studied formamidines result in behavioural changes, such as hyperactivity, that could be attributed to the interaction with neuronal type octopamine receptors (Dadai *et al.*, 1987). Therefore, it seems likely that only the neuronal octopamine receptor is the target of octopamine insecticides, as these behavioural changes could not result from interaction with octopamine receptors from non-neuronal tissues.

As the most interesting structural feature of this study, various substituents at position 1 of the phenolic ring were tested. Heterocyclic substitutions such as the 2-iminoimidazolidines or the 2-benzylimidazidines are the most effective. The spacing group between these two ringsystems is of great importance. Reduction of the spacing leads to a drastic decrease in affinity. The effect of further increasing the spacing by one group was not studied because the respective substances were not available. A further increase of the affinity should result because it has been shown for the cockroach nerve cord that a substance with this kind of structure also has very high affinity (XAMI; Orr *et al.*, 1991). Further studies are needed to clarify the pharmacological characteristics of octopamine receptors. It is inappropriate to combine pharmacological data from different octopamine receptor subtypes such as non-neuronal and neuronal octopamine receptors (Roeder, 1992; Nathanson, 1993) or from studies performed with octopamine receptor preparations from different insect species. Therefore, well-defined octopamine receptor preparations such as the locust neuronal octopamine receptor should be used to obtain quantitative pharmacological data of a variety of different classes of compounds. Studies like these might result in the development of substances with even higher affinity and specificity for octopamine receptors that could serve as new leads for better insecticides as well as better probes to study octopamine receptors in more detail.

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# The receptor occupation and plasma concentration of NKY-722, a water-soluble dihydropyridine-type calcium antagonist, in spontaneously hypertensive rats

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1 The occupation *in vivo* by NKY-722 of 1,4-dihydropyridine (DHP) calcium antagonist receptors in myocardium, aorta and cerebral cortex was investigated. At 1 and 3 h after oral administration of NKY-722 (3 mg kg$^{-1}$) in spontaneously hypertensive rats (SHR), there was a significant (44 and 41%, respectively) decrease in the number of myocardial (+)-[$^3$H]-PN 200–110 binding sites ($B_{max}$) compared to control values. A greater reduction of $B_{max}$ values was observed at 1 (86%), 3 (88%), 6 (63%) and 12 (46%) h later by a higher dose (10 mg kg$^{-1}$) of this drug. The occupation of myocardial 1,4-DHP calcium antagonist receptors after oral administration of NKY-722 correlated significantly with its plasma concentration. There was a significant decrease in cerebral cortical (+)-[$^3$H]-PN 200–110 binding ($B_{max}$) at 1 and 3 h after oral administration of NKY-722 (10 mg kg$^{-1}$).

2 Oral administration of nicardipine (10 mg kg$^{-1}$) in SHR caused a significant reduction of $B_{max}$ values for (+)-[$^3$H]-PN 200–110 binding in myocardium at 1 and 3 h later and in cerebral cortex at 1 h later.

3 The *in vivo* specific binding of (+)-[$^3$H]-PN 200–110 in particulate fractions of aorta of SHR was significantly (79 and 83%, respectively) reduced at 1 and 6 h after oral administration of NKY-722 (3 mg kg$^{-1}$), while myocardial (+)-[$^3$H]-PN 200–110 binding was decreased by 52% only at 1 h later. Also, nicardipine administration reduced *in vivo* (+)-[$^3$H]-PN 200–110 binding in aorta at 1 and 6 h later and in myocardium at 1 h later. On the other hand, the administration of both NKY-722 and nicardipine had no significant effect on *in vivo* (+)-[$^3$H]-PN 200–110 binding in cerebreal cortex.

4 It is concluded that NKY-722 may exert more selective and sustained occupation *in vivo* of 1,4-DHP calcium antagonist receptors in vascular tissues of SHR than in myocardial and brain tissues.

**Keywords:** NKY-722; calcium antagonist receptor; receptor occupation *in vivo*; aorta; myocardium

## Introduction

Calcium antagonists have been widely used for the treatment of angina pectoris, systemic hypertension and other cardiovascular diseases. Nifepidine, the prototype of the 1,4-dihydropyridine (DHP) calcium antagonist, has a short duration of cardiovascular effects. Thus, currently, novel 1,4-DHP derivatives possessing long-lasting antihypertensive effects and tissue selectivity have been introduced as 'second- and third-generation' agents (Kelly & O'Malley, 1992; Meredith & Elliott, 1992). The pharmacological effects of 1,4-DHP calcium antagonists are due to an interaction with specific receptors in the cardiovascular system. The binding properties of calcium antagonists to these receptors were characterized extensively *in vitro* in brain, myocardial, and smooth muscles (Bellemann *et al.*, 1981; Bolger *et al.*, 1982; Ehlert *et al.*, 1982; Gould *et al.*, 1982; Godfraind *et al.*, 1986). However, the *in vitro* receptor affinities of calcium antagonists may not reflect the pharmacological activity and the tissue selectivity *in vivo* of these drugs because the *in vitro* binding technique does not take various pharmacokinetic (absorption, distribution, elimination) and pharmacodynamic factors into account. Thus, *in vivo* characterization of calcium antagonist binding to the receptors in different tissues is important for the analysis of pharmacodynamics and pharmacokinetics of these drugs. However, little information is available regarding the comparison of receptor occupation *in vivo* by the 1,4-DHP derivatives in different tissues and its relationship to the drug plasma concentration.

NKY-722 [3-(4-allyl-1-piperazinyl)-2,2-dimethylpropyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate dihydrochloride], a novel water-soluble 1,4-DHP, exerts a potent and long-lasting antihypertensive effect in spontaneously hypertensive rats (SHR) and it has a vasoselective effect in isolated, blood-perfused heart preparations of the dog (Imagawa *et al.*, 1989; Osumi *et al.*, 1988; 1990; Wada *et al.*, 1994). However, the receptor binding properties of this drug have not been characterized previously. The purpose of the present study was to characterize the *in vivo* binding properties of NKY-722 to 1,4-DHP receptors in myocardial, vascular and brain tissues of SHR, particularly with respect to tissue selectivity, and to the relationship between its receptor occupation and plasma concentrations. The 1,4-DHP calcium antagonist receptors in these tissues have been measured by *in vitro* and *in vivo* binding techniques using (+)-[$^3$H]-PN 200–110 as a radio ligand (Lee *et al.*, 1984; Yamada *et al.*, 1990; 1992). As SHR have been most commonly utilized to study an antihypertensive effect of calcium antagonists, we have used this model of experimental hypertension in the present study.

## Methods

### *Drug administration*

Male SHR at 14 to 18 weeks of age (Charles River Japan Inc.) were housed three or four per cage in the laboratory with free access to food (normal rat chow) and water, and maintained on a 12 h dark/light cycle in a room with controlled temperature (24 ± 1°C) and humidity (55 ± 5%). They were fasted for 16 h before the administration of drugs, and

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administered orally with NKY-722 (3,10 mg kg$^{-1}$) or nicardipine hydrochloride (10 mg kg$^{-1}$) dissolved in water and in water containing ethanol (10%) and polyethylene glycol 400 (10%), respectively, as solvents. Half of control animals examined were given the former vehicle and the rest had the latter vehicle. There was no significant difference in control values between these vehicles in each experiment of the present study, and thus, the pooled data are presented. At 1 to 24 h after the drug administration, SHR were killed by taking the blood from descending aorta under light anaesthesia with ethyl ether, and the myocardium and brain were perfused with 0.9% saline from the aorta. Then, both tissues were removed, and blood vessels were trimmed. The plasma from rat blood was isolated by centrifugation, and stored at − 20°C until concentration of NKY-722 was determined.

### *Tissue preparation*

The myocardial tissues from SHR were minced with scissors and homogenized by a Kinematica Polytron homogenizer in 10 volumes of ice-cold 50 mM Tris HCl buffer (pH 7.4). The myocardial homogenate was centrifuged at 500 *g* for 10 min, and the supernatant fraction was centrifuged at 40,000 *g* for 15 min. The pellet was resuspended in the ice-cold buffer, and the suspension was centrifuged again at 40,000 *g* for 15 min. The resulting pellet was finally suspended in the buffer for use in the binding assay. The cerebral cortex was homogenized in 20 volumes of 50 mM Tris HCl buffer with a Polytron homogenizer, and the homogenate was centrifuged at 40,000 *g* for 15 min. The pellet was washed twice by centrifugation. The pellet was finally resuspended in the original volume of the buffer and used in the binding assay. All steps were performed at 4°C. Protein concentration was measured according to the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

### *( + )-[$^3$H]-PN 200–110 binding assay*

The binding assay of ( + )-[$^3$H]-PN 200–110 was performed according to the methods by Lee *et al.* (1984) and Yamada *et al.* (1990, 1992). Briefly, the membranes (400–600 μg of protein) prepared from rat myocardium and brain were incubated with different concentrations (0.03–0.57 nM) of ( + )-[$^3$H]-PN 200–110 in 50 mM Tris HCl buffer. Incubation was carried out in the dark with a sodium lamp for 60 min at 25°C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD, U.S.A.) through Whatman GF/B glass fibre filters, and filters were rinsed three times with 4 ml of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight in scintillation fluid (2 l of toluene, 1 l of Triton X-100, 15 g of 2,5-diphenyloxazole, 0.3 g of 1,4-*bis*[2-(5-phenyloxazoly)] benzene) and the radioactivity was determined in a liquid scintillation counter. Specific ( + )-[$^3$H]-PN 200–110 binding was determined experimentally from the difference between counts in the absence and presence of 1 μM nifedipine. All assays were conducted in duplicate.

### In vivo *labelling of calcium antagonist receptors*

*In vivo* labelling of calcium antagonist receptors using ( + )-[$^3$H]-PN 200–110 was performed as described by Yamada *et al.* (1992). NKY-722 (3 mg kg$^{-1}$) and nicardipine (10 mg kg$^{-1}$) were administered orally to SHR at 1 and 6 h before an i.v. injection of 555 kBq of ( + )-[$^3$H]-PN 200–110 (66.0 ng) into the femoral vein under temporary anaesthesia with ethyl ether. The blood was taken from the descending aorta of rats 10 min after the administration of ( + )-[$^3$H]-PN 200–110, and myocardial, aortic and brain (cerebral cortical) tissues were removed. These tissues were homogenized in ice-cold 50 mM Tris HCl buffer to a final tissue concentration of 10 mg ml$^{-1}$ (myocardium and aorta) and 20 mg ml$^{-1}$ (cerebral cortex) with Kinematica Polytron homogenizer. The particulate bound radioactivity was determined by rapid filtration of 1 ml (myocardium and cerebral cortex) and 1.5 ml (aorta) of homogenate over Whatman GF/C filters, which were washed subsequently with 3 ml of ice-cold buffer. Also, aliquots (1 or 1.5 ml) of the homogenate without filtering were measured as total radioactivity (bound + free). Total and particulate bound radioactivity were measured by liquid scintillation counter after the addition of scintillation fluids. Similarly, ( + )-[$^3$H]-PN 200–110 (555 kBq) was injected i.v. to control and nifedipine (40 mg kg$^{-1}$, i.p., 0.5 h pretreatment)-treated SHR, to determine total and non-specific binding in each tissue.

### *Determination of NKY-722 in plasma*

The plasma concentration of NKY-722 was determined by high-performance liquid chromatography (h.p.l.c.). Six volumes of a mixture of *n*-hexane and diethyl ether (3:1) and 0.1 N NaOH were added to the plasma samples (1.0 ml) containing nicardipine hydrochloride as internal standard. After being stirred, the mixture was centrifuged at 15,000 *g* for 10 min. The organic phase was transferred to the glass tube, 0.1 N HCl (0.2 ml) was added, and the mixture was vortexed. After the centrifugation, a water phase (0.1 ml) was used as the h.p.l.c. sample. The h.p.l.c. system was constructed with a pump (PU-980, Jasco), UV-detector (UV-970, Jasco), injector (Model 7125, Rheodyne) and integrator (Chromatocorder 807-IT, Jasco). The analysis was performed on an Cosmosil $C_{18}$ (10 μM, 4.6 mm × 250 mm ID, Nacarai tesque Inc.). The mobile phase for assay consisted of a mixture of 0.05 M acetate buffer (pH 4.0) and acetonitrile (52:48) at a flow rate of 1.1 ml min$^{-1}$. The column eluate was monitored at a wavelength of 350 nm.

### *Data analysis*

The analysis of binding data was performed as described previously (Yamada *et al.*, 1980). The apparent dissociation constant (*K*d) and maximal number of binding sites ($B_{max}$) for ( + )-[$^3$H]-PN 200–110 binding were estimated by Rosenthal analysis of the saturation data (Rosenthal, 1967). The ability of NKY-722 and nicardipine to inhibit specific ( + )-[$^3$H]-PN 200–110 (0.24 nM) binding *in vitro* was estimated by $IC_{50}$ values, which are the molar concentrations of unlabelled drug necessary for displacing 50% of the specific binding (estimated by log probit analysis). A value for the inhibition constant, $K_i$ was calculated from the equation, $K_i = IC_{50}/(1 + L/K_d)$, where L equals the concentration of ( + )-[$^3$H]-PN 200–110. The Hill coefficients for saturation data of ( + )-[$^3$H]-PN 200–110 binding and for inhibition of NKY-722 and nicardipine were obtained by the Hill plot analysis.

The occupancy (%) of myocardial calcium antagonist receptors by NKY-722 was calculated by the equation: $\{[B_{max}(\text{control}) - B_{max}(\text{NKY-722 or nicardipine})]/B_{max}(\text{control})\} \times 100$. The receptor occupancy by NKY-722 was plotted against its plasma drug concentrations (Cp) in SHR received orally the drug. The plot was found to obey Hill's equation:

$$\log[B/B_{max}\text{-}B)] = r\cdot\log(Cp) - \log(K')$$

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

### *Drugs*

( + )-[5-methyl-$^3$H]-PN 200–110 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate) (2605 GBq mmol$^{-1}$) was purchased from DuPont-NEN Co. Ltd. (Boston, MA). NKY-722 and nicardipine hydrochloride were donated by Kyoto Pharmaceutical Company (Kyoto, Japan) and Yamanouchi Pharmaceutical Company (Tokyo, Japan), respectively. Drugs were dissolved in distilled water or ethanol and diluted in 50 mM Tris HCl buffer for *in vitro* experiments. All solutions were

prepared daily. All other chemicals were obtained from commercial sources.

## Results

### In vitro *inhibition of specific ( + )-[$^3$H]-PN 200–110 binding in myocardial and brain membranes*

Specific binding of ( + )-[$^3$H]-PN 200–110 (0.03–0.57 nM) in myocardial and cerebral cortical membranes of SHR was saturable and Rosenthal analysis revealed a linear plot (data not shown), suggesting a single population of binding sites with $K_d$ values of 0.19 ± 0.01 (myocardium) and 0.11 ± 0.01 (cerebral cortex) nM (mean ± s.e.mean, $n = 8$). The $B_{max}$ values for ( + )-[$^3$H]-PN 200–110 in these tissues were 266 ± 14 (myocardium) and 171 ± 13 (cerebral cortex) fmol mg$^{-1}$ of protein. The Hill coefficients of ( + )-[$^3$H]-PN 200–110 binding in both tissues were close to unity. NKY-722 (0.3–30 nM) and nicardipine (0.3–30 nM) competed with ( + )-[$^3$H]-PN 200–110 for myocardial and cerebral cortical binding sites *in vitro*. The $K_i$ values for inhibition of ( + )-[$^3$H]-PN 200–110 binding by NKY-722 in SHR myocardium and cerebral cortex were 6.00 ± 1.44 and 1.68 ± 0.05 nM (mean ± s.e.mean, $n = 4$), respectively, and the values by nicardipine were 1.86 ± 0.24 and 0.53 ± 0.06 nM (mean ± s.e.mean. $n = 4$), respectively. The Hill coefficients for both drugs in these tissues were close to one (1.03–1.17).

### *Effects of oral administration of NKY-722 and nicardipine on myocardial and brain ( + )-[$^3$H]-PN 200–110 binding*

The effect of oral administration of NKY-722 and nicardipine on 1,4-DHP calcium antagonist receptors in myocardial and brain tissues of SHR was investigated. Following oral administration of NKY-722 at doses of 3 and 10 mg kg$^{-1}$ in SHR, there was a significant decrease in $B_{max}$ values for specific ( + )-[$^3$H]-PN 200–110 binding to myocardial membranes compared to the control values, as shown in Figure 1 and Table 1. The decreases in $B_{max}$ values at 1 and 3 h were 44 and 41%, respectively. Similarly, the decreases in $B_{max}$ values at 1, 3, 6 and 12 h after oral administration of higher dose (10 mg kg$^{-1}$) were 86, 88, 63 and 46%, respectively. Therefore, the reduction by NKY-722 was most pronounced at 1 to 3 h, and the effect decreased with time. The $B_{max}$ values at 6 (3 mg kg$^{-1}$) and 24 (10 mg kg$^{-1}$) h after the oral administration of NKY-722 were not significantly different from the control value, suggesting the disappearance of the effect of NKY-722. The $K_d$ values for myocardial ( + )-[$^3$H]-PN 200–110 binding were unchanged by oral administration of NKY-722 at a dose of 3 mg kg$^{-1}$, and they were significantly increased at 1 and 3 h after the administration at 10 mg kg$^{-1}$. Compared to the control values, the $K_d$ and $B_{max}$ values for ( + )-[$^3$H]-PN 200–110 binding in cerebral cortex of SHR were unchanged by oral administration of NKY-722 at the dose of 3 mg kg$^{-1}$, except an increase in the $K_d$ value at 6 h later, but there was a significant (41 and 43%, respectively) decrease in the $B_{max}$ values at 1 and 3 h after the drug administration at the dose of 10 mg kg$^{-1}$ (Table 2).

As shown in Table 3, there was a significant (57 and 48%, respectively) decrease in $B_{max}$ values for specific ( + )-[$^3$H]-PN 200–110 binding to myocardial membranes at 1 and 3 h after oral administration of nicardipine at the dose of 10 mg kg$^{-1}$ in SHR and a marked increase in the $K_d$ values at 1 h later. In addition, the administration of nicardipine showed a

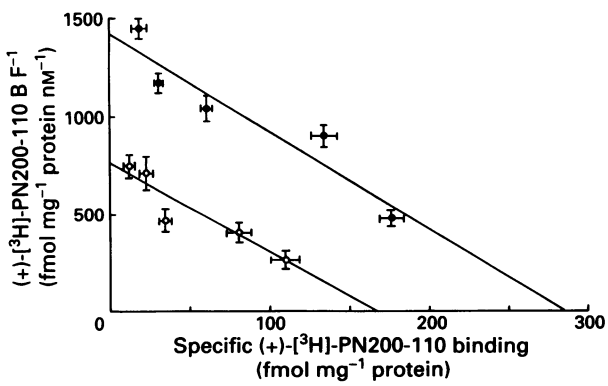


**Figure 1** Rosenthal plots of myocardial ( + )-[$^3$H]-PN 200–110 binding in control (●) and NKY-722 administered (○) SHR. SHR received orally NKY-722 (3 mg kg$^{-1}$), and were killed 3 h after the administration. Each point represents mean ± s.e.mean of 8 (control) and 6 (NKY-722) rats. Ordinate scale: bound over free (B F$^{-1}$) ( + )-[$^3$H]-PN 200–110 (fmol mg$^{-1}$ protein nM$^{-1}$). Abscissa scale: ( + )-[$^3$H]-PN 200–110 binding (fmol mg$^{-1}$ protein).

**Table 1** Effects of oral administration of NKY-722 on $K_d$ and $B_{max}$ values of specific ( + )-[$^3$H]-PN 200–110 binding to myocardial membranes of SHR

| | $K_d$ (nM) | $B_{max}$ (fmol mg$^{-1}$ protein) | *Receptor occupancy (%)* |
|---|---|---|---|
| Control | 0.19 ± 0.01 | 266 ± 14 | |
| NKY-722 | | | |
| 3 mg kg$^{-1}$ | | | |
| 1 h | 0.22 ± 0.03 | 150 ± 15*** | 43.6 |
| 3 h | 0.21 ± 0.02 | 158 ± 20*** | 40.6 |
| 6 h | 0.16 ± 0.01 | 218 ± 13 | 18.0 |
| 10 mg kg$^{-1}$ | | | |
| 1 h | 0.34 ± 0.05*** | 38.6 ± 0.7*** | 85.5 |
| 3 h | 0.37 ± 0.07** | 31.4 ± 3.3*** | 88.2 |
| 6 h | 0.19 ± 0.02 | 97.3 ± 8.9*** | 63.4 |
| 12 h | 0.15 ± 0.01* | 144 ± 15*** | 45.9 |
| 24 h | 0.25 ± 0.04 | 271 ± 14 | |

Values are mean ± s.e.mean of four to eight rats. Rats received 3 and 10 mg kg$^{-1}$ of NKY-722 orally, and they were killed at 1 to 24 h after the administration. Specific binding of ( + )-[$^3$H]-PN 200–110 (0.03–0.57 nM) to myocardial membranes was measured. The receptor occupancy (%) was calculated by the equation: $\{[B_{max}(\text{control}) - B_{max}(\text{NKY-722})]/B_{max}(\text{control})\} \times 100$. Asterisks show a significant difference from control values; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

**Table 2** Effects of oral administration of NKY-722 on $K_d$ and $B_{max}$ values of specific ( + )-[$^3$H]-PN 200–110 binding to cerebral cortical membranes of SHR

| | $K_d$ (nM) | $B_{max}$ (fmol mg$^{-1}$ protein) | *Receptor occupancy (%)* |
|---|---|---|---|
| Control | 0.11 ± 0.01 | 171 ± 13 | |
| NKY-722 | | | |
| 3 mg kg$^{-1}$ | | | |
| 1 h | 0.15 ± 0.01 | 198 ± 17 | |
| 3 h | 0.15 ± 0.01 | 168 ± 17 | |
| 6 h | 0.22 ± 0.01* | 212 ± 7 | |
| 10 mg kg$^{-1}$ | | | |
| 1 h | 0.07 ± 0.01 | 100 ± 4* | 41.5 |
| 3 h | 0.07 ± 0.001 | 97.7 ± 4.6* | 42.9 |
| 6 h | 0.09 ± 0.001 | 145 ± 6 | 15.2 |

Values are mean ± s.e.mean of four to eight rats. Rats received 3 and 10 mg kg$^{-1}$ of NKY-722 orally, and they were killed at 1 to 6 h after the administration. Specific binding of ( + )-[$^3$H]-PN 200–110 (0.03–0.57 nM) to cerebral cortical membranes was measured. The receptor occupancy (%) was calculated by the equation: $\{[B_{max}(\text{control}) - B_{max}(\text{NKY-722})]/B_{max}(\text{control})\} \times 100$. Asterisks show a significant difference from control values; *$P<0.01$.

significant (35%) decrease in $B_{max}$ value for cerebral cortical (+)-[$^3$H]-PN 200–110 binding 1 h later.

### *Occupation* in vivo *of calcium antagonist receptors*

The *in vivo* specific binding of (+)-[$^3$H]-PN 200–110 in tissues of SHR was measured. At 10 min after an i.v. injection of (+)-[$^3$H]-PN 200–110 (555 kBq) to control SHR, total amounts of radioactivity were 2596 ± 209, 2423 ± 125, and 1566 ± 116 Bq g$^{-1}$ tissue (mean ± s.e.mean, $n = 5$) in myocardium, aorta and cerebral cortex, respectively (Figure 2). The particulate bound radioactivity in these tissues, expressed as a percentage of total amount of radioactivity in each tissue, were 64 (myocardium), 48 (aorta) and 55 (cerebral cortex) %, respectively. To measure the *in vivo* nonspecific binding of (+)-[$^3$H]-PN 200–110 to these tissues, nifedipine (40 mg kg$^{-1}$, i.p.) was administered at 0.5 h before an i.v. injection of (+)-[$^3$H]-PN 200–110 (555 kBq) in SHR. As shown in Figure 2, the total radioactivity in each tissue of nifedipine-pretreated SHR was similar to that in control rats, but the particulate bound radioactivity was markedly reduced. The significant amount of specific (+)-[$^3$H]-PN 200–110 binding, therefore, defined as the difference in the particulate bound radioactivity between control and nifedipine-pretreated SHR, was demonstrated in the myocardium, aorta and cerebral cortex, as previously reported (Yamada *et al.*, 1992).

The *in vivo* specific binding of (+)-[$^3$H]-PN 200–110 in particulate fractions of myocardium and aorta of SHR was significantly (52 and 79%, respectively) decreased at 1 h after oral administration of NKY-722 (3 mg kg$^{-1}$), compared to each control value (Figure 3). A similar degree (83%) of reduction in aortic (+)-[$^3$H]-PN 200–110 binding was also observed at 6 h after oral administration of NKY-722; however, the myocardial (+)-[$^3$H]-PN 200–110 binding was almost identical to the control value at this time. Also, oral administration of nicardipine (10 mg kg$^{-1}$) showed a marked (83%) decrease in the *in vivo* specific binding of (+)-[$^3$H]-PN 200–110 in particulate fractions of SHR myocardium at 1 h later but not at 6 h later. At 1 and 6 h after the nicardipine administration, there was a significant (71 and 53%, respectively) decrease in aortic (+)-[$^3$H]-PN 200–110 binding, and thus, the extent of the reduction at 6 h was smaller than that at 1 h. On the other hand, the administration of both NKY-722 and nicardipine had little significant effect on the *in vivo* specific binding of (+)-[$^3$H]-PN 200–110 in particulate fractions of cerebral cortex (Figure 3).

### *Plasma concentration of NKY-722*

Plasma concentrations of NKY-722 in SHR (Table 1), used to measure myocardial (+)-[$^3$H]-PN 200–110 binding at 1–24 h after the oral drug administration (3, 10 mg kg$^{-1}$), were determined; they were (in ng ml$^{-1}$, mean ± s.e.mean, $n = 4$–6): 3 mg kg$^{-1}$, 60.1 ± 11.6 (1 h), 80.3 ± 4.4 (3 h),

**Table 3** Effects of oral administration of nicardipine on $K_d$ and $B_{max}$ values of specific (+)-[$^3$H]-PN 200–110 binding to myocardial and cerebral cortical membranes of SHR

| | $K_d$ (nM) | $B_{max}$ (fmol mg$^{-1}$ protein) | *Receptor occupancy (%)* |
|---|---|---|---|
| Myocardium | | | |
| Control | 0.19 ± 0.01 | 266 ± 14 | |
| Nicardipine (10 mg kg$^{-1}$) | | | |
| 1 h | 0.44 ± 0.04** | 115 ± 14** | 56.8 |
| 3 h | 0.35 ± 0.04 | 138 ± 18** | 48.1 |
| 6 h | 0.16 ± 0.01 | 221 ± 5 | 16.9 |
| Cerebral cortex | | | |
| Control | 0.11 ± 0.02 | 171 ± 13 | |
| Nicardipine (10 mg kg$^{-1}$) | | | |
| 1 h | 0.07 ± 0.01 | 111 ± 16* | 35.1 |
| 3 h | 0.08 ± 0.01 | 132 ± 4* | 22.8 |
| 6 h | 0.07 ± 0.01 | 160 ± 13 | 6.4 |

Values are mean ± s.e.mean of four to eight rats. Rats received 10 mg kg$^{-1}$ of nicardipine orally, and they were killed at 1 to 6 h after the administration. Specific binding of (+)-[$^3$H]-PN 200–110 (0.03–0.57 nM) to myocardial and cerebral cortical membranes was measured. The receptor occupancy (%) was calculated by the equation: {[$B_{max}$ (control) − $B_{max}$(nicardipine)]/$B_{max}$(control)} × 100. Asterisks show a significant difference from control values; *$P < 0.05$; **$P < 0.001$.

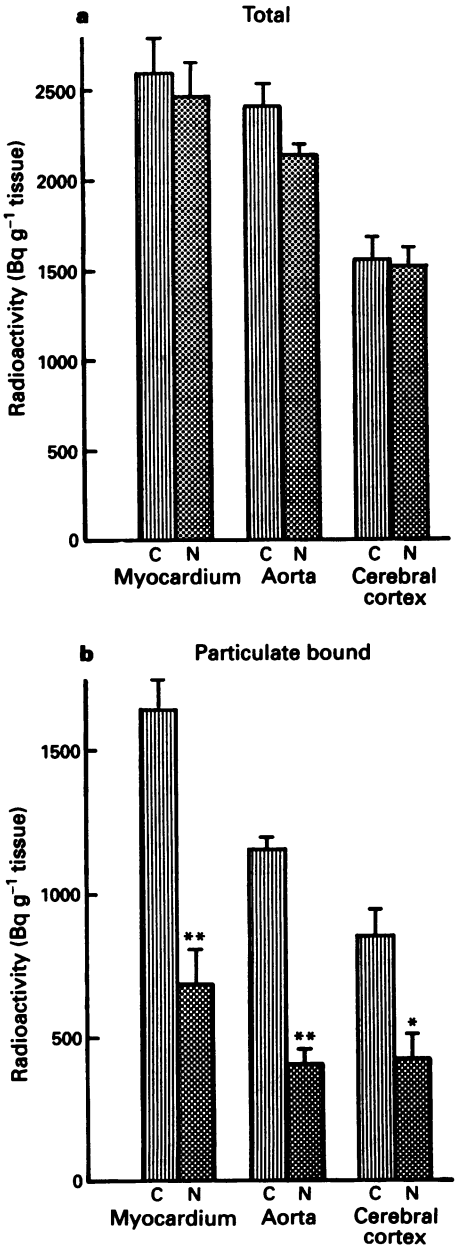


**Figure 2** Total and particulate bound radioactivity in myocardium, aorta and cerebral cortex of SHR after the i.v. injection of (+)-[$^3$H]-PN 200–110. (+)-[$^3$H]-PN 200–110 (555 kBq) was injected into the femoral vein in control (C) and nifedipine (N, 40 mg kg$^{-1}$, i.p., 0.5 h)-pretreated SHR, and they were killed 10 min later: (a) and (b) represent the total tissue radioactivity and particulate bound radioactivity, respectively. Each column represents mean ± s.e.mean of 5 control and 4 nifedipine-pretreated SHR. Asterisks show a significant difference from control value in each tissue; *$P < 0.05$; **$P < 0.001$.

5.0 ± 1.6 (6 h); 10 mg kg$^{-1}$, 355 ± 19 (1 h), 405 ± 18 (3 h), 112 ± 31 (6 h), 44.2 ± 6.6 (12 h), 16.5 ± 6.7 (24 h). As illustrated in Figure 4, the plasma concentration of NKY-722 in each SHR correlated significantly with the occupation by this drug of myocardial calcium antagonist receptors (Table 1).

## Discussion

The major findings of this study are that (1) NKY-722 occupied selectively cardiovascular 1,4-DHP calcium antagonist receptors in SHR and its occupation of myocardial receptors correlated significantly with the plasma concentration of this drug; and (2) NKY-722 produced more sustained decrease in the *in vivo* specific binding of (+)-[$^3$H]-PN 200–110 in aortic tissues than in myocardial and brain tissues.

The occupation by NKY-722 of 1,4-DHP calcium antagonist receptors in myocardium, aorta and cerebral cortex of SHR has been investigated in comparison with that by nicardipine. NKY-722 competed with (+)-[$^3$H]-PN 200–110 for the binding sites in myocardium and cerebral cortex of SHR *in vitro*, and this drug showed approximately three times higher binding affinity to the (+)-[$^3$H]-PN 200–110 binding sites in the cerebral cortex than in myocardium, as shown by $K_i$ values of 1.68 and 6.00 nM, respectively. The inhibitory effect of NKY-722 in both tissues was three times less potent than that of nicardipine. At 1 and 3 h after oral administration of NKY-722 (3 mg kg$^{-1}$), there was a significant (41–44%) reduction in myocardial (+)-[$^3$H]-PN 200–110 binding sites ($B_{max}$) in SHR compared with control values. The administration of a higher dose (10 mg kg$^{-1}$) of this drug produced a more sustained and greater (46–88%)

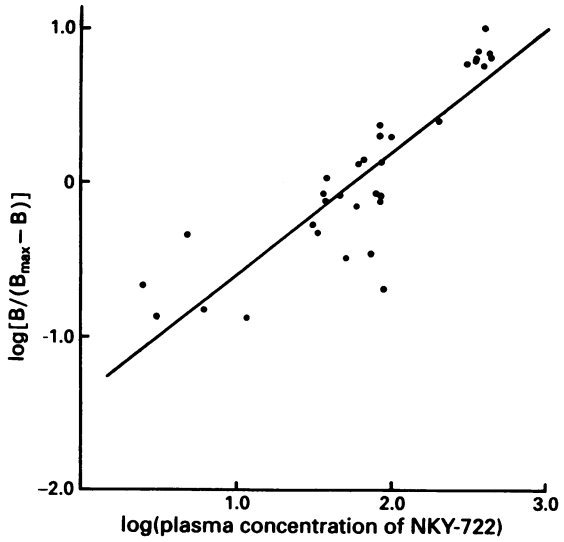


**Figure 4** Hill plots for the occupancy of myocardial 1,4-dihydropyridine calcium antagonist receptors as a function of plasma concentration of NKY-722 in SHR. The time-dependent changes in the receptor occupancy in SHR were analysed by a Hill equation in relation to the plasma concentration of NKY-722. The values of $B_{max}$ and $B$ were derived from the maximal number (fmol mg$^{-1}$ protein) of (+)-[$^3$H]-PN 200–110 binding sites in myocardial membranes of control and NKY-722-administered SHR, respectively (Table 1). The plasma concentrations (ng ml$^{-1}$) of NKY-722 in these SHR were measured: each point represents the values from each SHR received 3 and 10 mg kg$^{-1}$ of NKY-722. The points fit to the linear equation, $y = 0.79x - 1.40$, correlation coefficient $r = 0.87$, where y is the amount of receptor occupancy and x is the plasma concentration of NKY-722.

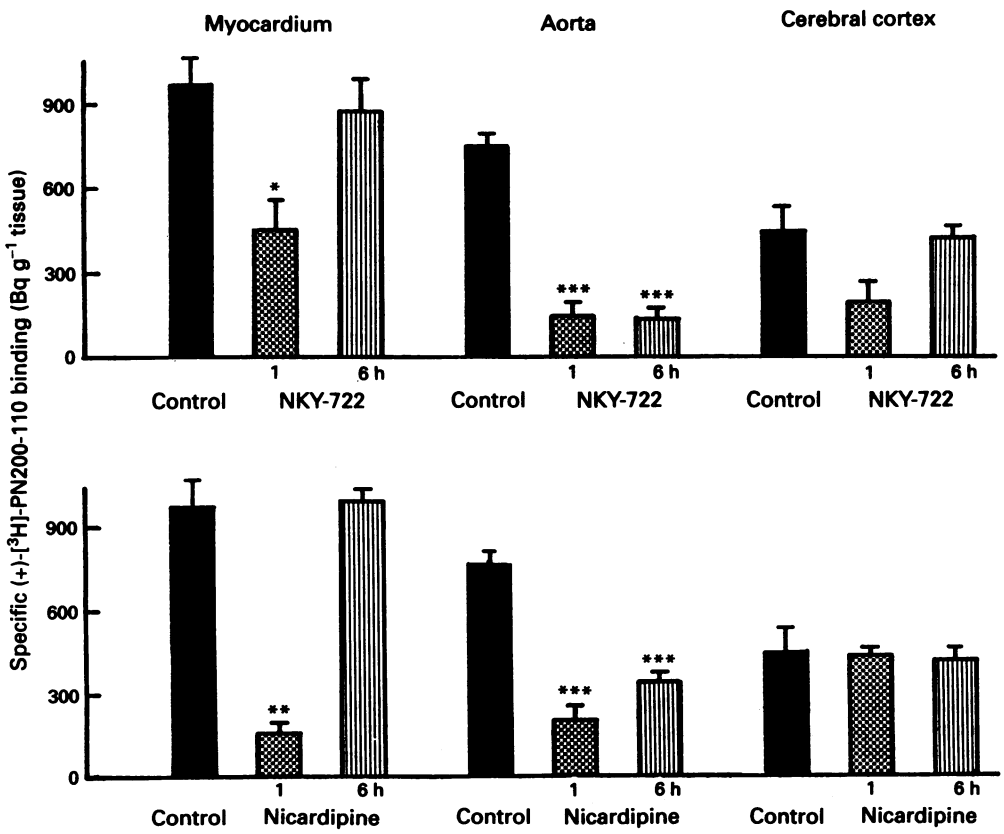


**Figure 3** *In vivo* inhibition of specific (+)-[$^3$H]-PN 200–110 binding in particulate fractions from myocardium, aorta and cerebral cortex of SHR at 1 and 6 h after oral administration of NKY-722 and nicardipine. (+)-[$^3$H]-PN 200–110 (555 kBq) was injected into the femoral vein in control, NKY-722 (3 mg kg$^{-1}$, p.o.)-and nicardipine (10 mg kg$^{-1}$, p.o.)-pretreated SHR, and they were killed 10 min later. Each column represents mean ± s.e.mean of 3–5 SHR. Asterisks show significant difference from control values; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

decrease in $B_{max}$ values for myocardial (+)-[$^3$H]-PN 200–110 binding at 1, 3, 6 and 12 h later. The extent of loss of the receptor was greatest at 1 and 3 h later and then decreased with time. The myocardial receptor density returned to the control level at 6 h (3 mg kg$^{-1}$) and 24 h (10 mg kg$^{-1}$) after NKY-722 administration. The $K_d$ values of myocardial (+)-[$^3$H]-PN 200–110 binding in SHR were unaltered by NKY-722 at the dose of 3 mg kg$^{-1}$, but they were significantly increased at 1 and 3 h later by this drug of 10 mg kg$^{-1}$. Thus, NKY-722 appears to produce mainly a change in the receptor density rathr than affinity of 1,4-DHP calcium antagonist receptors.

Oral administration of nicardipine (10 mg kg$^{-1}$) in SHR also produced a significant (48–57%) reduction in $B_{max}$ value for myocardial (+)-[$^3$H]-PN 200–110 binding at 1 and 3 h later but not at 6 h later. This inhibitory effect by nicardipine was similar to or slightly greater than the effect by NKY-722 at a dose of 3 mg kg$^{-1}$ in terms of extent and duration, but it was much weaker than that at a dose of 10 mg kg$^{-1}$. Such a difference between NKY-722 and nicardipine in the *in vivo* binding potency to myocardial 1,4-DHP calcium antagonist receptors may be responsible for the difference in both potency and duration of their pharmacological effects. In other words, NKY-722 (3 mg kg$^{-1}$, p.o.) produced a similar extent and duration of hypotensive effect in SHR as observed by nicardipine (10 mg kg$^{-1}$, p.o.) (Wada *et al.*, 1994). Thus, *in vitro* affinity of NKY-722 for myocardial 1,4-DHP binding sites was three times lower than that of nicardipine as described above, but the hypotensive effect of NKY-722 was approximately three times more potent. This observation might relate to the classical receptor theory by Stephenson (1956) that a drug with lower affinity *in vitro* could appear more potent *in vivo* due to its slower dissociation from the receptor site. In the cerebral cortex, the NKY-722 administration at 3 mg kg$^{-1}$ had little effect on (+)-[$^3$H]-PN 200–110 binding, and at 10 mg kg$^{-1}$, it produced a significant reduction of $B_{max}$ values for (+)-[$^3$H]-PN 200–110 binding at 1 and 3 h later. Thus, the *ex vivo* experiment with NKY-722 has indicated that this drug produced a selective occupation of myocardial, rather than cerebral cortical, 1,4-DHP calcium antagonist receptors in SHR.

The specific binding of (+)-[$^3$H]-PN 200–110 in particulate fractions from myocardial, aortic and brain tissues after an i.v. injection of (+)-[$^3$H]-PN 200–110 to SHR reflects *in vivo* labelling of 1,4-DHP calcium antagonist receptors in tissues (Yamada *et al.*, 1992). The *in vivo* specific binding of (+)-[$^3$H]-PN 200–110 in particulate fractions from myocardial and aortic tissues in SHR reduced significantly (52 and 79%, respectively) at 1 h after oral administration of NKY-722 (3 mg kg$^{-1}$), and the myocardial (+)-[$^3$H]-PN 200–110 binding restored to the control level at 6 h later. The time course of occupation *in vivo* of myocardial 1,4-DHP calcium antagonist receptors by NKY-722 was consistent with that in the *ex vivo* receptor binding experiment. Interestingly, a marked (83%) decrease in aortic (+)-[$^3$H]-PN 200–110 binding *in vivo* was observed even at 6 h after NKY-722 adminstration. Therefore, NKY-722 may produce a selective and sustained occupation *in vivo* of vascular 1,4-DHP calcium antagonist receptors in SHR. This finding agrees with the pharmacological data that NKY-722 was highly vasoselective. The dose of NKY-722 that produced 50% decrease in the force of contraction of the paced canine papillary muscle was about 100 times the dose that doubled coronary blood flow (Imagawa *et al.*, 1989). In contrast to a significant occupation of cardiovascular receptors, the *in vivo* specific binding of (+)-[$^3$H]-PN 200–110 in the cerebral cortex of SHR was not significantly reduced by NKY-722 administration. Inasmuch as NKY-722 competed with (+)-[$^3$H]-PN 200–110 for the binding sites in the cerebral cortex of SHR with higher affinity than in the myocardium *in vitro*, this observation, with the *ex vivo* binding data described above, suggests poor transport of this drug through the blood-brain barrier. The nicardipine administration also produced a sustained decrease in aortic (+)-[$^3$H]-PN 200–110 binding *in vivo*. In conclusion, the present study provides the first evidence demonstrating a significant selectivity of 1,4-DHP calcium antagonists in vascular tissues at the receptor level *in vivo*. Thus, the *in vivo* measurement of calcium antagonist receptor occupation may offer a better insight into the interaction at receptor level by calcium antagonists and into the elucidation of tissue selectivity of these drugs under physiological conditions.

Some 1,4-DHP derivatives are subject to extensive and variable first pass metabolism by the liver (Higuchi & Shiobara, 1980; Foster *et al.*, 1983) which reduces systemic bioavailability and drug efficacy after oral administration. The relationship between the cardiovascular effects of some 1,4-DHP derivative calcium antagonists and their plasma concentrations in experimental animals and in man has been investigated (Takata & Kato, 1986, Graefe *et al.*, 1988, Wu *et al.*, 1988). In the present study, the plasma concentration of NKY-722 in SHR was greatest at 1 and 3 h after the oral administration of this drug at doses of 3 and 10 mg kg$^{-1}$, and at 6 and 12 h later, it decreased markedly to approximately one-tenth of the maximum level. The plasma concentration of NKY-722 in SHR correlated significantly with the occupation of myocardial 1,4-DHP calcium antagonist receptors in the same rats (Figure 4). Recently, Wada *et al.* (1994) have shown that oral administration of NKY-722 (3 mg kg$^{-1}$) in SHR produced a potent hypotension which was pronounced at 1 to 6 h later and lasted until 8 h. However, at 6 h after oral administration of this dose of NKY-722, there was no significant occupation of myocardial 1,4-DHP calcium antagonist receptors in *ex vivo* and *in vivo* experiments, and the *in vivo* binding data demonstrated more sustained occupation by this drug of 1,4-DHP calcium antagonist receptors in vascular tissues of SHR than in myocardial tissues. Taken together, it is possible that the dissociation of NKY-722 from vascular 1,4-DHP receptors in SHR is significantly slower than the dissociation from myocardial receptors, depending heavily on the plasma concentration of this drug. Thus, a slowly dissociating blockade of vascular 1,4-DHP calcium antagonist receptors by NKY-722 may contribute to the potent and long-lasting hypotensive effect in SHR.

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# Inotropic changes induced by fluoroaluminates in rabbit left atrial muscles: possible involvement of G proteins

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**1** The effects of fluoroaluminate complexes (NaF plus $AlCl_3$) on force of contraction, cyclic AMP accumulation and phosphoinositide hydrolysis were examined in rabbit left atrial muscles.

**2** Fluoroaluminates (1–10 mM NaF + 10 μM $AlCl_3$) produced a biphasic inotropic response which was composed of an early small decline and subsequent increase in force of contraction. In the presence of the $Al^{3+}$ chelator, deferoxamine (100 μM), the positive inotropic response was completely abolished and a sustained negative inotropic response appeared, suggesting that only the positive inotropic response is due to the action of fluoroaluminates.

**3** The positive inotropic effect of fluoroaluminates was associated with a significant increase in the total duration of a single contraction; the time to peak tension and relaxation time were prolonged. In contrast, these parameters were substantially abbreviated by isoprenaline or histamine.

**4** When force of contraction was increased by isoprenaline or histamine, the addition of fluoroaluminates caused a marked negative inotropic effect, which was eliminated by pretreatment with pertussis toxin.

**5** Fluoroaluminates did not cause a significant increase in cyclic AMP content at concentrations of NaF in the range of 1–10 mM. However, the content of cyclic AMP was greatly elevated by fluoroaluminates when the atrial muscles were pretreated with pertussis toxin.

**6** Accumulation of [$^3$H]-inositol monophosphate in atrial muscle strips prelabelled with *myo*-[$^3$H]-inositol was significantly increased by fluoroaluminates at concentrations of NaF over 1 mM. The phosphoinositide response to fluoroaluminates remained unchanged with pertussis toxin pretreatment.

**7** These results indicate that, in rabbit left atrial muscles, fluoroaluminates produce a positive inotropic effect which may be mediated by $G_q$ but not by $G_s$ proteins; they produce a negative inotropic effect possibly through $G_i$ only when $G_s$ is activated with other agents.

**Keywords:** Fluoroaluminates; inotropic effects; cyclic AMP; phosphoinositide hydrolysis; G proteins; atrial myocardium

## Introduction

G proteins are transducers that convert extracellular signals to intracellular signals by coupling receptor-activation to different effector systems in the plasma membranes (Gilman, 1987). It is known that fluoride, in the presence of aluminium, is a potent activator of G proteins (Sternweis & Gilman, 1982). The active complex is thought to be the fluoroaluminate complex $AlF_4^-$ which is formed when fluoride and aluminium are present in solution together (Sternweis & Gilman, 1982; Martin, 1986). The cellular basis of this action is based upon a structural similarity of $AlF_4^-$ to $PO_4^{3-}$, thus enabling the former to interact with the nucleotide binding site of G proteins and to mimic the γ phosphate of guanosine triphosphate (GTP) (Bigay *et al.*, 1985; 1987). Thus, fluoroaluminates possibly cause dissociation of α from βγ subunits of G proteins in a manner similar to a proposed mechanism of action of a stable analogue of GTP, GTPγS (Harden, 1989), and may be considered to be a nonhydrolyzable and reversible GTP analogue (Poll *et al.*, 1989). Hence, fluoroaluminates have been used as a pharmacological probe to establish the significance of G protein activation in several cellular systems (Blackmore *et al.*, 1985; Cockcroft & Taylor, 1987; Paris & Pouyssegur, 1987).

Long before fluoroaluminates were recognized as activators of G proteins, fluoride was known to produce a positive inotropic action on the heart (Loewi, 1955; Katzung *et al.*, 1957; Covin & Berman, 1959; Berman, 1966). Because aluminium is a contaminant of glassware (Sternweis & Gilman, 1982), it is possible that in earlier studies demonstrating the positive inotropic effect of fluoride, fluoroaluminates rather than fluoride were causing the increased cardiac contractility.

The present study was undertaken to characterize effects of fluoroaluminate complexes (NaF plus $AlCl_3$) on force of contraction in rabbit isolated left atrial muscles, assuming that fluoroaluminates may cause inotropic changes by activating G proteins directly *via* their GTP binding site. Since fluoroaluminates could conceivably activate $G_s$ and $G_p$ and thereby stimulate adenylate cyclase and phospholipase C, respectively, depending on cell types (Sternweis & Gilman, 1982; Blackmore *et al.*, 1985; Harden, 1989), the abilities of fluoroaluminates to promote adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation and inositol phosphate accumulation in the atrial muscles were also examined.

## Methods

### *Measurement of inotropic responses*

New Zealand White rabbits of either sex weighing 1.6–2 kg were killed by a blow on the head. The hearts were quickly removed and transferred to a dissection bath filled with oxygenated warm Krebs-Henseleit solution. The composition of the solution was (mM): NaCl 119, $CaCl_2$ 2.5, KCl 4.8, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 24.9 and glucose 10.0. The left atrium was carefully dissected, cut into halves and both halves were used (Hattori *et al.*, 1991). The preparations were mounted vertically in a water-jacketed organ bath containing 50 ml of Krebs-Henseleit solution. The solution in the bath was bubbled with 95% $O_2$ and 5% $CO_2$ and its temperature was maintained at 30 ± 1°C. The lower end of

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the muscle was fixed onto a pair of hook electrodes for stimulation and the other end was connected by a silk thread to a force transducer (Nihon Kohden, TB-612T, Tokyo, Japan). Isometric tension developed in the preparation was recorded on a thermal array recorder (Nihon Kohden, RTA-1200) through a preamplifier (Nihon Kohden, RP-5). The resting tension applied to the preparation was adjusted to 2 g. The atrial muscle was driven electrically by rectangular pulses of 3 ms duration with an intensity of 1.5 times the diastolic threshold at a frequency of 1 Hz. The pulses were delivered by an electronic stimulator (Sanei-Sokki, 3F46, Tokyo, Japan) through an isolation unit (Sanei-Sokki, 5361). The preparations were allowed to equilibrate for at least 60 min before the experiments were begun.

The inotropic effect produced by NaF in the presence of $AlCl_3$ was expressed as a percentage change in the basal force of contraction obtained before adding NaF. $AlCl_3$ (10 $\mu$M) was given 10 min before the addition of NaF. When added alone, $AlCl_3$ had no significant effect on force of contraction.

In a series of experiments carried out to examine the effects of fluoroaluminates on the positive inotropic response to isoprenaline or histamine, these agonists were added after the administration of $AlCl_3$, and then NaF was applied when the tension developed reached a steady level. It was confirmed that the positive inotropic effects of these agonists were maintained at a steady level for at least 20 min after application.

In some experiments, left atrial muscles were obtained from rabbits pretreated with pertussis toxin (PTX). The animals received a single ear-vein injection of PTX (10 $\mu g\,kg^{-1}$) in a volume of 0.4 to 0.5 ml 48 h before they were killed. PTX was dissolved in 0.9% NaCl. The effectiveness of the dose of PTX was assessed by evaluating the negative inotropic effect of carbachol which was nearly completely abolished in left atria prepared from the PTX-treated rabbits.

### *Cyclic AMP assays*

Left atria were prepared and mounted in the organ bath in the same way as described above. The muscles were removed from the organ bath at 20 min after the addition of NaF unless otherwise specified, and were immediately frozen with a clamp which had been cooled with liquid nitrogen. The frozen samples were stored at − 80°C until homogenized.

The frozen tissues were weighed and homogenized in 1 ml of ice-cold 6% trichloroacetic acid. The homogenate was centrifuged at 3000 r.p.m. for 10 min at 4°C, and the supernatant was reserved. The pellet was resuspended in 1 ml of cold 6% trichloroacetic acid and centrifuged again. After addition of 10 $\mu$l of 1 N HCl, the combined supernatant fractions were extracted 4 times with 1 ml of water-saturated ether, heated at 80°C for 30 min to evaporate the residual ether, then lyophilized overnight and resuspended in distilled water. The cyclic AMP contents were determined by the sensitive radioimmunoassay method described by Honma *et al.* (1977) using a Yamasa cyclic AMP kit (Yamasa Shoyu Company, Choshi, Japan).

### *Phosphoinositide measurements*

Phosphoinositide hydrolysis was determined by measuring the incorporation of label from *myo*-[$^3$H]-inositol into inositol monophosphate ($IP_1$) as previously described (Hattori *et al.*, 1989). Briefly, rabbit left atrial strips were labelled with 4 $\mu Ci\,ml^{-1}$ *myo*-[$^3$H]-inositol (24.4 $Ci\,mmol^{-1}$, New England Nuclear, Boston, MA, U.S.A.) for 120 min in 3 ml Krebs-Henseleit solution gassed with 95% $O_2$ and 5% $CO_2$ at a temperature of 37°C. LiCl (final concentration 10 mM) was then added, and 5 min later NaF or carbachol was added. When NaF was used as a stimulant, $AlCl_3$ (10 $\mu$M) was added 5 min before the LiCl. Following 30 min of incubation with NaF or carbachol, the tissue was transferred to a tube containing 675 $\mu$l of ice-cold chloroform/methanol/HCl (100:200:1, v/v/v) and homogenized after adding 180 $\mu$l of 5 mM EDTA. The homogenizer tip was rinsed with 570 $\mu$l of chloroform/methanol/HCl/5 mM EDTA (100:200:1:80), and the rinsing fluid was added to the original solution. After the addition of 375 $\mu$l of chloroform and 75 $\mu$l of 5 mM EDTA, the samples were centrifuged at 3900 r.p.m. for 20 min. An aliquot of the aqueous layer was removed, neutralized with borax and run on ion-exchange columns for separation of [$^3$H]-$IP_1$ as described by Berridge *et al.* (1982). The radioactively labelled products were counted in 15 ml of Scintisol EX-H (Dojin, Kumamoto, Japan) by a liquid scintillation counter.

### *Drugs*

The drugs used were as follows: NaF (Nakalai, Kyoto, Japan); $AlCl_3$ (Wako, Osaka, Japan); deferoxamine mesylate, (−)-isoprenaline hydrochloride, carbachol chloride and 3-isobutyl-1-methyl-xanthine (IBMX) (Sigma, St. Louis, MO, U.S.A.); histamine dihydrochloride (Merck, Darmstadt, Germany); and PTX (List Biological Laboratories, Campbell, CA, U.S.A.). All compounds except PTX (see above) and IBMX were dissolved in distilled water. IBMX was prepared in dimethyl sulphoxide. Further dilutions to the proper concentrations were made with Krebs-Henseleit solution. Ascorbic acid (0.1 mM) was added to the solution of isoprenaline to retard its auto-oxidation.

### *Statistics*

All data were expressed in terms of the means ± s.e.mean. Statistical assessment of the data was made by Student's paired and unpaired $t$ tests or two-way analysis of variance (ANOVA) with repeated measurements. A significant difference was assumed to exist if the $P$ value was less than 0.05.

## Results

### *Inotropic changes induced by fluoroaluminates*

In the presence of 10 $\mu$M $AlCl_3$, which had no significant change in force of contraction, NaF (1–10 mM) induced a biphasic inotropic response in rabbit left atrial muscles electrically driven at 1 Hz. The time course of inotropic changes produced by 5 mM NaF is shown in Figure 1. After the addition of NaF, force of contraction initially decreased. The initial phase lasted for at least 5 min before the positive inotropic response was evident. The maximal level of the positive inotropic response generally occurred within 20 min, although it was attained at 25 to 40 min after the addition of NaF at lower concentrations (1–2 mM). As depicted in Figure 2, the initial negative inotropic response and subsequent positive inotropic response to NaF showed different concentration-dependencies. The initial, transient depression of force of contraction appeared to increase with increasing NaF concentration. On the other hand, the maximum increase in force of contraction was obtained with 2 mM NaF. At concentrations higher than 2 mM, the positive inotropic effect of NaF gradually decreased. Resting tension was mildly but significantly raised by 7.5 and 10 mM NaF after ~15 min of exposure (data not shown).

The chelator, deferoxamine, at a concentration of 100 $\mu$M (Ackrill & Day, 1984) completely abolished the positive inotropic response to 5 mM NaF in the presence of 10 $\mu$M $AlCl_3$ (Figure 1). However, the negative inotropic response to NaF was resistant to deferoxamine. Thus, NaF produced a sustained negative inotropic effect in the presence of deferoxamine. The positive inotropic responses to other agents such as isoprenaline and histamine were not affected by deferoxamine (data not shown). In the absence of $AlCl_3$ NaF still produced a biphasic inotropic response, although the positive inotropic component was enhanced by $AlCl_3$. Since the

glassware was used in the present experiments, the positive inotropic effect of NaF alone was probably due to fluoroaluminates formed by the complexing of fluoride with aluminium present as a contaminant of glass. In the presence of $AlCl_3$, 10 mM NaCl did not cause any change in force of contraction (data not shown).

When NaF caused a positive inotropic effect, the duration of isometric contraction curve was significantly prolonged (Figure 3). In the presence of 10 μM $AlCl_3$, 5 mM NaF increased the time to peak tension from 92 ± 2 to 98 ± 2 ms ($n = 6$, $P < 0.01$) and the half relaxation time from 66 ± 2 to 77 ± 3 ms ($n = 6$, $P < 0.01$). In contrast, the positive inotropic effects of isoprenaline and histamine were associated with a marked abbreviation of these parameters (Figure 3). Thus, the time to peak tension was shortened by 10 nM isoprenaline from 90 ± 4 to 78 ± 2 ms ($n = 5$, $P < 0.05$) and the half relaxation time from 60 ± 3 to 40 ± 2 ms ($n = 5$, $P < 0.01$). Also, the time to peak tension was shortened by 10 μM histamine from 90 ± 4 to 76 ± 3 ms ($n = 4$, $P < 0.05$) and the half relaxation time from 65 ± 2 to 45 ± 2 ms ($n = 4$, $P < 0.01$).

To assess the possible involvement of PTX-sensitive G proteins, the influence of PTX pretreatment on the inotropic changes induced by NaF in the presence of $AlCl_3$ was then examined. As shown in Figure 4, NaF (5 mM) caused an initial decrease in force of contraction even in atrial muscles from the animals pretreated with PTX (10 μg $kg^{-1}$, 48 h). However, in the PTX-pretreated muscles, the negative inotropic phase was more transient and the positive inotropic phase appeared more rapidly and was enhanced. A two-way ANOVA test indicated the control and PTX-pretreated curves to be clearly different from each other.

When the positive inotropic effect of 10 nM isoprenaline reached a steady level, the addition of 5 mM NaF, in the presence of 10 μM $AlCl_3$, exerted a marked negative inotropic action (Figure 5a). The marked negative inotropic action of NaF was rapid in onset compared to the positive inotropic response seen in Figure 1. NaF (5 mM) also greatly reduced the increased force of contraction by 10 μM histamine (Figure 5b). The suppressant effect of NaF on the positive inotropic responses to isoprenaline and histamine was significantly attenuated by pretreatment with PTX (Figure 5).

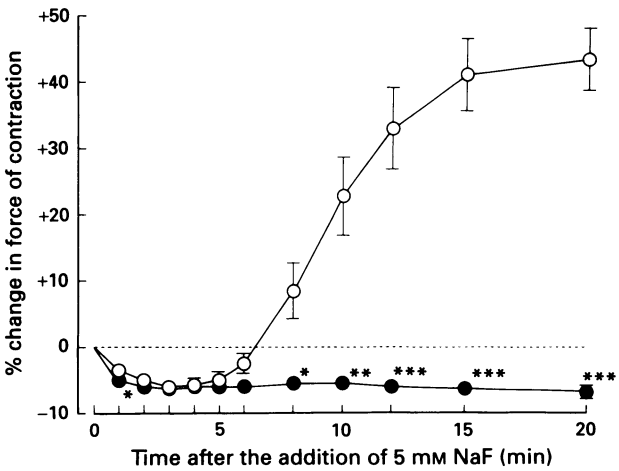


**Figure 1** Effect of deferoxamine on the time course of changes in force of contraction after the application of 5 mM NaF in the presence of 10 μM $AlCl_3$. Deferoxamine (100 μM) was allowed to act for 30 min before the addition of NaF and experiments were carried out in its presence. (○) Control; (●) 100 μM deferoxamine. Points (means ± s.e.mean, $n = 7$) represent the change in force of contraction expressed as a percentage of basal values recorded before the addition of NaF. Basal force of contraction was 1476 ± 85 mg ($n = 14$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs the corresponding control values.

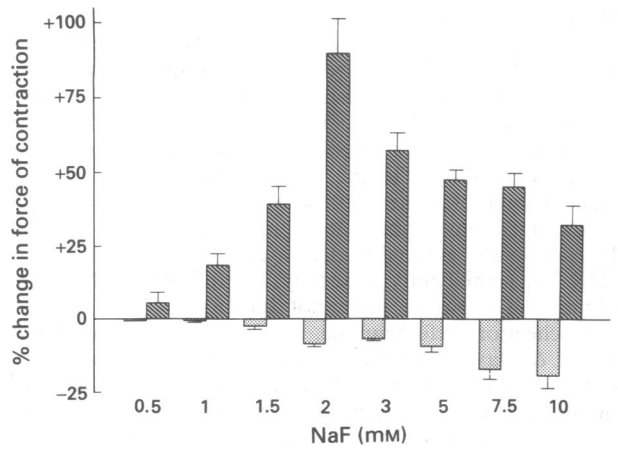


**Figure 2** Changes in force of contraction induced by various concentrations of NaF in the presence of 10 μM $AlCl_3$. First column of each pair indicates the initial negative inotropic response, and second column of each pair shows the subsequent positive inotropic response. Columns represent means ± s.e.mean of 6–9 experiments. Basal force of contraction was 1445 ± 51 mg ($n = 54$).

## *Effect of fluoroaluminates on cyclic AMP content*

The cyclic AMP contents of rabbit left atrial muscles at a range of times after the addition of 4 mM NaF, in the

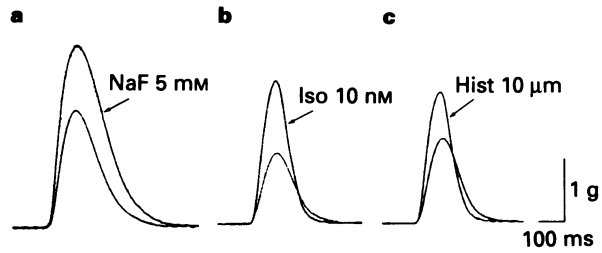


**Figure 3** Representative tracings showing the changes in time course of isometric contractions induced by 5 mM NaF (a), 10 nM isoprenaline (Iso) (b) and 10 μM histamine (Hist) (c).

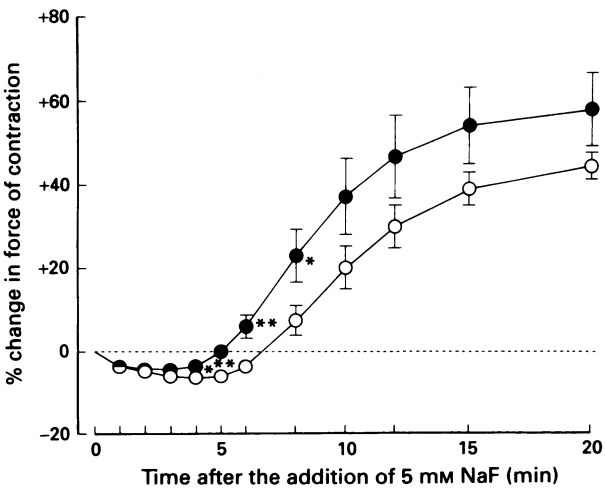


**Figure 4** Influence of pretreatment with pertussis toxin (PTX) on the time course of changes in force of contraction after the application of 5 mM NaF in the presence of 10 μM $AlCl_3$. (○) Control; (●) PTX-pretreated. Points (means ± s.e.mean, $n = 7$–8) represent the changes in force of contraction expressed as a percentage of basal values recorded before the addition of NaF. Basal force of contraction was not significantly different between control (1535 ± 131 mg, $n = 8$) and PTX-pretreated muscles (1416 ± 118 mg, $n = 7$). $^*P < 0.05$; $^{**}P < 0.01$ vs the corresponding control values.

presence of 10 μM $AlCl_3$, are shown in Figure 6. The content of cyclic AMP was not significantly changed by treatment with NaF for up to 20 min. The lack of effect of NaF was not due to rapid breakdown of cyclic AMP, because even in the presence of IBMX, a phosphodiesterase inhibitor, NaF failed to increase cyclic AMP content. In the presence of 10 μM IBMX, the contents of cyclic AMP were 0.51 ± 0.08 ($n = 4$) and 0.47 ± 0.05 pmol $mg^{-1}$ wet wt. ($n = 4$) 20 min after the addition of 10 μM $AlCl_3$ alone and 4 mM NaF plus 10 μM $AlCl_3$, respectively.

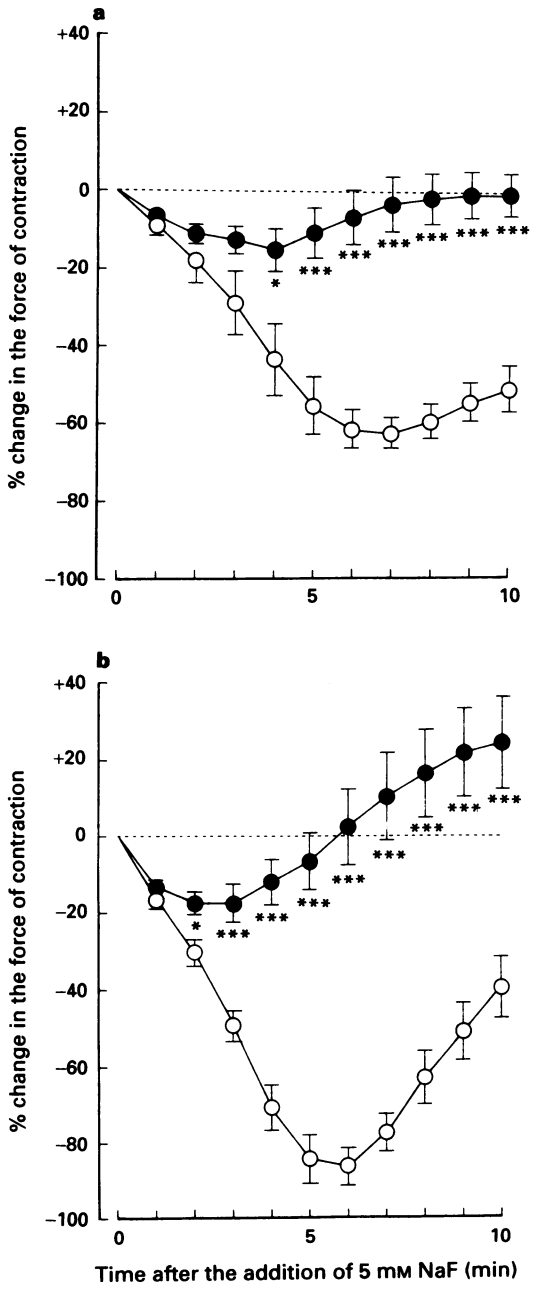


**Figure 5** Influence of pretreatment with pertussis toxin (PTX) on the time course of the suppressive effect of 5 mM NaF, in the presence of 10 μM $AlCl_3$, on the positive inotropic responses to 10 nM isoprenaline (a) and 10 μM histamine (b). (○) Control; (●) PTX-pretreated. Each point (means ± s.e.mean, $n = 6-7$) represents the percentage change in force of contraction when the increases in force of contraction caused by isoprenaline or histamine just before the addition of NaF were taken as 100%. Isoprenaline (10 nM) and histamine (10 μM) increased force of contraction by 114 ± 32% ($n = 6$) and 79 ± 9% ($n = 7$) in control muscles and by 74 ± 11% ($n = 6$) and 103 ± 27% ($n = 7$) in PTX-pretreated muscles, respectively. $^*P < 0.05$; $^{***}P < 0.001$ vs the corresponding control values.

Figure 7 shows the effect of varying concentrations of NaF, in the presence of 10 μM $AlCl_3$, on cyclic AMP content determined 20 min after the administration of NaF. NaF at concentrations up to 10 mM had no significant effect on cyclic AMP content. However, the tissue cyclic AMP content was greatly increased by 3 mM NaF in the PTX-pretreated muscles (Figure 7).

### *Effect of fluoroaluminates on [$^3$H]-$IP_1$ formation*

The effect of varying concentrations of NaF, in the presence of $AlCl_3$, on [$^3$H]-$IP_1$ formation in rabbit left atrial strips prelabelled with *myo*-[$^3$H]-inositol is shown in Figure 8. NaF increased [$^3$H]-$IP_1$ formation in a concentration-dependent manner. A significant increase in the formation of [$^3$H]-$IP_1$ compared with the basal value occurred at concentrations of NaF ≥ 2 mM. The level of [$^3$H]-$IP_1$ formation produced by 5 mM NaF was comparable to that induced by 10 μM carbachol, used as a reference. The inositol phosphate response to NaF was unaffected by PTX pretreatment. In PTX-pretreated muscle strips, the [$^3$H]-$IP_1$ levels before and after the addition of 10 mM NaF were 5.8 ± 0.6 and 24.2 ± 7.1 d.p.m. $mg^{-1}$ wet wt., respectively ($n = 3$, for each group).

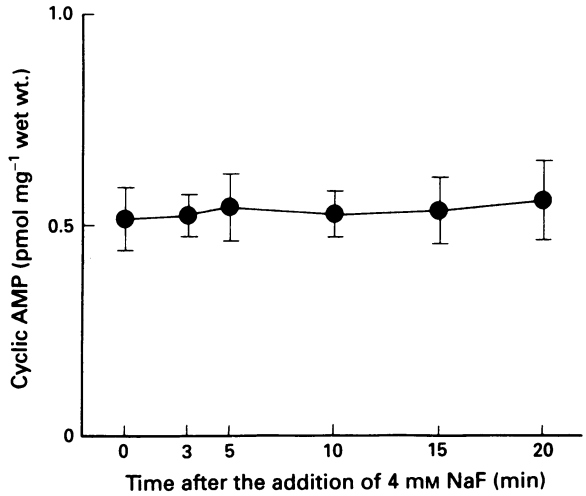


**Figure 6** Time course of changes in cyclic AMP contents after the application of 4 mM NaF in the presence of 10 μM $AlCl_3$. Points are means ± s.e.mean of 5–9 experiments.

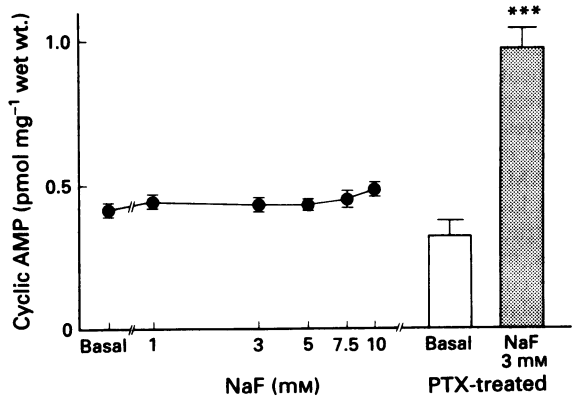


**Figure 7** Effect of varying concentrations of NaF, in the presence of 10 μM $AlCl_3$, on cyclic AMP contents. The basal response and the response to 3 mM NaF, in the presence of 10 μM $AlCl_3$, in PTX-pretreated muscles are shown as the open column and stippled column, respectively. The cyclic AMP contents were determined 20 min after the addition of NaF. The values are shown as means ± s.e.mean of 5–7 experiments. $^{***}P < 0.001$ vs the basal value obtained in PTX-pretreated muscles.

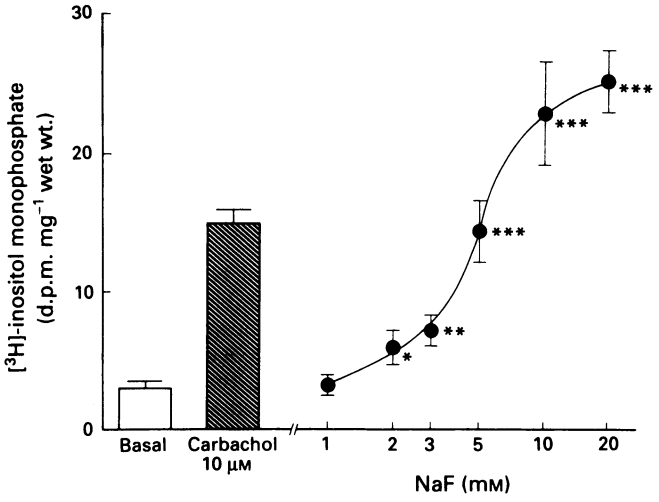


**Figure 8** Effect of increasing concentrations of NaF, in the presence of 10 μM $AlCl_3$, on the accumulation of [$^3$H]-$IP_1$. The levels of [$^3$H]-$IP_1$ were measured following a 30 min exposure of the muscle strips to NaF or carbachol. The basal response is shown by open column, and the response to 10 μM carbachol by hatched column. The values are shown as means ± s.e.mean of 5–6 experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$ vs the basal value.

## Discussion

In the present study, NaF, in the presence of $AlCl_3$, caused a biphasic inotropic response in rabbit left atrial muscles, an effect that was characterized by an initial decreasing phase and a late developing positive inotropic phase. The positive inotropic response was completely blocked by deferoxamine, a chelator of $Al^{3+}$ (Ackrill & Day, 1984). On the other hand, the negative inotropic response was sustained in the presence of deferoxamine. These results indicate that the positive inotropic response is due to the action of fluoroaluminates, while the negative inotropic effect appeared to be caused by fluoride alone.

Fluoroaluminates produced a significant increase in force of contraction at concentrations of NaF in the range of 1–10 mM. The maximum increase was observed with 2 mM NaF. Coincidence of the concentrations at which NaF effectively activates G proteins in other tissues (Gilman, 1987; Harden, 1989) and causes a positive inotropic effect (this study) implicates the involvement of a G protein in the genesis of the latter effect. Although fluoroaluminates can activate $G_s$ and thus stimulate adenylate cyclase in *in vitro* experiments (Sternweis & Gilman, 1982), the positive inotropic response to fluoroaluminates was not accompanied by any increase in intracellular content of cyclic AMP. Blackmore *et al.* (1985) have observed that, in rat hepatocytes, cyclic AMP levels were slightly but significantly decreased by NaF at low concentrations (<10 mM), but were increased at concentrations over 20 mM. Also, in bovine tracheal smooth muscles, fluoroaluminates failed to increase cyclic AMP levels at NaF concentrations of less than 20 mM (Hall *et al.*, 1990). One explanation for this lack of effect of fluoroaluminates on cyclic AMP levels may be a preponderance of $G_i$ over $G_s$ in intact cells and tissues. As a result, simultaneous activation of $G_s$ and $G_i$ by fluoroaluminates could cause inhibition of adenylate cyclase, leading to no change or a slight decrease in cyclic AMP. Fluoroaluminates would also be expected to stimulate other G proteins besides $G_s$ and $G_i$. Thus, another possible explanation is that the βγ complexes produced by dissociation of other G proteins interfered with activation of $G_s$ (Katada *et al.*, 1984). The present finding that fluoroaluminates produced an increase in cyclic AMP content in left atrial muscles from PTX-pretreated rabbits supports the possibility that fluoroaluminates primarily stimulate $G_i$ to inhibit the production of cyclic AMP. We observed that the positive inotropic effect of fluoroaluminates was associated with an increase in the total duration of a single contraction; the time to peak tension and relaxation time were significantly prolonged. These changes in time course of isometric contractions were in strong contrast to those produced by stimulation of β-adrenoceptors and histamine $H_2$-receptors. The marked abbreviation of the relaxation time by activation of these receptors is considered to be due to the cyclic AMP-mediated phosphorylation of functional proteins in both the sarcoplasmic reticulum and the myofibrils (Tsien, 1977). Taken together, it is reasonable to conclude that $G_s$ is not involved in the positive inotropic effect of fluoroaluminates.

The ability of fluoroaluminates to prolong the duration of a single contraction is similar to those of $\alpha_1$-adrenoceptor agonists, angiotensin II and endothelin-1 (Endoh & Blinks, 1988; Ishihata & Endoh, 1993; Hattori *et al.*, 1993). These agonists *via* their specific receptors activate a G protein (perhaps $G_q$), which triggers activation of phospholipase C, and thereby catalyses hydrolysis of membrane phosphoinositides (Fleming *et al.*, 1992). Indeed, these agonists have no effect on intracellular cyclic AMP levels and instead stimulate phosphoinositide hydrolysis in the myocardium (Brown *et al.*, 1985; Ishihata & Endoh, 1993; Hattori *et al.*, 1993). In the present study, fluoroaluminates stimulated [$^3$H]-$IP_1$ formation in rabbit left atrial strips prelabelled with *myo*-[$^3$H]-inositol. This phosphoinositide response was observed at the same concentrations of NaF as reported in hepatocytes and a number of smooth muscles (Blackmore *et al.*, 1985; Marc *et al.*, 1988; Zeng *et al.*, 1989; Hall *et al.*, 1990), although the phosphoinositide response to fluoroaluminates could be demonstrated at somewhat higher concentrations of NaF than that required for the positive inotropic response. Thus, as previously indicated (Blackmore *et al.*, 1985; Harden, 1989), the phosphoinositide response to fluoroaluminates in rabbit left atrial muscles is thought to be a result of stimulation of phospholipase C by direct activation of $G_q$. In view of striking similarities between the characteristic of the positive inotropic effect produced by fluoroaluminates and those by $\alpha_1$-adrenoceptor agonists, angiotensin II as well as endothelin-1, the involvement of $G_q$ in the positive inotropic effect of fluoroaluminates may be inferred. However, the role of the phosphoinositide system in the regulation of myocardial contractility remains unsettled (Movsesian *et al.*, 1985; Ehrlich & Watras, 1988; Hattori *et al.*, 1989). Furthermore, the relationship between the stimulation of phosphoinositide hydrolysis and the positive inotropic effect in response to $\alpha_1$-adrenoceptor stimulation is still the subject of controversy (Endou *et al.*, 1991; Endoh *et al.*, 1991). Nonetheless, it seems likely that fluoroaluminates and activation of $\alpha_1$-, angiotensin II and endothelin receptors may share a common signal transduction pathway, possibly involving stimulation of $G_q$, to produce a positive inotropic effect in myocardium.

When the positive inotropic effect was induced by isoprenaline or histamine, the subsequent addition of NaF, in the presence of $AlCl_3$, caused a marked negative inotropic action. The positive inotropic effects of isoprenaline and histamine are principally due to an elevation of cyclic AMP resulting from β-adrenoceptor- and $H_2$-receptor-mediated activation of adenylate cyclase *via* $G_s$ (Fleming *et al.*, 1992; Levi *et al.*, 1991). Pretreatment with PTX drastically eliminated the suppressive effect of fluoroaluminates on the positive inotropic responses to isoprenaline and histamine, suggesting that this effect is presumably due to activation of $G_i$ by fluoroaluminates. The results are in good agreement with the previous reports showing that fluoroaluminates inhibit adenylate cyclase activity when it is stimulated with other agents in intact tissues (Blackmore *et al.*, 1985; Marc *et al.*, 1988; Hall *et al.*, 1990). Thus, the present study implies that, in rabbit left atrial muscles, fluoroaluminates produce a

negative inotropic effect by activating $G_i$ when $G_s$ is stimulated with other agents.

It is well known that activation of PTX-sensitive G protein(s) stimulates atrial inwardly rectifying $K^+$ channels and thereby reduces atrial force of contraction directly (Brown & Birnbaumer, 1990). However, we did not clearly observe a PTX-sensitive negative inotropic action of fluoroaluminates *per se* in rabbit left atrial muscles. At the present time, we do not have a clear understanding of this contradictory result. Luetje *et al.* (1988) have shown that $G_o$, which is a PTX substrate in addition to $G_i$, is expressed in the heart at a very low level, mainly in atria and less in ventricle. Furthermore, in ferret ventricular muscle which is an exception in that it responds to muscarinic agonists with a pronounced negative inotropy and a marked abbreviation of action potential duration (Boyett *et al.*, 1988), the content of $G_o$ is found to be extremely high (Endoh *et al.*, 1993). Thus, $G_o$ might play a predominant role in activation of atrial muscarinic $K^+$ channels. If so, the present data that fluoroaluminates exerted a PTX-sensitive negative inotropic effect only when the force of contraction was increased by isoprenaline or histamine could reflect different sensitivities of $G_i$ and $G_o$ to fluoroaluminates in rabbit atria.

In summary, the present study suggests that G proteins are likely to be involved in the inotropic changes induced by fluoroaluminates in rabbit left atrial muscles. Fluoroaluminates produce a positive inotropic effect which may be mediated by $G_q$ but not by $G_s$, while producing a negative effect possibly through $G_i$ only when $G_s$ is activated with other agents.

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# Effect of R56865 on cardiac sarcoplasmic reticulum function and its role as an antagonist of digoxin at the sarcoplasmic reticulum calcium release channel

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1 The effect of R56865 (N-[1-[4-(4-fluorophenoxy)-butyl]-4-piperidinyl]-*N*-methyl-2-benzothiazolamine) on cardiac sarcoplasmic reticulum (SR) $Ca^{2+}$-release channel function was investigated. The effect of R56865 on [$^3$H]-ryanodine and [$^3$H]-digoxin binding to SR vesicles and its effect on the ATP-stimulated $^{45}Ca^{2+}$ uptake into SR vesicles was also studied.

2 R56865 (0.5–50 μM) had no effect on single-channel open probability (Po) when added to native cardiac SR $Ca^{2+}$-release channels, incorporated into planar phospholipid bilayers, that had previously been activated by 10 μM $Ca^{2+}$. The single-channel conductance (93 pS) and the $Ca^{2+}/Tris^+$ permeability ratio (12.5) were also unaffected by R56865.

3 R56865 failed to affect the rapid $Ca^{2+}$-induced efflux of $^{45}Ca^{2+}$ from cardiac SR vesicles. The initial efflux rate at an extravesicular [$Ca^{2+}$] of 0.1 μM was 176 ± 33 nmol $^{45}Ca^{2+}$ $mg^{-1}$ protein $s^{-1}$ ($n = 5$). Addition of 0.5–50 μM R56865 to the efflux solution did not affect the initial efflux rate or the total amount of $^{45}Ca^{2+}$ released from the vesicles.

4 The specific binding of [$^3$H]-ryanodine to SR vesicles can be viewed as a marker for SR $Ca^{2+}$-release channel activation. R56865 (0.05–50 μM) did not change the amount of specific [$^3$H]-ryanodine bound at 10 μM activating $Ca^{2+}$. Taken together these data (points 2, 3 and 4) suggest that R56865 does not affect the $Ca^{2+}$ activation of the cardiac SR $Ca^{2+}$-release channel.

5 R56865 (0.5–50 μM) decreased the ATP-stimulated uptake of $^{45}Ca^{2+}$ into cardiac SR vesicles. The total amount of $^{45}Ca^{2+}$ taken up into the vesicles was decreased by 26–37% and the initial rate of uptake was also reduced.

6 R56865 decreased the binding of [$^3$H]-digoxin to cardiac SR vesicles. It inhibited binding of [$^3$H]-digoxin to both sites on SR membranes. However, it acted as a mixed type of non-competitive antagonist, as it increased the $K_d$ and reduced the $B_{max}$ for [$^3$H]-digoxin. Additionally, when SR $Ca^{2+}$-release channels incorporated into bilayers were activated by 1 nM digoxin at 10 μM $Ca^{2+}$, 5 μM R56865 decreased single-channel Po to that seen prior to activation by digoxin, confirming that R56865 is an antagonist at the cardiac glycoside receptor site on the cardiac SR $Ca^{2+}$-release channel.

7 The results presented here show that R56865 decreases $^{45}Ca^{2+}$ uptake into SR vesicles and also non-competitively decreases the binding of cardiac glycosides to their activation sites on the SR $Ca^{2+}$-release channel. Since this compound fails to affect other aspects of the functioning of the SR, we conclude that inhibition of $Ca^{2+}$-uptake into the SR and/or antagonism of digoxin binding to its high affinity sites on the cardiac SR $Ca^{2+}$-release channel may contribute to the protection against glycoside-induced toxicity seen with R56865. Indeed the reduction of $Ca^{2+}$ uptake into the SR during $Ca^{2+}$ overload may contribute to the protective action of R56865 against arrhythmias induced by veratridine or by ischaemia. Notwithstanding this, it is possible that the multitude of other actions on specific ion translocation processes, as well as the possible non-specific membrane effects of R56865, may be as responsible for the protection against arrhythmias as the effects on SR function we have documented here. However, the simultaneous interference with more than one of these processes by R56865 may be required for its anti-arrhythmic action.

**Keywords:** Sarcoplasmic reticulum; cardiac glucosides; anti-arrhythmic drugs; $Ca^{2+}$-release channel

## Introduction

Cardiac glycosides have recently been reported to bind to and activate sheep sarcoplasmic reticulum (SR) $Ca^{2+}$-release channels incorporated into planar lipid bilayers and also to release $Ca^{2+}$ from SR vesicles, effects which are specific to the cardiac isoform of the channel and which occur at clinically relevant concentrations (McGarry & Williams, 1993). We have also demonstrated that [$^3$H]-digoxin binds to two sites on the cardiac channel and that the lack of effect of glycosides on the skeletal channel is due to the absence of a glycoside binding site on skeletal SR and not because of different transduction mechanisms between cardiac and skeletal $Ca^{2+}$-release channels (S.J. McGarry & A.J. Williams, unpublished observations). It is possible that activation of the cardiac channel by digoxin contributes to or is responsible for the increase in contraction of cardiac muscle. Additionally, this action may underlie or contribute to the manifestation of toxic effects seen with this drug.

R56865 (N-[1-[4-(4-fluorophenoxy)-butyl]4-piperidinyl]-*N*-methyl-2-benzothiazolamine) at sub micromolar concentrations protects against, or raises the threshold for the onset of, the toxic effects of glycoside administration (Vollmer *et al.*, 1987; Damiano *et al.*, 1991). The protection against cardiac glycoside-induced toxicity (the appearance of delayed after depolarizations, arrhythmias and an increase in muscle tension) has been shown to be mediated by R56865 exerting its effect at a site distinct from the sarcolemmal $Na^+/K^+$ ATPase (Heers *et al.*, 1991) i.e. it does not displace glycosides

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from their binding site(s) on $Na^+/K^+$ ATPase nor does R56865 itself affect the functioning of $Na^+/K^+$ ATPase. R56865 has also been shown to inhibit ischaemia-reperfusion arrhythmias (Garner *et al.*, 1990) and to protect against triggered propagated contractions and triggered arrhythmias induced by caffeine or Bay K8644 (Daniels & ter Keurs, 1992). Similarly, it is protective against the mechanical and electrical consequences of veratridine administration (Wilhelm *et al.*, 1991).

R56865 appears to have many actions on ion channels. It has been demonstrated to block the $Na^+$ current (Verdonck *et al.*, 1991; Wilhelm *et al.*, 1991), to block the $Na^+$ activated $K^+$ channel (Luk & Carmeliet, 1990) and to depress the transient inward current (Leyssens *et al.*, 1988). It has also been shown to prevent the ultrastructural changes associated with $Ca^{2+}$ overload (Teigtmeier *et al.*, 1992). More recently, it was demonstrated to decrease catecholamine release from bovine adrenal chromaffin cells, an effect that may also contribute to its anti-arrhythmic action (Garcez-Do-Carmo *et al.*, 1993). However, R56865 does not affect the L-type $Ca^{2+}$ channel or the inward rectifier (Himmel *et al.*, 1990) and it appears to exert no direct action via the $Na^+/Ca^{2+}$ exchanger, even at very high concentrations (Ichikawa *et al.*, 1994). Thus it is obvious that this compound may exert its protective action against arrhythmias induced by various means by acting through several mechanisms.

The lack of selectivity of R56865 may be compounded by its high lipid solubility. It also interacts with $\alpha_1$-adrenoceptors and with dihydropyridine binding sites in rat cortex (Koch *et al.*, 1988) and it displays anti-histamine activity in guinea-pig ileum (data in Finet *et al.*, 1989). These effects are seen at concentrations of R56865 that display anti-arrhythmic activity.

The effect of R56865 on sarcoplasmic reticulum function in multicellular preparations is difficult to assess given its other actions and its effects on isolated sarcoplasmic reticulum have not been investigated. Given the novel actions of cardiac glycosides on the SR $Ca^{2+}$-release channel documented above, we have studied the effect of R56865 on cardiac SR $Ca^{2+}$ uptake and on the functioning of the cardiac SR $Ca^{2+}$-release channel.

## Methods

### *Preparation of SR membrane vesicles*

The preparation of sheep cardiac SR membrane vesicles was as described by Sitsapesan & Williams (1990).

### *Single-channel methods*

Lipid bilayers containing phosphatidylethanolamine in decane (30 mg $ml^{-1}$) were formed across a 200 μm diameter hole in the partition between two fluid-filled styrene copolymer chambers, referred to as *cis* and *trans*. The *trans* chamber was held at ground and the *cis* chamber clamped at holding potentials relative to this. Current flow through the bilayer was measured with an operational amplifier as a current-voltage converter (Miller, 1982). Initially, both chambers contained 50 mM choline chloride, 10 mM HEPES and 5 mM $CaCl_2$, with the pH adjusted to 7.4 with Tris. HSR vesicles were added to the *cis* chamber. The chamber was stirred and the choline chloride concentration increased to give a 7:1 gradient *cis-trans* to promote vesicle fusion with the bilayer, fusion being marked by the appearance of $Cl^-$-selective channels (Smith *et al.*, 1985). SR vesicles incorporated into the bilayer in a fixed orientation such that the cytosolic face of the $Ca^{2+}$-release channel was directed towards the *cis* chamber and the luminal face to the *trans* chamber. Following vesicles fusion, the *cis* and *trans* chambers were perfused with solution allowing resolution of only $Ca^{2+}$-release channel currents. The *cis* chamber was perfused with 250 M HEPES, 125 mM Tris, pH 7.4; and the *trans* with 250 mM glutamic acid, 10 mM HEPES, with the pH adjusted to 7.4 with $Ca(OH)_2$, giving a $[Ca^{2+}]$ in the *trans* chamber of 67 mM. The concentration of $Ca^{2+}$ in the *cis* chamber was buffered to the desired level by the addition of $CaCl_2$ and EGTA. The concentrations of each were calculated by EQCAL (Biosoft, Cambridge). Experiments were performed at room temperature (22 ± 1°C).

### *Data acquisition, analysis and display*

Single-channel data were displayed on an oscilloscope and recorded on video tape. Current recordings were replayed, low-pass filtered using an 8-pole Bessel filter (Frequency Devices 902) at 1 kHz and digitized at 2 kHz using an AT based system, Satori (Intracel, Cambridge). Channel open probability (Po) and open and closed lifetimes were determined by 50% threshold analysis at a holding potential of 0 mV. Single-channel Po was determined from $Po = T_{open}/(T_{open} + T_{closed})$ where $T_{open}$ and $T_{closed}$ are the mean open and closed lifetime durations, respectively. Digitized single-channel traces were transferred from Satori as plot files into CorelDraw 4 (Corel Corporation) and labelled for display. Lifetimes, accumulated from 3 min of steady state recordings, were stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to a probability density function by the method of maximum likelihood (Colquhoun & Sigworth, 1983). Lifetimes lasting less than 1 ms were incompletely resolved under these data acquisition conditions and were therefore excluded from the fitting procedure. A missed events correction was applied (Colquhoun & Sigworth, 1983) and a likelihood ratio test was used to compare fits to double and triple exponentials (Blatz & Magleby, 1986).

Single-channel conductance was obtained from the slope of the linear regression lines drawn through single-channel current amplitude data monitored at holding potentials within the range (+40 to −40 mV). Reversal potentials were obtained from extrapolations of these lines. The $Ca^{2+}/Tris^+$ permeability ratio was calculated from the equation given by Fatt & Ginsborg (1958).

### *ATP-stimulated $^{45}Ca^{2+}$ uptake into SR vesicles*

HSR vesicles were incubated in a solution containing KCl 100 mM, HEPES 20 mM, ATP 1 mM, $MgCl_2$ 5 mM and $^{45}CaCl_2$ 10 μM at 22°C. At times varying from 30 s to 20 min, aliquots containing 100 μg of protein were removed. The aliquots were placed on 0.65 μm pore diameter Millipore filters under vacuum and washed with a solution containing KCl 100 mM, HEPES 20 mM, $MgCl_2$ 5 mM, $CaCl_2$ 5 mM and EGTA 1 mM (non-release buffer). This solution removed any extravesicular $^{45}Ca^{2+}$ without causing the release of loaded intravesicular $^{45}Ca^{2+}$. The remaining radioactivity on the filters was counted in a liquid scintillation counter after immersion of the filters in 10 ml Packard scintillant.

### *Rapid $^{45}Ca^{2+}$ efflux from SR vesicles*

The release of $^{45}Ca^{2+}$ from SR vesicles actively loaded with $^{45}CaCl_2$ was investigated using the Bio-Logic rapid filtration apparatus (Intracel, Cambridge), and a modification of the method of DuPont (1984). Aliquots of vesicles actively loaded with $^{45}Ca^{2+}$, as outlined above, containing approximately 200 μg of protein, were placed on 0.65 μm pore diameter filters pre-soaked in non-release buffer. Vesicles were washed with 5 ml non-release buffer under vacuum to remove extravesicular $^{45}Ca^{2+}$. The filter was then placed in the efflux apparatus and washed for controlled times (10–1000 ms) with a solution consisting of KCl 100 mM, HEPES 20 mM, EGTA 1 mM and $CaCl_2$ 695 μM (free $[Ca^{2+}] = 0.1$ μM). The remaining radioactivity on the filters was counted in a liquid scintillation counter as outlined above. Initial efflux rates

were calculated from the derivative of the single exponential decay curve best fitted to each set of data using INPLOT4 (GraphPad Software). Each experiment was conducted in duplicate and results are displayed as mean ± s.e.mean.

### *Binding assays*

Binding of [$^3$H]-ryanodine to HSR membranes was conducted essentially as described by Holmberg & Williams (1990). For equilibrium binding, HSR membranes (100 μg protein) were incubated with either 5 mM [$^3$H]-ryanodine (76 Ci mmol$^{-1}$) for 90 min or 8.6 nM [$^3$H]-digoxin (27 Ci mmol$^{-1}$) for 20 min at 37°C in a solution containing KCl 1 M and PIPES 25 mM, pH 7.4, in a final volume of 1 ml. R56865 was added at the beginning of each assay. Non-specific binding was determined by the addition of 1 μM unlabelled ryanodine or 200 μM unlabelled digoxin. Saturation binding of [$^3$H]-digoxin was conducted in identical conditions with 0.1–250 nM [$^3$H]-digoxin. All assays were performed in triplicate with at least five different preparations with the appropriate control. Binding was terminated by the addition of 5 ml ice cold binding buffer followed by immediate filtration through a Whatman GF/B filter pre-soaked in binding solution. The filter was then washed with three further 5 ml volumes of buffer before being placed in 10 ml Packard MV scintillant and the remaining radioactivity on the filter counted in a liquid scintillation counter.

Curves were fitted to the data by least squares non-linear regression with INPLOT4 (GraphPad Software) and are displayed as mean ± s.e.mean.

### *Association and dissociation rate experiments*

The association-rate experiments were conducted using a Bio-Logic rapid filtration apparatus, described by DuPont (1984). The injection syringe was loaded with 8.6 nM [$^3$H]-digoxin in binding solution in a total volume of 20 ml, which had been pre-heated to 37°C. HSR membranes containing 100 μg of protein were diluted into 1 ml of binding solution at 37°C. A Millipore 0.65 μm pore diameter filter, pre-soaked in binding solution, was then placed in the filtration apparatus. The vesicles in the 1 ml of solution were adsorbed onto the filter under vacuum and washed for controlled times from 10–2000 ms with the binding solution containing [$^3$H]-digoxin. The filter was then washed with three 5 ml volumes of ice-cold binding solution and placed in 10 ml Packard MV scintillant and the remaining radioactivity on the filter counted in a liquid scintillation counter. R56865 was added to the injection syringe at each concentration. Non-specific binding was determined by repeating the experiments with 200 μM digoxin also present in the injection syringe.

HSR membranes containing 100 μg of protein were incubated in binding solution with 8.6 nM [$^3$H]-digoxin for 20 min at 37°C and dissociation was initiated by addition of a 20 fold excess of binding solution or by addition of R56865. The solution containing the membranes was then filtered through Millipore 0.65 μm pore diameter filters and the radioactivity remaining on the filters counted as outlined above. All assays were performed in triplicate with the appropriate control. Association and dissociation rates were analysed by the method of Bennett & Yamamura (1978).

### *Drugs used*

R56865 was from Janssen Pharmaceuticals, Germany and was dissolved as a 5 mM stock solution in 100% ethanol. In all experiments, the concentration of ethanol did not exceed 1% and was without effect. Digoxin was obtained from Sigma (Poole, Dorset). $^{45}CaCl_2$ (2.24 mCi ml$^{-1}$), [$^3$H]-ryanodine (76 Ci mmol$^{-1}$) and [$^3$H]-digoxin (27 Ci mmol$^{-1}$) were from NEN (Stevenage, Herts). Phosphatidylethanolamine was from Avanti Polar Lipids, Alabama, U.S.A.

## Results

### *Single-channel studies*

R56865 (0.5–50 μM) had no effect on single sheep cardiac SR $Ca^{2+}$-release channels in lipid bilayers (Figure 1). Channels were voltage-clamped at 0 mV and activated by the addition of 10 μM $Ca^{2+}$ to the cytosolic face of the channel. R56865 was ineffective when added to the cytosolic or luminal face of the channel. At 10 μM $Ca^{2+}$, single-channel Po was 0.043 ± 0.018 ($n = 4$); in the presence of 50 μM R56865, the Po was 0.035 ± 0.020 ($n = 4$). Similarly, the single-channel conductance and $Ca^{2+}$/$Tris^+$ permeability ratio were 93 ± 2 pS ($n = 5$) and 12.5 ± 0.3 ($n = 5$) in 10 μM $Ca^{2+}$ and were unchanged on addition of 50 μM R56865. Higher concentrations of R56865 were not tested as these are well in excess of those shown to exert an anti-arrhythmic action. A further reason is to avoid the possibility of solvent effects and non-specific membrane actions of the drug.

### *Rapid $^{45}Ca^{2+}$ efflux experiments*

R56865 also failed to affect the rapid efflux of $^{45}Ca^{2+}$ from cardiac SR vesicles. At an extravesicular [$Ca^{2+}$] of 0.1 μM, concentrations of R56865 up to 50 μM were without effect. At 0.1 μM $Ca^{2+}$, the efflux of $^{45}Ca^{2+}$ followed single exponential decay kinetics, with an initial rate of 176 ± 33 nmol $^{45}Ca^{2+}$ mg$^{-1}$ protein s$^{-1}$ ($n = 5$). Neither the initial rate of release nor the total amount of $^{45}Ca^{2+}$ released was significantly changed at any concentration of R56865 tested. Table 1 shows the initial efflux rates at a range of concentrations of R56865.

### *[$^3$H]-ryanodine binding*

The binding of [$^3$H]-ryanodine to native HSR membranes, which is widely used as a marker for SR $Ca^{2+}$-release channel Po (Chu *et al.*, 1991) was similarly unaffected by R56865. At concentrations from 0.05–50 μM, R56865 did not significantly alter the binding from the control value in 10 μM $Ca^{2+}$ of 5.21 ± 0.65 pmol $^3$H mg$^{-1}$ protein (Figure 2). Interestingly, the related compound flunarizine was also unable to affect binding ($n = 6$, not shown).

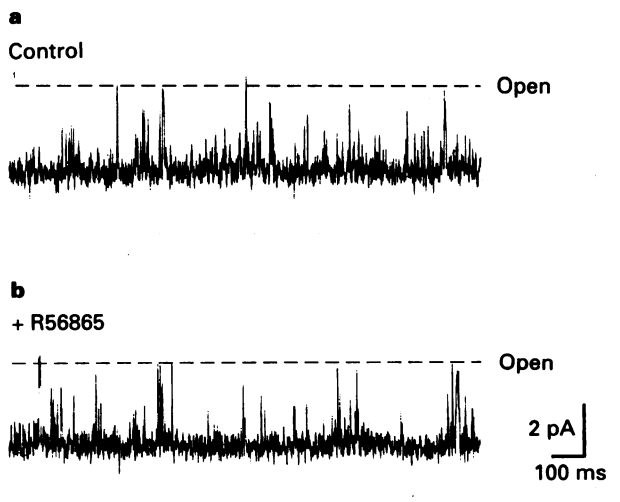


**Figure 1** Representative portions of data from a single SR $Ca^{2+}$-release channel held at 0 mV. The channel was activated by 10 μM $Ca^{2+}$ on the cytosolic face of the channel (a). Addition of up to 50 μM R56865 (b) to the *cis* chamber failed to affect the open probability (Po) of $Ca^{2+}$-release channels. In this channel, Po was 0.031 before and 0.028 after addition of 50 μM R56865. Single-channel Po was calculated from 3 min of continuous recordings as described in the Methods section. Dotted lines indicate channel open level.

**Table 1** The initial efflux rates, in the presence and absence of R56865, for the release of $^{45}Ca^{2+}$ from cardiac SR vesicles induced by 0.1 μM $Ca^{2+}$

| *Treatment* | *Initial efflux rate* (nmol $^{45}Ca^{2+}$ $mg^{-1}$ protein $s^{-1}$) |
|---|---|
| Control (0.1 μM) $Ca^{2+}$ | 176 ± 33 ($n = 5$) |
| +0.5 μM R56865 | 156 ± 27 ($n = 4$) |
| +5 μM R56865 | 167 ± 22 ($n = 5$) |
| +50 μM R56865 | 171 ± 17 ($n = 5$) |

All experiments were performed in duplicate and are the mean ± s.e.mean of the stated number of experiments. All the data were fitted to single and double exponentials and the best fits determined using an *F*-test. Each set of data was best fitted by a single exponential decay curve and the initial rate was determined as the derivative of the curve.

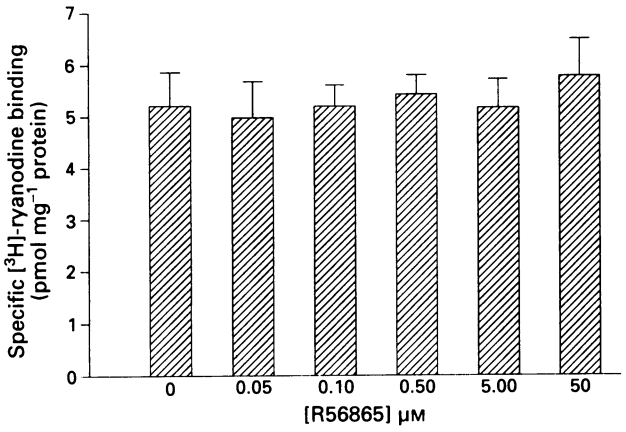


**Figure 2** Lack of effect of R56865 on the $Ca^{2+}$ stimulation of [$^3$H]-ryanodine binding to the cardiac SR membranes. Incubation of HSR vesicles with 5 nM [$^3$H]-ryanodine in binding solution containing 10 μM $Ca^{2+}$ for 90 min at 37°C yielded specific binding of 5.21 ± 0.65 pmol $mg^{-1}$ protein ($n = 6$). This was unchanged on pre-incubation of vesicles with 0.05–50 μM R56865. Each column is the mean ± s.e.mean of six different experiments conducted in triplicate.

### *Inhibition of ATP-stimulated $^{45}Ca^{2+}$ uptake into SR vesicles*

Figure 3 shows the inhibition of the ATP-stimulated uptake of $^{45}Ca^{2+}$ into HSR vesicles by 5 μM R56865. The initial rate of uptake and total amount of $^{45}Ca^{2+}$ taken up was reduced by 0.5–50 μM R56865 (Figure 4). As it inhibits both the initial rate and total amount of uptake, R56865 appears to act as a non-competitive inhibitor of uptake, although a more detailed study would be required to assess this. However, R56865 could only maximally inhibit total uptake by approximately 37% at 50 μM, suggesting that it is a relatively poor inhibitor of uptake.

### *Displacement of [$^3$H]-digoxin from SR membranes*

Cardiac glycosides increase the Po of single cardiac SR $Ca^{2+}$-release channels and [$^3$H]-digoxin binds to two sites on cardiac HSR membranes, probably on the SR $Ca^{2+}$-release channel (McGarry & Williams, 1993, and unpublished observations). Equilibrium binding studies with R56865 show that it decreases the binding of [$^3$H]-digoxin to both its sites on HSR membranes with apparent $K_i$s of 9.95 nM and 3.50 μM ($n = 11$) (Figure 5a). However, a more detailed analysis of the effect shows that R56865 dose-dependently decreases the density of binding sites for [$^3$H]-digoxin. Maximum binding density was decreased from approximately 11 pmol $mg^{-1}$ protein in control to 1 pmol $mg^{-1}$ protein in the presence of

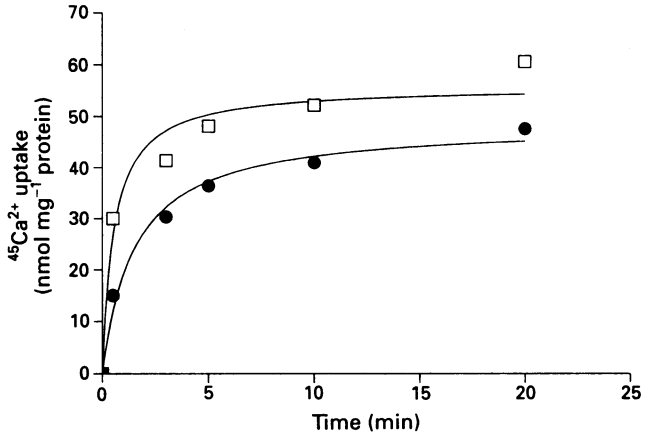


**Figure 3** Inhibition of the ATP-stimulated uptake of $^{45}Ca^{2+}$ into cardiac HSR vesicles by 5 μM R56865. R56865 (●) was added 5 min prior to the addition of 10 μM $^{45}Ca^{2+}$ (total $[Ca^{2+}] = 20$ μM) and reduced total uptake as well as initial rate of uptake. The data are the mean of five different experiments conducted in triplicate. Standard errors are omitted for clarity and are no more than ± 7% of the given values.

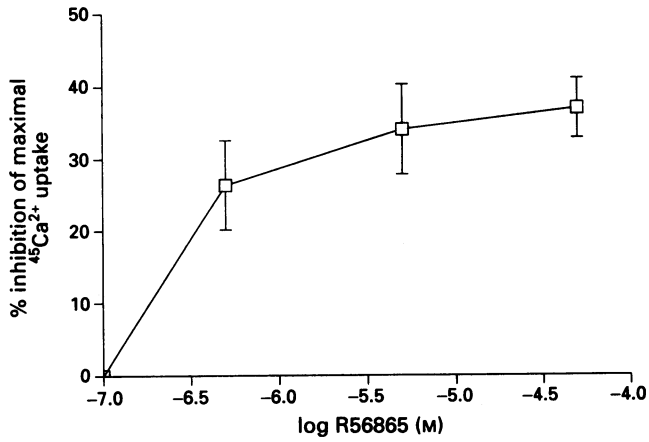


**Figure 4** The concentration-dependent reduction of the ATP-stimulated $^{45}Ca^{2+}$ uptake into SR vesicles by 0.5–50 μM R56865. All data are the mean ± s.e.mean of five different experiments conducted in triplicate.

50 μM R56865. Additionally, the $K_d$ was increased approximately 10 fold (Figure 5b). Interestingly, flunarizine was unable to decrease digoxin binding to SR membranes ($n = 5$, not shown).

### *R56865 increases the rate of dissociation of [$^3$H]-digoxin from SR membranes with no effect on the rate of association*

The rate of association of [$^3$H]-digoxin with HSR membranes is extremely fast and a rapid filtration apparatus must be used to measure it accurately. The rate of association proceeds with a $K_{+1}$ of $2.64 \times 10^8$ $M^{-1}$ $s^{-1}$ ($n = 5$). This is unaffected by 50 μM R56865 added at the beginning of the experiment ($K_{+1}$ $2.55 \times 10^8$ $M^{-1}$ $s^{-1}$, $n = 5$). However, R56865 dose-dependently increased the rate of dissociation of [$^3$H]-digoxin from SR membranes when compared to the rate of dissociation obtained by dilution of the reaction medium (Figure 6).

### *R56865 decreases the digoxin-induced increase in $Ca^{2+}$-release channel Po*

As R56865 decreases the binding of [$^3$H]-digoxin to its sites on HSR membranes, it is expected that it would reduce the

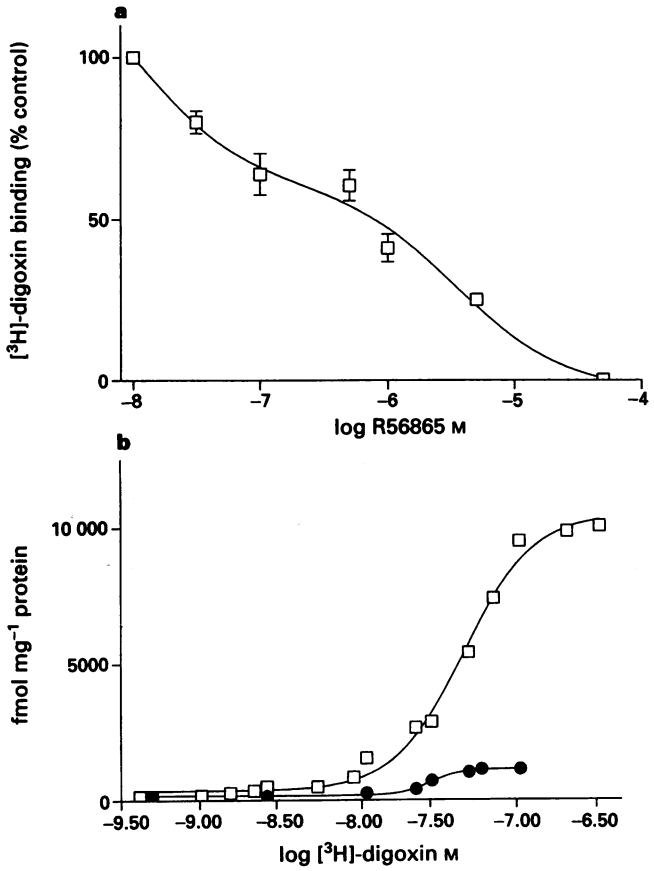


**Figure 5** (a) Equilibrium binding of 8.6 nM [$^3$H]-digoxin to cardiac HSR membranes in the presence of R56865. The data indicate that R56865 dose-dependently displaces [$^3$H]-digoxin specifically from both of its binding sites. The data are the mean ± s.e.mean of eleven different experiments conducted in triplicate. The two site inhibition curve was fitted to the data using INPLOT4 (GraphPad Software). (b) R56865 (50 μM) (●) displaces [$^3$H]-digoxin non-competitively from its blinding sites on cardiac HSR membranes. The $B_{max}$ is dose-dependently decreased by R56865 and the $K_d$ is dose-dependently increased compared to control (□). The data are the mean of at least nine different experiments conducted in tripicate. Errors are within 5% of the given values.

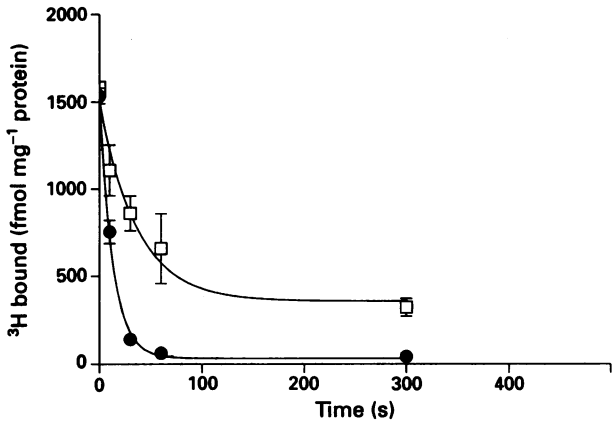


**Figure 6** R56865 (●) increases the rate of dissociation of bound [$^3$H]-digoxin in comparison to the rate of dissociation induced by a 20 fold dilution of the reaction buffer (□). R56865 had no effect on the rate of association of [$^3$H]-digoxin with SR membranes ($n = 5$, not shown). Data were best fitted to single exponential decay curves using INPLOT4 and are displayed as the mean ± s.e.mean of five different experiments conducted in triplicate.

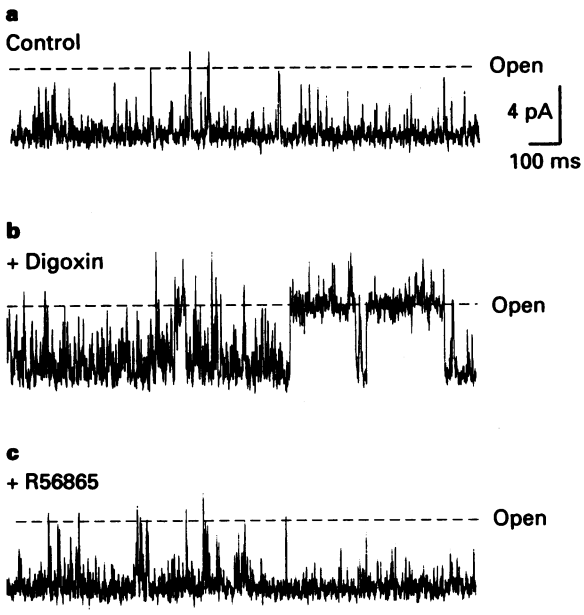


**Figure 7** Representative SR $Ca^{2+}$-release channels voltage clamped at 0 mV in 10 μM activating *cis* $Ca^{2+}$. In this example, two channels were present in the bilayer. At 10 μM $Ca^{2+}$ (a) the open probability (Po) was 0.040. Addition of 1 nM digoxin to the *cis* chamber increased Po to 0.256 (b). Further additoin of 5 μM R56865 in the continued presence of 1 nM digoxin reduced Po to 0.021 (c). Po was calculated from 3 min of continuous recordings as outlined in the Methods. The first open level is indicated by the dotted line.

increase in single-channel Po induced by digoxin. This is demonstrated in Figure 7. In channels activated by 10 μM $Ca^{2+}$, 1 nM digoxin added to the cytosolic face of the channel increased Po from 0.045 ± 0.024 to 0.267 ± 0.089 ($n = 4$). Addition of 5 μM R56865 to the cytosolic face of the channel in the continued presence of digoxin immediately decreased Po to approximately that seen with 10 μM $Ca^{2+}$ alone (0.022 ± 0.010, $n = 4$).

## Discussion

The anti-arrhythmic drug R56865 had no effect on the Po or conductance of single cardiac SR $Ca^{2+}$-release channels incorporated into lipid bilayers at 10 μM activating cytosolic $Ca^{2+}$. It was ineffective at sub-micromolar concentrations, in the range that it affords protection against arrhythmias, and at relatively high concentrations (up to 50 μM). This finding was confirmed by the lack of effect of R56865 on the $Ca^{2+}$-induced release of $^{45}Ca^{2+}$ from cardiac SR vesicles over the same concentration range. Similarly, R56865 failed to affect the binding of [$^3$H]-ryanodine (a marker for $Ca^{2+}$-release channel Po (Chu *et al.*, 1991)) to SR membranes in 10 μM $Ca^{2+}$. Together these data show that R56865, even at high concentrations, does not directly affect the conductance or the $Ca^{2+}$ activation of the cardiac SR $Ca^{2+}$-release channel. It could be surmised that R56865 does not bind to the SR $Ca^{2+}$-release channel; however, it is possible that it binds to a site on the channel that does not affect these parameters.

Wilhelm *et al.* (1991) showed that R56865 inhibits afterdepolarizations and aftercontractions due to veratridine administration and that only part of this protection was due to inhibition of the $Na^+$ current. It was suggested that R56865 may inhibit the oscillatory release of $Ca^{2+}$ from the SR. Oscillatory $Ca^{2+}$ release from the SR, due to $Ca^{2+}$ overload, may lead to the appearance of afterdepolarizations and arrhythmias and may be induced by cardiac glycosides (Vollmer *et al.*, 1987; Damiano *et al.*, 1991), veratridine

(Wilhelm *et al.*, 1991), ischaemia (Garner *et al.*, 1990) or caffeine or Bay K8644 (Daniels & ter Keurs, 1992). As R56865 does not alter the functioning of the SR $Ca^{2+}$-release channel to affect $Ca^{2+}$ release, another possible mechanism by which it may act would be if it decreased the uptake of $Ca^{2+}$ into the SR. This would lead to a decreased release of $Ca^{2+}$ from the SR during $Ca^{2+}$ overload and therefore, a suppression of the arrhythmias. Our data show that R56865 decreases the ATP-stimulated uptake of $Ca^{2+}$ into SR vesicles. Although the data suggest that the drug may be a non-competitive inhibitor of uptake, they also show that R56865 is a relatively poor inhibitor, with a maximum inhibition of total uptake of approximately 37% at 50 μM R56865.

The reduction of $Ca^{2+}$ uptake into the SR by R56865 during $Ca^{2+}$ overload, thereby reducing spontaneous $Ca^{2+}$ release, may be a mechanism by which it protects against arrhythmias in general i.e. arrhythmias caused by a variety of stimuli. However, Daniels & ter Keurs (1992) suggested, from data obtained using ventricular trabeculae, that R56865 decreases SR $Ca^{2+}$ release, possibly by increasing the threshold for $Ca^{2+}$-induced $Ca^{2+}$ release, but does not suppress SR $Ca^{2+}$ uptake. We find the opposite of this, that R56865 decreases $Ca^{2+}$ uptake into the SR but does not affect the threshold for the $Ca^{2+}$ activation of the cardiac SR $Ca^{2+}$-release channel or for $Ca^{2+}$-induced release of $^{45}Ca^{2+}$ from SR vesicles.

Cardiac glycosides may induce toxicity by acting through several mechanisms: inhibition of the $Na^+/K^+$ ATPase (increasing $[Na^+]_i$ then $[Ca^{2+}]_i$) and by directly increasing SR $Ca^{2+}$ release by acting on the SR $Ca^{2+}$-release channel (McGarry & Williams, 1993). However, both of these mechanisms, and possibly others, may need to be activated to induce inotropy and/or toxicity. From the data presented here and elsewhere (VerDonck *et al.*, 1991; Wilhelm *et al.*, 1991), it is apparent that R56865 may protect against or reduce glycoside toxicity by acting through several means: inhibition of the $Na^+$ current, although this may only partically account for the protective effect, as in the protection against veratridine toxicity (VerDonck *et al.*, 1991; Wilhelm *et al.*, 1991), reduction of intracellular $Na^+$ would antagonize the rise in intracellular $Na^+$ due to inhibiton of the $Na^+/K^+$ APTase by glycosides. This may help reduce toxicity. Additionally, R56865 may reduce SR $Ca^{2+}$ uptake, subsequent SR $Ca^{2+}$ oscillatory release and therefore, arrhythmias. These are effects that would also afford protection against arrhythmias induced by the agents listed above.

If R56865 decreases SR $Ca^{2+}$ uptake and blocks cardiac $Na^+$ channels, then this may also be negatively inotropic. It is possible that these effects in resting (control) conditions are not pronounced enough to be negatively inotropic and it is only in situations where there is $Ca^{2+}$ overload (induced by a variety of possible stimuli) that these effects are detectable or apparent. However, it is possible, and in fact probable, that several electrophysiological processes may have to be inhibited by R56865 to protect against arrhythmias and that inhibition of each by a small degree contributes to the overall protection.

In cardiac glycoside-induced toxicity, R56865 has an additional action as it also decreases the binding of glycosides to the SR $Ca^{2+}$-release channel, reducing their potentiation of $Ca^{2+}$-induced $Ca^{2+}$ release. [$^3$H]-digoxin binds to two sites on SR membranes, with fast rates of association and dissociation (S.J. McGarry & A.J. Williams, unpublished observations). R56865 displaces [$^3$H]-digoxin from both its sites, which probably reside on the SR $Ca^{2+}$-release channel. The displacement is effectively irreversible, with R56865 decreasing the density of binding sites as well as increasing the $K_d$ for [$^3$H]-digoxin. R56865 does not affect the rate of association of [$^3$H]-digoxin with its sites, but it dose-dependently increases the rate of dissociation. Whether these effects of R56865 are mediated by its binding to the cardiac glycoside sites on the $Ca^{2+}$-release channel protein or by R56865 binding to other sites on the channel, that do not affect its conductance or gating which changes the conformation of the glycoside binding sites, is unclear. These results were confirmed with single-channel studies which show that R56865 abolishes the increase in single SR $Ca^{2+}$-release channel Po induced by 1 nM digoxin. Hence, there is a correlation between displacement of [$^3$H]-digoxin and reduction of the effect of digoxin on single SR $Ca^{2+}$-release channel Po by R56865. This also supports the suggestion that [$^3$H]-digoxin is binding to the $Ca^{2+}$-release channel protein and not to an accessory SR protein. However, the possibility that [$^3$H]-digoxin binds to a tightly associated protein cannot be discounted.

In summary, we have shown that R56865 does not affect the conductance or gating of the cardiac SR $Ca^{2+}$-release channel. It does not affect the $Ca^{2+}$ activation of single-channel activity, the $Ca^{2+}$-induced release of $^{45}Ca^{2+}$ from SR vesicles or the $Ca^{2+}$ stimulation of [$^3$H]-ryanodine binding to SR membranes. It reduces the ATP-stimulated uptake of $^{45}Ca^{2+}$ into SR vesicles, an effect that may contribute to its general anti-arrhythmic action. Additionally, R56865 is an antagonist of cardiac glycosides at their binding sites on the SR $Ca^{2+}$-release channel. This effect may add to the protection provided against glycoside-induced arrhythmias. Notwithstanding this, it is possible that the multitude of other actions on specific ion translocation processes, as well as the non-specific membrane actions of R56865, may be as responsible for the protection against arrhythmias as the effects on SR function we have documented here. However, the simultaneous interference with more than one of these processes by R56865 may be required for its anti-arrhythmic action.

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# Inhibition of calcium release from the sarcoplasmic reticulum of rabbit aorta by hydralazine

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1 The mechanism of hydralazine-induced vasorelaxation was investigated in rabbit isolated aorta, by determining its ability to interfere with force development under a variety of conditions.

2 Hydralazine relaxed phenylephrine-contracted aorta with half maximal relaxation at 17 μM and maximal relaxation above 100 μM. At 200 μM, hydralazine had little effect on contractions induced by 25 mM or 50 mM $K^+$.

3 Hydralazine was equally effective at inhibiting contractile responses to phenylephrine in the absence or presence of extracellular $Ca^{2+}$. Responses to phenylephrine in $Ca^{2+}$-free solution were blocked to the same degree whether hydralazine was applied during filling of the sarcoplasmic reticulum (SR) $Ca^{2+}$ stores or after filling was complete. Caffeine-induced contractions were less sensitive to block by hydralazine.

4 Thapsigargin, cyclopiazonic acid, ryanodine, nifedipine and diltiazem all failed to block the inhibitory effect of hydralazine on tonic contractions to phenylephrine in the presence of extracellular $Ca^{2+}$. However, when cyclopiazonic acid was applied either with diltiazem or ryanodine, substantial inhibition of the hydralazine response was observed.

5 We propose that tonic contractions to phenylephrine are largely maintained by $Ca^{2+}$ cycling through the SR, with $Ca^{2+}$ entering the smooth muscle cell being sequestered by the SR eventually to leak out through $IP_3$-activated channels close to the contractile proteins. Sequestration of $Ca^{2+}$ would employ two pathways, one sensitive to inhibitors of the SR $Ca^{2+}$-ATPase and the other to Ca antagonists. We further suggest that, in the presence of extracellular $Ca^{2+}$ and phenylephrine, the leakage of $Ca^{2+}$ through $IP_3$-activated channels is significantly reduced only if both routes for SR $Ca^{2+}$ accumulation are blocked or the $Ca^{2+}$-ATPase is blocked while the SR is made leaky with ryanodine.

6 We conclude that the main action of hydralazine is to block the $IP_3$-dependent release of $Ca^{2+}$ from the sarcoplasmic reticulum. Thus conditions that diminish the contribution of $IP_3$-induced $Ca^{2+}$ release to tension can inhibit the hydralazine-induced vasorelaxation.

**Keywords:** Hydralazine; vasodilatation; rabbit aorta; smooth muscle; calcium release; calcium stores; sarcoplasmic reticulum; cyclopiazonic acid; ryanodine

## Introduction

The antihypertensive drug, hydralazine, has been in clinical use for over 40 years, but its mechanism of action is still poorly understood. It acts directly on the vasculature and inhibits responses to a wide variety of vasoconstrictor agents (Kirpekar & Lewis, 1957). Hydralazine can relax vascular muscle *in vitro* after the endothelial cell layer has been removed, implying that its main action is at the level of the smooth muscle cell. Its effects are not correlated with an increase in tissue guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels (Diamond & Janis, 1978; 1980; Yen *et al.*, 1989), so it acts through a mechanism distinct from the nitrovasodilator drugs. Several studies conclude that hydralazine interferes with $Ca^{2+}$ handling by the smooth muscle cell. For example, using the $Ca^{2+}$ indicator aequorin, DeFeo & Morgan (1989) showed that the hydralazine-induced relaxation of ferret aorta was accompanied by a fall in the intracellular $Ca^{2+}$ concentration. Furthermore, hydralazine did not alter the relationship between the intracellular $Ca^{2+}$ concentration and the force developed by aortic strips (DeFeo & Morgan, 1989). Thus hydralazine appears to relax smooth muscle solely by reducing the intracellular $Ca^{2+}$ concentration, and not by chaning the $Ca^{2+}$ sensitivity of the contractile proteins. Further support for this conclusion is provided by the finding that hydralazine had no effect on the contraction of chemically skinned renal arteries, brought about by the addition of Ca-calmodulin and ATP (Kreye *et al.*, 1983).

Potential mechanisms that would lead to a fall in the intracellular $Ca^{2+}$ concentration include inhibition of $Ca^{2+}$ influx or $Ca^{2+}$ release from intracellular stores, or stimulation of $Ca^{2+}$ efflux or $Ca^{2+}$ accumulation into the stores. It has been proposed that hydralazine inhibits $Ca^{2+}$ influx, based on the observation in rabbit aorta that it inhibits $K^+$-stimulated contractures and the net uptake of $^{45}Ca^{2+}$ during these contractures (McLean *et al.*, 1978b). However, most of the studies that show a significant inhibition of $K^+$-contractures employed high concentrations (>0.1 mM) of hydralazine (Barron *et al.*, 1977; McLean *et al.*, 1978a,b; Orallo *et al.*, 1991), well above the levels that would be reached in plasma after a therapeutic dose (Talseth, 1976). There seems to be general agreement from studies employing low concentrations of hydralazine, that the drug is more effective at inhibiting contractures induced by receptor stimulation (McLean *et al.*, 1978a; Lipe & Moulds, 1981; Khayyal *et al.*, 1981; Higashio & Kuroda, 1988a; Yen *et al.*, 1989). Moreover, Lipe & Moulds (1981) noted that hydralazine was most potent at inhibiting contractions of human vascular muscle that showed the least requirement for extracellular $Ca^{2+}$. Consistent with this, contractions induced by noradrenaline in $Ca^{2+}$-free solutions are sensitive to block by hydralazine (McLean *et al.*, 1978a; Ebeigbe & Aloamaka, 1985; Higashio & Kuroda, 1988b; Orallo *et al.*, 1991). Thus hydralazine may relax vascular muscle by interfering with the intracellular storage of $Ca^{2+}$, rather than by inhibiting $Ca^{2+}$ influx.

The aim of this study was to clarify the cellular mech-

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anisms by which hydralazine induces vasodilatation. We have examined the possible contributions of an effect on $Ca^{2+}$ influx, mediated either by direct modulation of Ca channels or through membrane hyperpolarization, and an effect on intracellular $Ca^{2+}$ storage by the sarcoplasmic reticulum (SR). We conclude that the main action of hydralazine is to inhibit the agonist-induced release of $Ca^{2+}$ from the SR.

## Methods

Male New Zealand white rabbits (2–3 kg) were killed by a lethal i.v. injection of sodium pentobarbitone (80 mg $kg^{-1}$; Ceva, Watford, Hertfordshire) and exsanguinated. The descending thoracic aorta was excised, placed in physiological salt solution then freed of connective tissue. The endothelial cell layer was removed by gently rubbing the intimal surface. Although initial experiments indicated that removal of the endothelium was successful, we did not routinely check for its absence. The vessel was cut into rings (1–2 mm wide) or transverse strips (1–2 mm wide, 0.8–1 cm long), which were mounted in a chamber (~0.5 ml volume) for isometric tension recording with a Harvard Apparatus (South Natick, MA, U.S.A.), model 60-2998 transducer. Tissues were placed under an optimal basal tension of 1.25 g and continuously perfused at 1 ml $min^{-1}$ with physiological salt solution at 35 to 37°C. They were allowed to equilibrate for 1 h before experiments commenced.

### *Experimental protocols*

To determine the relationship between hydralazine concentration and the induced smooth muscle relaxation, tissues were precontracted by changing the perfusion solution to one containing phenylephrine (1 μM). Once tension had reached a stable level, the tissue was perfused with hydralazine in the continued presence of phenylephrine, and the resulting relaxation was measured as a percentage of the phenylephrine-induced tone. A similar procedure was followed to investigate the effectiveness of hydralazine on $K^+$-contracted tissues. Potential inhibitors of the hydralazine-induced relaxation were tested against tissues precontracted with phenylephrine, with the inhibitors perfused for up to 1 h before hydralazine was added. In this case the relaxation response to hydralazine was measured as a percentage of the phenylephrine-induced tension recorded in the presence of the inhibitor, immediately before hydralazine was applied. After obtaining a steady response to hydralazine, the tissue was relaxed to baseline by perfusion with the normal physiological salt solution.

Contractions due to the release of $Ca^{2+}$ from intracellular stores were induced by exposing the tissue either to caffeine (10 mM) or phenylephrine (1 μM) in the absence of extracellular $Ca^{2+}$. Tissues were perfused with $Ca^{2+}$-free solution containing 1 mM EGTA (ethyleneglycol-*bis*-(β-aminoethyl)-N,N,N',N'-tetraacetic acid) for various times before, during and after a brief application of caffeine or phenylephrine. They were then perfused with normal physiological salt solution for 10 min to refill the stores, and the cycle was repeated until reproducible responses were obtained.

Results are expressed as mean ± s.e.mean. Statistical comparisons among groups of data were made using the GraphPAD Instat software to perform one way analysis of variance (ANOVA), with $P$ values corrected by the Bonferroni method. When comparing only 2 groups of data, an unpaired $t$ test was used.

### *Drugs and solutions*

The physiological salt solution had the following composition (mM): NaCl 112, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, $NaH_2PO_4$ 0.5, $KH_2PO_4$ 0.5, $NaHCO_3$ 15, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 5, glucose 10, phenol red 0.02 and it was constantly bubbled with a 95% $O_2$, 5% $CO_2$ gas mixture (pH 7.2). The $Ca^{2+}$-free solutions were prepared by omitting $CaCl_2$ and adding 1 mM EGTA, with the pH readjusted to 7.2 with NaOH after equilibration with 95% $O_2$, 5% $CO_2$. Solutions with varying concentrations of $K^+$ were prepared by equimolar substitution of KCl for NaCl. They also contained 1 μM phentolamine to prevent smooth muscle stimulation by noradrenaline released from nerve terminals in the tissue.

Phenylephrine hydrochloride, hydralazine hydrochloride and diltiazem hydrochloride, all from Sigma Chemical Co. Ltd., Poole, Dorset, ryanodine from Calbiochem Novabiochem, Nottingham and phentolamine mesylate from Ciba Laboratories, Horsham, Sussex were prepared as 10–100 mM stock solutions in distilled water. Stock solutions of cyclopiazonic acid (100 mM; Calbiochem), thapsigargin (1 mM; Sigma) and nifedipine (10 mM; Sigma) were prepared by dissolving them in 100% dimethylsulphoxide. In each case, serial dilutions were made with the appropriate experimental solution. Dimethylsulphoxide, at the highest concentration achieved during experiments (0.1%), had no effect itself on the hydralazine response. Caffeine (Sigma) was dissolved directly in the experimental solution.

### *Hydralazine stability in solution*

Hydralazine has been reported to degrade with a half-life of 30 min in oxygenated salt solutions at pH 7.4 (Worcel, 1978). We therefore diluted hydralazine into the experimental solution just before applying it to tissue. However, responses to hydralazine usually took at least 30 min to develop. We therefore performed control experiments on four muscle strips, prepared from adjacent segments of the same rabbit aorta, to determine the extent to which degradation of the drug might have influenced our results. The strips were contracted to a steady level of tension with 1 μM phenylephrine before perfusing hydralazine (50 μM) until a maximum response was reached. The hydralazine perfusing one strip was exchanged every 5 min with freshly prepared solution and induced a relaxation of 62%. The other three strips were challenged consecutively with the same hydralazine solution. Perfusion of the first of these strips began as soon as the hydralazine was prepared, and continued with the same solution until the maximum relaxation (55%) was reached. The second and third strips were perfused with the same solution, beginning 1.3 and 3.3 h later, and this resulted in relaxations of 69% and 60%, respectively. While there was some variation among the responses to hydralazine, this did not correlate with the length of time for which the hydralazine had been present in the oxygenated solution. Thus degradation of hydralazine did not appear to be significant in the present experiments.

## Results

When applied to isolated aorta in the presence of physiological saline, phenylephrine (1 μM) caused a slowly developing contraction, which eventually reached a steady level of tension that was maintained for several hours. When applied during this sustained phase of tension, hydralazine induced a concentration-dependent relaxation as shown in Figure 1a. The concentration producing 50% relaxation ($IC_{50}$) was 17 μM, with the maximum relaxation achieved at around 200 μM hydralazine. However, even in the presence of a maximal concentration of hydralazine, aortic strips rarely relaxed completely, but retained around 20% of the initial phenylephrine-induced tone. The response to hydralazine was slow in onset, taking up to 1 h to develop fully even at the highest concentrations. In addition, the hydralazine-induced relaxations were slow to reverse; in many tissues, recovery was incomplete even after 3 h of washing. For this reason, hydralazine was tested only once on each tissue, with the

effects of various conditions determined by comparing the mean responses of several tissues. The relaxation response to hydralazine was frequently, but not always, preceded by a transient contraction, which was pronounced in some tissues (see Figure 4). We have not investigated this response further.

As can be seen in Figure 1b, hydralazine was much less effective in relaxing tissues when they were precontracted with high $K^+$ solutions. At 200 μM, hydralazine produced only 20 ± 6% ($n = 3$) inhibition of 25 mM $K^+$-induced contractures and 21 ± 5% ($n = 3$) inhibition of 50 mM $K^+$-induced contractures. Thus the response to hydralazine was the same regardless of the degree to which the extracellular $K^+$ concentration was elevated to cause contraction.

### *Role of stored Ca in the response to hydralazine*

Rabbit aorta contracts in the absence of $Ca^{2+}$ when exposed to agents that release $Ca^{2+}$ from intracellular stores. Possible actions of hydralazine on $Ca^{2+}$ stores were evaluated by testing its effects on responses to caffeine, which directly releases $Ca^{2+}$ from intracellular stores (Herrmann-Frank *et al.*, 1991), and phenylephrine, in $Ca^{2+}$-free solution containing 1 mM EGTA. The caffeine- and phenylephrine-releasable stores have been shown to overlap completely in the rabbit aorta (Leijten & van Breemen, 1984). Consistent with this, responses to 10 mM caffeine in $Ca^{2+}$-free conditions were blocked completely by prior exposure to 1 μM phenylephrine ($n = 2$) and by 90% after previous exposure to caffeine ($n = 2$). Similarly, contractions to 1 μM phenylephrine were suppressed by 81 ± 6% ($n = 4$) or 63 ± 2% ($n = 4$) when preceded by exposure to phenylephrine or caffeine, respectively. Contractions induced by phenylephrine were never completely blocked. Even after 1 h continuous exposure to phenylephrine in $Ca^{2+}$-free solution, 18 ± 2% ($n = 4$) of the initial tension remained, suggesting a $Ca^{2+}$-independent component.

As illustrated in Figure 2, 100 μM hydralazine reduced responses to 2 min applications of 10 mM caffeine in $Ca^{2+}$-free solution by 44 ± 7% ($n = 5$). However, hydralazine was more effective ($P < 0.01$) in inhibiting contractions to $Ca^{2+}$ released from intracellular stores when they were triggered by receptor activation with phenylephrine. When phenylephrine (1 μM) was substituted for caffeine in the above protocol, 100 μM hydralazine reduced the resulting contractions by 71 ± 4% ($n = 6$), which is similar to the inhibition of phenylephrine contractions observed in the presence of extracellular $Ca^{2+}$. Figure 3 shows results of experiments designed to test whether this effect of hydralazine was due to an action

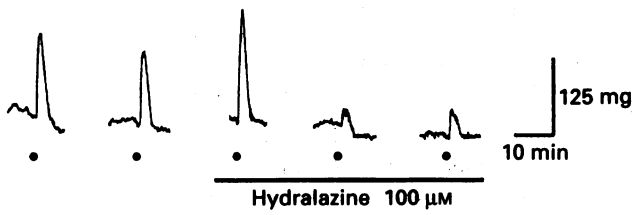


**Figure 2** The effect of hydralazine on contractile responses to caffeine. The tissue was perfused with $Ca^{2+}$-free solution containing 1 mM EGTA for 10 min before, during and for 5 min after 2 min applications of 10 mM caffeine (●). Normal $Ca^{2+}$ solution was then reintroduced for 10 min to refill the sarcoplasmic reticulum = $Ca^{2+}$ stores. This cycle was repeated several times before and after the addition of 100 μM hydralazine, which is indicated by the bar. In this preparation, the response to caffeine was reduced by 65% in the presence of hydralazine. The mean inhibition in 5 preparations was 44 ± 7%.

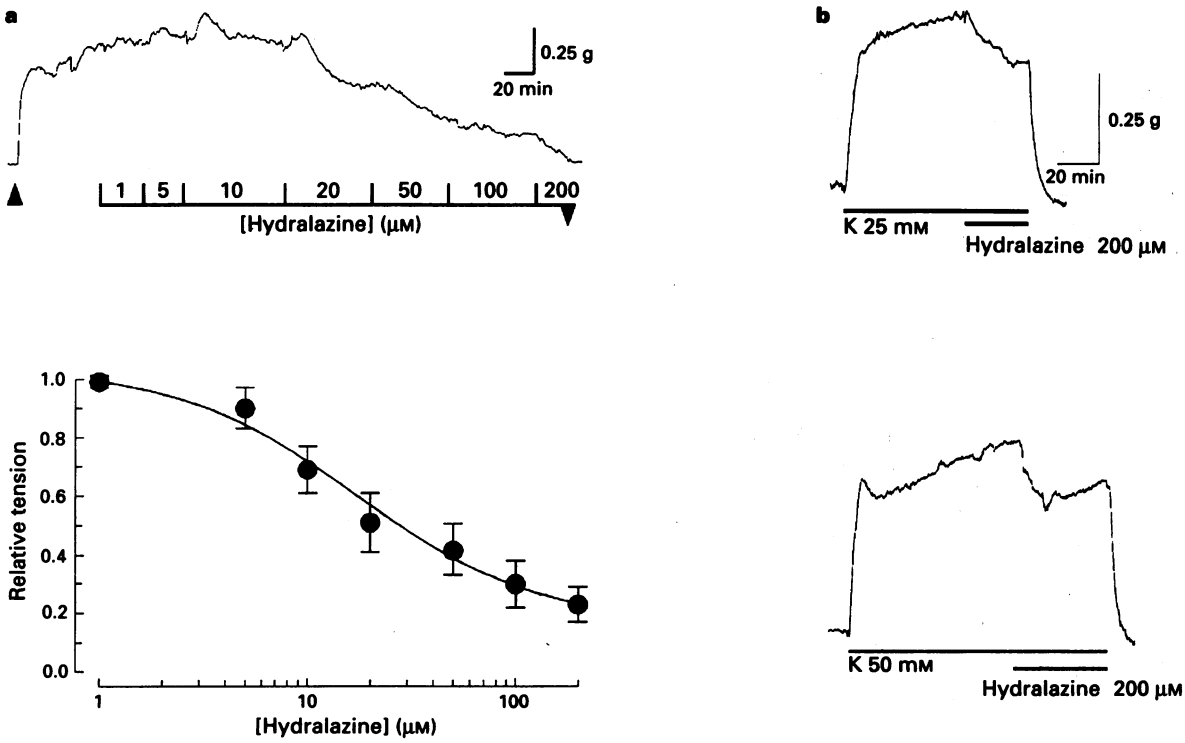


**Figure 1** Concentration dependence of hydralazine-induced relaxation of precontracted rabbit aorta. (a) Top panel, increasing concentrations of hydralazine, cumulatively applied to a ring of aorta that was precontracted with 1 μM phenylephrine, caused increasing relaxation. The application and removal of phenylephrine is indicated by the arrowheads. Bottom panel, relationship between hydralazine concentration and the loss of phenylephrine-induced tension. Points represent means with s.e.mean of 6–8 observations. The curve shows the best fit to a standard hyperbolic equation, from which the concentration of hydralazine giving a half maximal response ($IC_{50}$) was estimated as 17 μM. (b) At 200 μM, hydralazine produced only a small relaxation when tissues were precontracted with 25 mM $K^+$ (top panel) or 50 mM $K^+$ (bottom panel).

on the release or refilling of the $Ca^{2+}$ stores. In Figure 3a, extracellular $Ca^{2+}$ was depleted by exposing the tissue to $Ca^{2+}$-free solution for 10 min, followed by a 5 min application of phenylephrine (1 μM), a 30 min wash in $Ca^{2+}$-free solution and a 10 min exposure to normal physiological saline in order to refill the intracellular $Ca^{2+}$ stores. Once the responses to phenylephrine were reproducible (usually 4–6 cycles), 100 μM hydralazine was added for 25 min immediately before the next refilling period in normal saline, and throughout the filling period. This should have allowed development of hydralazine block before the stores were refilled. The subsequent response to phenylephrine was reduced by 69 ± 3% ($n = 3$), which is similar to the inhibition observed using the shorter protocol. In Figure 3b, hydralazine exposure was restricted to the period after the stores had been filled. To enable a similar duration of exposure to hydralazine, the protocol was adjusted so that tissues were perfused with $Ca^{2+}$-free solution for 30 min prior to the phenylephrine contraction and 10 min afterwards. In this situation, hydralazine (100 μM) inhibited the phenylephrine contraction by 68 ± 2% ($n = 5$). Since this value does not differ from that observed when hydralazine was applied before the store-refilling period, or when applied in the presence of extracellular $Ca^{2+}$, a major part of the action of hydralazine must be to inhibit the phenylephrine-induced release of $Ca^{2+}$ from intracellular stores or the action of released $Ca^{2+}$.

### *Effects of modulators of sarcoplasmic reticulum function*

To investigate further the role of SR $Ca^{2+}$ stores in the action of hydralazine, we examined how its effects were influenced by a variety of agents known to alter SR function. These experiments were carried out in the presence of extracellular $Ca^{2+}$, using the normal physiological saline. Muscle contraction was initiated and maintained by applying 1 μM phenylephrine. Once a steady level of tension was achieved, hydralazine was applied at 100 μM, a concentration shown to produce near maximal relaxation under control conditions (Figure 1a). This resulted in a 76 ± 3% ($n = 11$) reduction of the sustained level of tension (Figure 4a). Drugs that modulate SR function were perfused across phenylephrine-contracted tissues for 1 h before adding 100 μM hydralazine, and the response to hydralazine was measured as the percentage loss of tension relative to that recorded immediately before hydralazine application. An example of such an experiment, examining the effect of ryanodine on the hydralazine response, is shown in Figure 4b. The results obtained with a number of different modulators of SR function are summarised in Figure 5.

At the concentration used (10 μM), ryanodine opens $Ca^{2+}$-permeable channels in the SR membrane, making the SR leaky to $Ca^{2+}$ (Hwang & van Breemen, 1987). Consistent with this, we found that 10 μM ryanodine blocked transient contractions to phenylephrine in $Ca^{2+}$-free solution by 71 ± 2% ($n = 3$). Thus when ryanodine was applied to phenylephrine-contracted aorta in the presence of extracellular $Ca^{2+}$, an increase in tension of 46 ± 5% ($n = 3$) was observed, presumably reflecting an elevated cytoplasmic $Ca^{2+}$ concentration. However, pretreatment with ryanodine failed to prevent the response to hydralazine (Figure 4b and 5a). Thapsigargin and cyclopiazonic acid are both thought to inhibit directly the $Ca^{2+}$-ATPase in the SR membrane, thereby limiting $Ca^{2+}$ uptake (Seidler *et al.*, 1989; Thastrup, 1990). Thapsigargin at 1 μM, and cyclopiazonic acid at 30 μM, have previously been shown to substantially block enzyme activity and responses of rabbit aorta to phenylephrine in $Ca^{2+}$-free media (Luo *et al.*, 1993). In our hands, 30 μM cyclopiazonic acid suppressed phenylephrine-induced contractions in $Ca^{2+}$-free solution by 78 ± 3% ($n = 3$). In normal saline, both thapsigargin (1 μM) and cyclopiazonic acid (30 μM) had variable effects on phenylephrine-induced tension. No effect was observed in two out of six preparations exposed to thapsigargin and in two out of eight preparations exposed to cyclopiazonic acid. In three preparations, thapsigargin caused relaxation of 26 ± 10%, but it increased tension by 29% in another. Cyclopiazonic acid caused relaxation in two aortic strips with a mean of 12%, while increasing tension in the four remaining strips by 60 ± 26%.The reason for this variability is unclear. It may

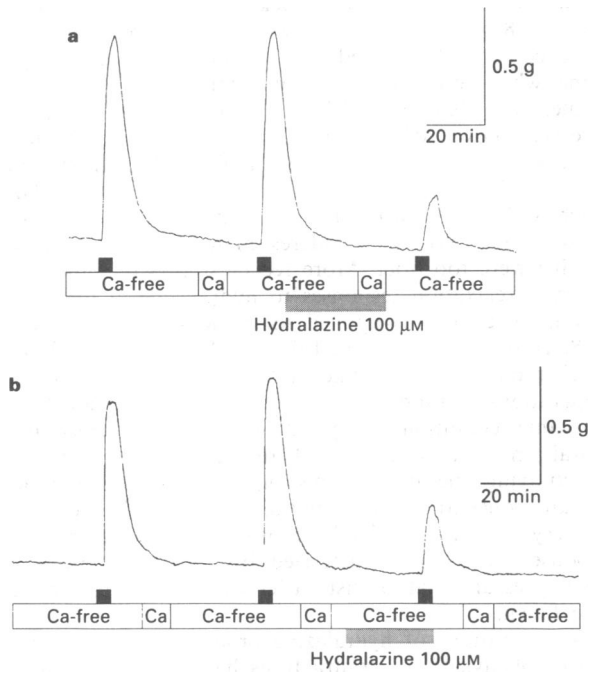


**Figure 3** The effect of hydralazine 100 μM on contractions induced by phenylephrine (1 μM) in $Ca^{2+}$-free solution, containing EGTA (1 mM). Transient contractions to phenylephrine, applied for 5 min (indicated by short black bars) following 10 min (a) or 30 min (b) exposure to $Ca^{2+}$-free solution, were reproducible, provided stores were refilled between applications by 10 min exposure to $Ca^{2+}$-containing physiological solution. (a) Phenylephrine application was followed by 30 min in $Ca^{2+}$-free solution before restoring the extracellular $Ca^{2+}$ for 10 min. Hydralazine was applied for the last 25 min in $Ca^{2+}$-free solution and during store refilling (shaded bar). (b) Phenylephrine application was followed by 10 min in $Ca^{2+}$-free solution before refilling the stores. Hydralazine was applied with the phenylephrine, in addition to 25 min before the phenylephrine application.

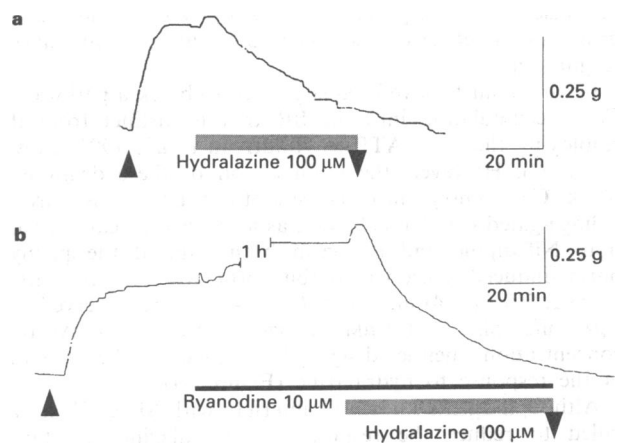


**Figure 4** The effects of 100 μM hydralazine on tonic contractions to phenylephrine, applied in the absence (a) or presence (b) of ryanodine (10 μM). The application and removal of phenylephrine (1 μM) are shown by the arrowheads. Hydralazine and ryanodine exposures are indicated by bars. Ryanodine was continuously perfused during the 1 h gap indicated in the record in (b).

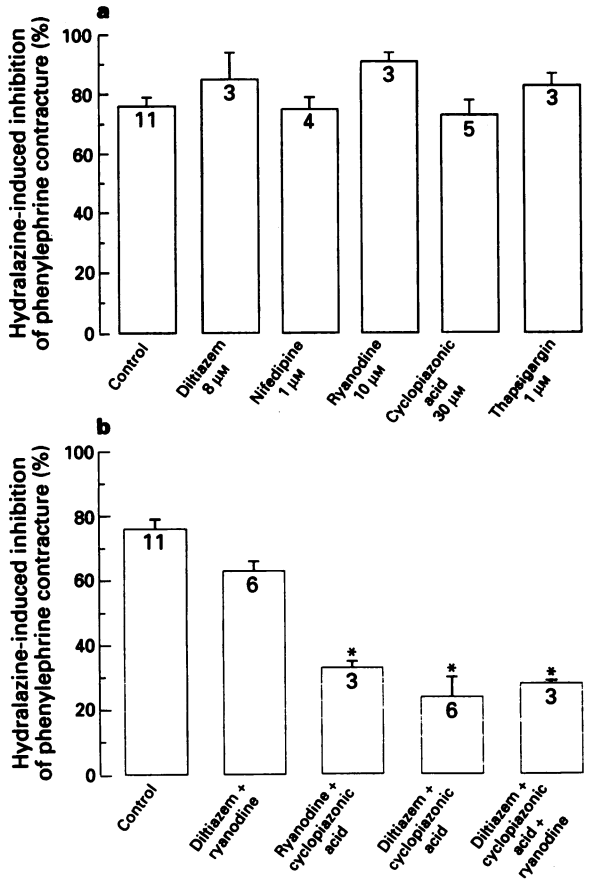


**Figure 5** Effects of inhibitors of $Ca^{2+}$ influx and sarcoplasmic reticulum function on the response to 100 μM hydralazine. Control columns show the percentage reduction by hydralazine of tonic phenylephrine-induced contractions in normal physiological saline. (a) Responses to hydralazine following exposure of the tissue to diltiazem (8 μM), nifedipine (1 μM), ryanodine (10 μM), cyclopiazonic acid (30 μM) or thaspsigargin (1 μM). (b) Responses to hydralazine following exposure to combinations of these drugs, applied together. *$P < 0.001$ as compared with control, from ANOVA with Bonferroni correction.

reflect inconsistency in the degree of endothelium removed from different tissues, since thapsigargin has been shown to relax vascular muscle through an action on the endothelium (Matsuyama *et al.*, 1993). Nevertheless, despite the variable responses to thapsigargin and cyclopiazonic acid, neither drug had any effect on the relaxation induced by hydralazine (Figure 5a).

Ca antagonists have been suggested to block a pathway for $Ca^{2+}$ accumulation into the SR that is distinct from that employing the $Ca^{2+}$-ATPase (Bourreau *et al.*, 1991; Low *et al.*, 1992). However, the main action of these drugs is to block $Ca^{2+}$ entry into the smooth muscle cells through voltage-gated Ca channels, and as a result they cause relaxation. Nifedipine and diltiazem both reduced the phenylephrine-induced tension in rabbit aorta. Maximal relaxations of 23 ± 5% ($n = 4$) and 18 ± 9% ($n = 3$) were observed with 1 μM nifedipine and 8 μM diltiazem, respectively. At these concentrations, neither drug had any effect on the amplitude of the response to hydralazine (Figure 5a).

Although agents known to interfere with SR $Ca^{2+}$ storage failed to reduce the response to hydralazine, substantial block was observed when pairs of these drugs, acting through independent mechanisms, were applied together (Figure 5b). The relaxation elicited by 100 μM hydralazine was reduced to only 24 ± 6% ($n = 6$) after exposing aortic strips simultaneously to both cyclopiazonic acid and diltiazem for 1 h, which caused a 22 ± 13% ($n = 3$) loss of the phenylephrine-induced tone before hydralazine was added. A similar reduction in the hydralazine response was observed if thapsigargin was substituted for cyclopiazonic acid or nifedipine was substituted for diltiazem (not shown). The response to hydralazine was also substantially attenuated in the combined presence of cyclopiazonic acid and ryanodine, which had little effect on the phenylephrine-induced contraction before hydralazine was added (2 ± 16% relaxation; $n = 4$). This combination caused an 82 ± 3% ($n = 4$) inhibition of phenylephrine-induced contractions in $Ca^{2+}$-free solution, slightly more than either drug alone. Diltiazem and ryanodine together reduced tension by 41 ± 8% ($n = 3$), but this combination had only a small inhibitory effect on the response to hydralazine. The most potent combination appeared to be cyclopiazonic acid with diltiazem, and no additional block of the hydralazine response was observed upon adding ryanodine (Figure 5b). When ryanodine, cyclopiazonic acid and diltiazem were applied simultaneously, responses to phenylephrine in $Ca^{2+}$-free solution were blocked by 79 ± 5% ($n = 4$). When applied to tissues contracted with phenylephrine in the presence of normal $Ca^{2+}$, an initial 47 ± 5% ($n = 3$) loss of tension was observed followed by a gradual increase, such that after 60 min tension was 10 ± 3% higher than before the drugs were added.

## Discussion

It has been suggested that different arterial preparations vary in responsiveness to hydralazine (Khayyal *et al.*, 1981; Kreye, 1984). In the rabbit renal artery, the $IC_{50}$ for hydralazine inhibition of noradrenaline contractures was around 0.1 μM (Khayyal *et al.*, 1981). This contrasts with studies on the rabbit aorta, which suggest that hydralazine has little effect below mM concentrations (Kirpekar & Lewis, 1957; McLean *et al.*, 1978a). In our hands, however, the rabbit aorta proved to be consistently relaxed by μM concentrations of hydralazine when tested against tonic contractions to phenylephrine. The $IC_{50}$ was 17 μM, with a maximal response observed above 100 μM. The difference between the hydralazine sensitivities reported here and in the earlier studies may be related to the slow onset of action of hydralazine, which took up to 1 h to produce its maximal effect. Responsiveness could have been underestimated if exposures to hydralazine were too brief. More recent studies on rabbit aorta observed relaxation responses to hydralazine at μM concentrations when it was applied for at least 1 h (Cook *et al.*, 1988; Higashio & Kuroda, 1988a,b). Furthermore, Higashio & Kuroda (1988a) found that noradrenaline-contracted preparations of rabbit renal artery and aorta, studied under the same conditions, respond with similar sensitivity to hydralazine. Rabbit aorta is therefore an appropriate model for studying the mechanisms of action of therapeutically relevant concentrations of hydralazine. The slow onset and recovery from hydralazine block is consistent with the clinical response, which is characterized by a gradual lowering of blood pressure that persists after blood levels of the drug have fallen.

Concentrations of hydralazine producing maximal inhibition of phenylephrine contractures had little effect on tonic contractions induced by elevating the extracellular $K^+$ concentration. This agrees with several studies employing a variety of arterial preparations (McLean *et al.*, 1978a, Khayyal *et al.*, 1981; Lipe & Moulds, 1981; Hermsmeyer *et al.*, 1983; Higashio & Kuroda, 1988a; Yen *et al.*, 1989). Reports claiming similar potency in blocking $K^+$-induced and agonist-induced contractures (Ebeigbe & Aloamaka, 1985; Orallo *et al.*, 1991) applied hydralazine for only a few minutes or used excessive concentrations, so the conclusions are uncertain. Overall, the evidence indicates that hydralazine primarily blocks a step in the pathway linking the activation of membrane receptors to contraction. Since $K^+$-induced tension mainly reflects $Ca^{2+}$ influx through voltage-gated Ca chan-

nels, it is unlikely that Ca-channel blockade contributes significantly to hydralazine-induced vasodilatation. This conclusion is supported by the finding that Ca antagonists were without effect on the response to hydralazine. The Ca antagonist, D600, similarly failed to prevent hydralazine-induced relaxations in rat tail artery (Hermsmeyer *et al.*, 1983). Also, in agreement with a previous study on dihydralazine (Thirstrip & Nielsen-Kudsk, 1992), we conclude that hydralazine does not act as a K channel opener in rabbit aorta. Vasodilators that open K-channels, for example cromakalim, characteristically block contractions to low ($<35$ mM) but not high ($>50$ mM) concentrations of $K^+$ (reviewed in Gurney, 1994). Hydralazine was equally ineffective in inhibiting high $K^+$ contractures regardless of the $K^+$ concentration used.

Hydralazine blocked phenylephrine-induced contractions equally well in the presence or absence of extracellular $Ca^{2+}$. Thus it may interfere with $Ca^{2+}$ handling by intracellular stores. The suppression of caffeine-induced contractions by hydralazine supports such a mechanism. Caffeine activates $Ca^{2+}$ permeable channels in the SR of vascular muscle, with mediate $Ca^{2+}$-induced release of $Ca^{2+}$ (Herrmann-Frank *et al.*, 1991). These are distinct from the channels that mediate SR $Ca^{2+}$ release when the cell is stimulated by agonists such as phenylephrine. By acting on specific membrane receptors, agonists stimulate the formation of inositol 1,4,5-trisphosphate ($IP_3$), which binds to and opens specific $IP_3$-receptor channels in the SR membrane (Ehrlich & Watras, 1988). The caffeine-sensitive and $IP_3$-sensitive $Ca^{2+}$ stores overlap completely in rabbit aorta (Leijten & van Breemen, 1984). Thus caffeine and $IP_3$ release the same pool of $Ca^{2+}$, but the $Ca^{2+}$ leaves the SR through different channels. Since hydralazine reduced contractions to phenylephrine in $Ca^{2+}$-free solution more effectively than caffeine contractions, it may preferentially block a step in the $IP_3$-dependent pathway. An action on $Ca^{2+}$ release is further supported by the finding that hydralazine exerted its full effect on phenylephrine contractions in $Ca^{2+}$-free solution, even when it was applied after the stores had been pre-filled with $Ca^{2+}$. Whether or not hydralazine has additional effects on $Ca^{2+}$ accumulation into the stores could not easily be determined. The slow reversibility of hydralazine block meant that, even when tissue exposure to the drug was restricted to the store-filling period, its action would have persisted during the subsequent test with phenylephrine.

Agents known to deplete the SR of $Ca^{2+}$ would be expected to block the action of a drug that interferes with the release of stored $Ca^{2+}$. That cyclopiazonic acid, thapsigargin and ryanodine can deplete the SR of $Ca^{2+}$ is suggested by the fact that they cause pronounced inhibition of phenylephrine-induced contractions in $Ca^{2+}$-free solution. However, when applied by themselves, all of these agents failed to influence the hydralazine-induced relaxation. We were surprised to find though, that when cyclopiazonic acid was present together with either ryanodine or diltiazem, strong inhibition of the hydralazine response developed. The simplest explanation for this finding is that depletion of the SR is not readily achieved in the presence of extracellular $Ca^{2+}$ and phenylephrine stimulation, but requires interference with more than one route of $Ca^{2+}$ exchange with the SR. The results are consistent with an inhibitory action of hydralazine on $IP_3$-dependent $Ca^{2+}$ release. This interpretation requires, however, that the maintained phenylephrine-induced contraction largely reflects the sustained, $IP_3$-dependent release of SR $Ca^{2+}$, which is balanced by uptake through two separate pathways, one requiring the SR $Ca^{2+}$-ATPase and the other blockable by Ca antagonists.

There is substantial evidence to support an SR $Ca^{2+}$ cycling mechanism in the maintenance of tension in rabbit aorta. Although tonic tension clearly depends on $Ca^{2+}$ influx (Lodge & van Breemen, 1985), $Ca^{2+}$ entering the smooth muscle cell may first be accumulated into the peripheral SR (van Breemen, 1977). Receptor stimulation, by enhancing $IP_3$ production, induces a permeable SR, through which $Ca^{2+}$ could cycle to be released in the vicinity of the myofilaments. This might well be important for maintaining tension in rabbit aorta, where $IP_3$ is produced continuously during exposure to α-adrenoceptor agonists for many minutes (Coburn *et al.*, 1988; Kajikuri & Kuriyama, 1990). During sustained tension, the cytoplasmic $Ca^{2+}$ concentration would then depend on the relative rates of SR uptake and release, as well as on $Ca^{2+}$ influx and efflux across the sarcolemma.

Separate pathways, sensitive either to cyclopiazonic acid or Ca antagonists, have been proposed for $Ca^{2+}$ uptake into smooth muscle SR (Bourreau *et al.*, 1991; Low *et al.*, 1992). Direct evidence for a low affinity pathway, distinct from the $Ca^{2+}$-ATPase, has been presented for porcine aortic smooth muscle (Missiaen *et al.*, 1991). The high affinity $Ca^{2+}$-ATPase would provide the main route for $Ca^{2+}$ uptake (Missiaen *et al.*, 1991). Thus, inhibiting its activity with cyclopiazonic acid prevented the stores from refilling after they had been discharged by phenylephrine in $Ca^{2+}$-free solution. Nevertheless, the low affinity uptake may persist after blockade of the $Ca^{2+}$-ATPase when intracellular $Ca^{2+}$ levels are elevated, for example when the tissue is stimulated with phenylephrine in the presence of extracellular $Ca^{2+}$. Under these conditions, the low affinity pathway may be able to maintain $Ca^{2+}$ cycling through the SR.

When cyclopiazonic acid and diltiazem were present together, both SR uptake pathways would have been blocked, leading to store depletion and a diminished contribution of $IP_3$-dependent $Ca^{2+}$ release to tension. This can explain why the response to hydralazine was suppressed by cyclopiazonic acid and diltiazem, only when they were present together. Similarly, although ryanodine would have made the SR leaky, with no other blockers present $Ca^{2+}$ reuptake could have maintained SR $Ca^{2+}$ levels and $IP_3$-dependent $Ca^{2+}$ release at near normal levels. The block of the hydralazine response observed when ryanodine was present with cyclopiazonic acid could reflect depletion of the SR, due to $Ca^{2+}$ leaking out faster then it was accumulated. In contrast, the high affinity $Ca^{2+}$-ATPase may be able to maintain SR filling when diltiazem and ryanodine are present together, explaining why this combination only slightly reduced the hydralazine response.

The fact that diltiazem and cyclopiazonic acid were just as effective in inhibiting the hydralazine response as all three SR modulators together, suggests that blocking both routes for $Ca^{2+}$ sequestration maximally depletes the stores even in the presence of extracellular $Ca^{2+}$ and phenylephrine. The residual response to hydralazine observed in these conditions may reflect incomplete emptying of the SR stores. Consistent with this, phenylephrine contractions in $Ca^{2+}$ free solution were never completely abolished, even with cyclopiazonic acid, ryanodine and diltiazem all present together. On the other hand, the contraction remaining under these conditions may not be due to released $Ca^{2+}$, since even after prolonged ($>1$ h) exposure to phenylephrine in $Ca^{2+}$-free/1 mM EGTA solution, some tension remained. Such apparently $Ca^{2+}$-independent actions of α-agonists have been seen before (Bolton, 1979) and may be mediated by protein kinase C (Khalil *et al.*, 1992). Thus the residual response to hydralazine after store depletion could represent a separate action, perhaps on the pathways involved in activation of the contractile proteins. While previous work argues against such an action (Kreye *et al.*, 1983; DeFeo & Morgan, 1989), it cannot be ruled out.

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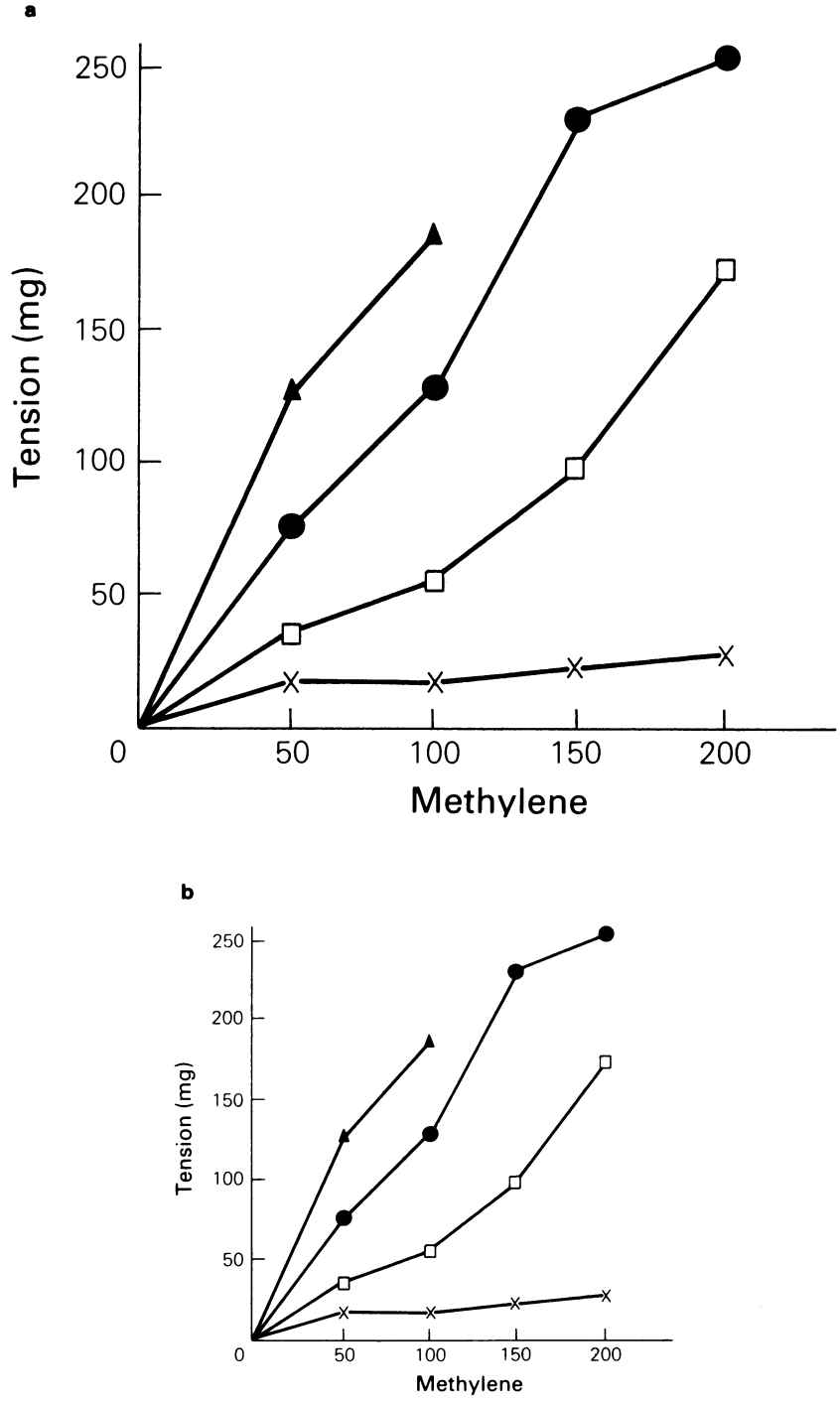


**Figure 1** (a) Artwork as drawn. (b) Artwork reduced to 60 per cent of its original size for publication in the Journal.

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# ABBREVIATIONS AND SYMBOLS

## Physico-chemical quantities

The *British Journal of Pharmacology* uses the SI symbols for units. The following prefixed for multiples of units should be used:

| *Multiplier* | *Prefix* | *Symbol* |
|---|---|---|
| $10^{-1}$ | deci | d |
| $10^{-2}$ | centi | c |
| $10^{-3}$ | milli | m |
| $10^{-6}$ | micro | μ |
| $10^{-9}$ | nano | n |
| $10^{-12}$ | pico | p |
| $10^{-15}$ | femto | f |
| $10^{-18}$ | atto | a |
| *Multiplier* | *Prefix* | *Symbol* |
| $10^{3}$ | kilo | k |
| $10^{6}$ | mega | M |
| $10^{9}$ | giga | G |
| $10^{12}$ | tera | T |

Thus, micron = μm; ångstrom = 0.1 nm. Mixed prefixes are not permissible, thus m μg should be ng. The symbols d ($10^{-1}$) and c ($10^{-2}$) should be restricted to those occasions on which there is a strongly felt need for them (e.g. cm).

## Use of the solidus

The solidus should be avoided as far as possible and the negative index substituted, e.g. mg $kg^{-1}$ rather than mg/kg; pmol $mm^{-2}$ $min^{-1}$ rather than pmol/$mm^2$/min.

# SYMBOLS

Symbols denoting physical quantities are usually printed as italic capitals (indicated by single underline in typescript). A dash over the symbol indicates a mean value; a dot over the symbol indicates a time derivative. Suffixes may be used to indicate 'where' and 'what'. They are printed as inferiors on the line. Multiple suffixes should be avoided if a simpler symbol adequately defined is unambiguous, but if necessary should be separated by commas e.g. $P_{A,\,CO_2}$ denotes partial pressure of $CO_2$ alveolar air.

# CHEMICAL AND BIOLOGICAL ABBREVIATIONS

Authors should also consult *Nomenclature Guidelines for Authors* contained in this issue of the Journal. The abbreviations listed may be used without definition *except* those for chemicals, drugs and enzymes which must be written in full at first mention in the title, summary and again in the text. At first mention they should be followed by the abbreviation in brackets. Subsequently, the abbreviation alone may be used.

The list of abbreviations for chemical, drug and enzyme names is clearly not comprehensive and includes only a few commonly used examples.

**Use abbreviations sparingly as extensive use can make the text hard to follow.**

**Physico-chemical quantities**

| *Quantity* | *Preferred unit* | *Symbol* |
|---|---|---|
| Amount (of substance) | mole | mol |
| Capacitance | farad | F |
| Concentration | moles per litre | M or mol $l^{-1}$ |
| Current | ampere | A |
| Electrical conductance | siemens | S |
| Electromotive force | volt | V |
| Flow (blood or other liquid) | litres per second (or min) | 1 $s^{-1}$ or 1 $min^{-1}$ |
| Flow (air or other gas) | litres per second (or min) | 1 $s^{-1}$ or 1 $min^{-1}$ |
| Force | newton | N |
| Frequency of regular event | hertz | Hz |
| Length | metre | m |
| Mass | gram | g |
| Power | watt | W |
| Pressure (or partial pressure) | pascal* | Pa |
| Radioactivity | becquerel or curie | Bq (60 d.p.m.) or Ci ($3.7 \times 10^{10}$ Bq) |
| Resistance (electrical) | ohm | Ω |
| Temperature | degree celsius | °C |
| Time | second (preferred) | s |
| | minute | min |
| | hour | h |
| Volume (blood or other liquid) | litre | l |
| Volume (air or other gas) | litre | l |
| Work | joule | J |

* mm of mercury (mmHg) are allowed if conventional, and if mercury manometer is used for calibration.

**Chemical and biological abbreviations**

| | |
|---|---|
| acetylcholine | ACh |
| acetylcholinesterase | AChE |
| adenosine 3′:5′-cyclic monophosphate | cyclic AMP |
| adenosine 5′-phosphate | AMP |
| adenosine triphosphatase | ATPase |
| γ-aminobutyric acid | GABA |
| analysis of variants | F |
| adrenaline | Ad |
| analytical standard of reagent purity | A.R. |
| anhydrous | anhyd. |
| approximate(ly) | approx. |
| approximately equals | ≈ |
| aqueous | aq. |
| arg-vasopressin | AVP |
| boiling point | b.p. |
| bovine serum albumin | BSA |
| cardiovascular system | CVS |
| catechol-*O*-methyl transferase | COMT |
| central nervous system | CNS |
| cerebrospinal fluid | CSF |
| chi-squared (statistics) | $\chi^2$ |
| clearance | *c* |
| coenzyme A | CoA |
| concentrated | conc. |
| correlation coefficient | *r* |
| cubic | cu. |
| degree of freedom (statistics) | d.f. |
| deoxyribonucleic acid | DNA |
| deoxyribonuclease | DNase |
| dextro-(absolute configuration) | D- |
| dextro-(optical rotation) | (+)- |
| diameter | diam. |
| diameter, inside | i.d. |
| diameter, outside | o.d. |
| diffusion coefficient | *D* |
| 3,4-dihydroxyphenylalanine | DOPA |
| 3,4-dihydroxyphenylethylamine | dopamine |
| direct current | d.c. |
| disintegration per minute | d.p.m. |
| dissociation constant | $K_D$ |
| dissociation constant, negative logarithm of | pK |
| distilled | dist. |
| dry ice | solid $CO_2$ |
| edition | edn |
| editor(s) | ed. |
| effective concentration | $EC_{50}$ |
| effective dose, median | $ED_{50}$ |
| electrocardiogram | ECG |
| electrocorticogram | ECoG |
| electroconvulsive therapy | ECT |
| electroencephalogram | EEG |
| electromyogram | EMG |
| electron spin resonance | e.s.r. |
| endothelial-derived relaxing factor | EDRF |
| epithelial-derived relaxing factor | EpDRF |
| equilibrium constant | *K* |
| equivalent (general use) | equiv. |
| erythrocyte | r.b.c. |
| erythrocyte sedimentation rate | ESR |
| ethylenediaminetetracetic acid | EDTA |
| excitatory postsynaptic potential | e.p.s.p. |
| experiment | expt |
| experimental | exptl |

| | |
|---|---|
| fatty acids, nonesterified | NEFA |
| figure(s) (with reference number) | Figure(s) |
| figure (diagram) | figure |
| gas-liquid chromatography | g.l.c. |
| glomerular filtration rate | GFR |
| haemoglobin | Hb |
| half-life | $t_{\frac{1}{2}}$ |
| high-frequency | h.f. |
| high performance liquid chromatography | h.p.l.c. |
| human serum albumin | HSA |
| hydrogen-ion concentration | $[H^+]$ |
| hydrogen-ion activity, negative logarithm of (hydrogen-ion exponent) | pH |
| 6-hydroxydopamine | 6-OHDA |
| N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] | HEPES |
| 5-hydroxyindoleacetic acid | 5-HIAA |
| 5-hydroxytryptamine | 5-HT |
| immunoglobulins | IgA, IgD, IgE, IgG, IgM |
| inhibitor constant | $K_i$ |
| inhibitory concentration | $IC_{50}$ |
| inhibitory postsynaptic potential | i.p.s.p. |
| insoluble | insol. |
| international unit | iu |
| intra-arterial | i.a. |
| intracellular fluid | ICF |
| intradermal | i.d. |
| intramuscular | i.m. |
| intraperitoneal | i.p. |
| intracerebroventricular | i.c.v. |
| intravenous | i.v. |
| isotope (atomic mass) e.g. iodine-131 | $^{131}I$ |
| isotopically substituted compounds e.g. | [$^{14}$C]-ethanol |
| laevo-(absolute configuration) | L- |
| laevo-(optical rotation) | (−)- |
| lethal dose, median | $LD_{50}$ |
| leukotriene | LT |
| logarithm to base e | $\log_e$ or ln |
| logarithm to base 10 | $\log_{10}$ |
| maximum | max. |
| mean arterial pressure | MAP |
| mean value of (statistics) | $\bar{x}$ |
| melting point | m.p. |
| meta | *m*- |
| Michaelis constant | $K_M$ |
| minimum | min. |
| mobility (electrophoresis) | *m* |
| monoamine oxidase | MAO |
| noradrenaline | NA |
| nuclear magnetic resonance | n.m.r. |
| number | no. or No. |
| number of observations (statistics) | *n* |
| ortho | *o*- |
| packed cell volume | PCV |
| page/pages | p./pp. |
| para- | *p*- |
| paragraph | para. or ¶ |
| parts per millon | p.p.m. |
| per cent | % |
| platelet activating factor | PAF |
| posterior | post. |
| probability (significance level in a statistical test) | *P* |
| radioimmunoassay | RIA |
| rectus (configuration by the sequence rule) | **R** |
| red blood corpuscle | RBC |
| relative band speed to front (chromatography) | $R_F$ |
| relative molecular mass | $M_r$ |
| relative retention time (gas chromatography) | $t_r$ |
| renal plasma flow | RPF |
| resistance (respiratory) | *R* |
| respiratory conductance | Sgaw |
| revolutions per minute | r.p.m. |
| ribonucleic acid | RNA |
| section | § |
| sedimentation coefficient (ultracentrifugation) | *s* |
| sinister (configuration by the sequence rule) | **S** |
| soluble | sol. |
| solution | soln. |
| Spearman rank coefficient | $r_s$ |
| standard deviation (of observed sample) | s.d. |
| standard error (of estimate mean value) | s.e.mean |
| standard error (of sampling) | s.e. |
| standard temperature and pressure | STP |
| subcutaneous | s.c. |
| sum (statistical): of hypothetical population | $\Sigma$ |
| of observed sample | S or $\Sigma$ |
| temperature | temp. |
| thin layer chromatography | t.l.c. |
| time, clock – 24 h clock used e.g. 18 h 30 min | *t* |
| time constant | $\tau$ |
| 2-amino-2-hydroxymethyl-propan-1,3-diol | Tris |
| ultraviolet | u.v. |
| unit | u |
| vacuum | vac. |
| valency | e.g. $Fe^{2+}$; Fe(II) protoporphyrin |
| volume by volume | v/v |
| wavelength | $\lambda$ |
| weight | wt. |
| weight by volume | w/v |

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# NOMENCLATURE GUIDELINES FOR AUTHORS

## With effect from 1 January 1995

The Nomenclature Working Party (NWP) of the Editorial Board of the *British Journal of Pharmacology* has consulted many acknowledged experts in an effort to clarify and standardize receptor and other nomenclature systems for use by Editors until a complete set of recommendations of the IUPHAR Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) are published.

NWP is unanimous in its view that, with rare exceptions, the Journal should use spellings, names and abbreviations that have been chosen by international bodies convened for the purpose.

For receptor nomenclature, with few exceptions, the Journal generally follows the guidelines laid down in the current *Trends in Pharmacological Sciences* Receptor and Ion Channel Nomenclature Supplement and the reports of NC-IUPHAR published in *Pharmacological Reviews*.

### 1 *Definition of receptors and subtypes*

Receptors and their subtypes are defined in terms of the relative potencies of agonists and selectivities of antagonists in functional studies, by the binding of such ligands and to structural information, where available.

### 2 *Format of receptor names*

It was agreed that, until NC-IUPHAR provides full recommendations:

(a) Editors will permit with reluctance new nomenclature systems in papers accepted for publication if and only if there are compelling reasons to introduce a new terminology (or modify an accepted one). The criteria upon which the new receptor type or subtype are defined must be given, together with adequate explanations of the relationship between the previous nomenclature (fully referenced) and the proposed one.

N.B. The new nomenclature should not appear in the Title, Short Title or Keywords, unless qualified by the adjective putative (e.g. . . . mediated by the putative $M_5$ muscarinic acetylcholine receptor.

(b) Only well-established and universally accepted subtype names (e.g. muscarinic and nicotinic acetylcholine receptors; α-and β-adrenoceptors) will be acceptable without any reference to the originator of these terms. In cases of controversy concerning further subdivision of the subtype, full referencing must be given.

(c) When receptors are expressed from DNA/RNA that has been introduced into cells and these receptors display a similar pharmacological profile to the native receptors, they should be denoted by use of lower case, e.g. m1 for expressed receptor and $M_1$ for native receptor. The stoichiometry of the expressed receptor should be indicated, where appropriate, e.g. for an immature muscle nicotinic acetylcholine receptor, it might be $(\alpha 1)_2\ \beta 1\gamma\delta$.

**(d)** Receptor subtypes should be designated by means of a subscript numeral or capital letter. Some double subscripts (i.e. numerical plus letter) are acceptable.

### 3 *Types of receptor*

The NWP accepts that there are additional receptors to those described below which can be considered as well established. In many cases, however, their existence has been confirmed only in cloning studies and it is as yet unclear how they relate to similar subdivisions proposed on the grounds of differences in agonist and antagonist potencies in various tissues.

(a) *Acetylcholine receptors* The two principal subfamilies are muscarinic and nicotinic acetylcholine receptors (the abbreviations muscarinic AChRs and nicotinic AChRs are acceptable, but not mAChR or nAChR, which may be confused with muscle and neuronal subtypes).

*Muscarinic acetylcholine receptors* The principle subtypes are $M_1$, $M_2$, $M_3$ and $M_4$.

*Nicotinic acetylcholine receptors* The principal subgroups are muscle and neuronal nicotinic acetylcholine receptors.

(b) *Adenosine receptors* Known also as $P_1$ purinoceptors (see purinoceptors, 3u). (See Fredholm, B.B., *et al.*, (1994) *Pharmacol. Rev.*, **46**, 145–156.)

(c) *Adrenoceptors* The principal subtypes are $\alpha_1$-, $\alpha_2$-, $\beta_1$-, $\beta_2$- and $\beta_3$-adrenoceptors. Additional subtypes must be fully referenced. (See Bylund, D.B., *et al.*, (1994) *Pharmacol. Rev.*, **46**, 121–136.)

(d) *Angiotensin receptors* The principle subtypes are $AT_1$ and $AT_2$.

(e) *Bombesin receptors* Proposed subtypes such as $BB_1$, $BB_2$ may be used but must be fully referenced.

(f) *Bradykinin receptors* The principle subtypes are $B_1$ and $B_2$ receptors.

(g) *Calcitonin gene-related peptide (CGRP) receptors* Proposed CGRP receptor subtypes must be fully referenced.

(h) *Cannabinoid receptors* These should follow the style $CB_1$, $CB_2$ and be fully referenced.

(i) *Cholecystokinin (CCK) receptors* The principal subtypes are $CCK_A$ and $CCK_B$ receptors.

(j) *Dopamine receptors* $D_1$ and $D_2$ dopamine receptors are recognised. Other subtypes must be fully referenced.

(k) *Endothelin receptors* The principle subtypes are $ET_A$ and $ET_B$ receptors. (See Masaki, T., *et al.*, (1994) *Pharmacol. Rev.*, **46**, 137–142.)

(l) *Excitatory amino acid receptors* Three ionotropic subtypes are recognised and named: (1) NMDA receptors; (2) AMPA receptors, and (3) kainate receptors. A second class is the metabotropic (mGlu) receptor family. Further subtypes of excitatory amino acid receptors must be fully referenced.

(m) *γ-Aminobutyric acid (GABA) receptors* The principal subtypes are $GABA_A$ and $GABA_B$ receptors. Regulatory sites on the $GABA_A$ receptor should be referenced.

(n) *Histamine receptors* The principle subtypes are $H_1$, $H_2$ and $H_3$ receptors.

(o) *5-Hydroxytryptamine (5-HT) receptors* The principle subtypes are 5-$HT_1$, 5-$HT_2$, 5-$HT_3$ and 5-$HT_4$. Further

subdivisions require full referencing. (See Hoyer, D., *et al.*, (1994) *Pharmacol. Rev.*, **46**, 157–203).

(p) *Leukotriene receptors* When first mentioned, the style leukotriene (LT) receptor should be used, thereafter LT receptor. Receptors should be designated according to the leukotriene that selectively or preferentially binds to them. All leukotriene receptor subtypes should be fully referenced.

(q) *Neuropeptide Y (NPY) receptors* Proposed subtypes should be fully referenced.

(r) *Opioid receptors* The principal subtypes are μ-, δ- and κ-opioid receptors. Other proposed subtypes should be fully referenced.

(s) *Oxytocin receptors* (see Vasopressin and oxytocin receptors).

(t) *Prostanoid receptors* The principal types are DP, EP, FP, IP and TP receptors. When first mentioned, the style prostanoid (XP) receptor should be used, thereafter XP receptor (where X denotes the type). Proposed subtypes should be referred to as $XP_n$, (e.g. $EP_1$, $EP_2$) and referenced. (See Coleman, R.A., *et al.*, (1994) *Pharmacol. Rev.*, **46**, 205–229).

(u) *Purinoceptors* The principal subtypes are $P_1$ and $P_2$ receptors. Subdivision of $P_1$ into $A_1$, $A_2$ and $A_3$ subtypes and of $P_2$ into $P_{2X}$ and $P_{2Y}$ are permitted. Other subtypes e.g. $P_{2T}$, $P_{2Z}$ should be fully referenced. (See Fredholm, B.B., *et al.*, (1994) *Pharmacol Rev.*, **46**, 143–156.)

(v) *Somatostatin (SS) receptors* Proposed subtypes should be fully referenced.

(w) *Tachykinin receptors* The term tachykinin is preferred to neurokinin. The principle subtypes are $NK_1$, $NK_2$ and $NK_3$ receptors.

(x) *Vasoactive intestinal peptide (VIP) receptors* Proposed subtypes should be fully referenced.

(y) *Vasopressin and oxytocin receptors* The principle subtypes are $V_{1A}$, $V_{1B}$, $V_2$ and OT receptors.

**4** *Naming of ion Channels*

The four main ion channels are named according to the abbreviation of the ion normally carrying the current (i.e. K channels, Na channels, Ca channels and Cl channels). In naming channels the charge on the ion is understood and need not be stated. Further subtypes, e.g. $K_{ATP}$ must be fully referenced. For Ca channels, see Spedding, M. & Paoletti, P. (1992) *Pharmacol. Rev.*, **44**, 363–376.

**5** *Naming of nerve fibres*

Many nerve fibres are now known to release more than one transmitter, and future work may show that this is in fact the general rule. In that case, the concept of the same transmitter being released either at different developmental stages or under various experimental conditions would no longer hold, and single adjectives that imply this (e.g. cholinergic, noradrenergic) would become inappropriate when applied to nerve fibres, as distinct from transmitter functions. For the present, those nerve fibres that are known to function by releasing more than one identified transmitter may be described accordingly; for example, noradrenergic-purinergic, cholinergic-peptidergic (in alphabetical order, the order implying no priority of function). N.B. The suffix 'ergic' should continue to be applied only to nerve fibres and to the transmission event, in accordance with Dale's intentions. For example, 'cholinergic' indicates that the nerve fibre, or the transmission, functions under particular conditions through the release of a choline-like substance. The suffix should not be used loosely to mean 'pertaining to'. Hence, for example, the expression 'cholinergic receptor' (rather than acetylcholine receptor) is an inappropriate use of the term. Transmission events involving nitric oxide may be referred to as *nitrergic*. However, *nitrergic* may be used to describe axons only when there is sufficient evidence that nitric oxide is released from them as a neurotransmitter.

(a) *Catecholamine releasing nerve fibres* The adjective to be applied to nerve fibres that release dopamine as a transmitter is dopaminergic (not DAergic).

Nerve fibres that are known to function by releasing noradrenaline are to be described as noradrenergic. The term adrenergic should now be reserved for nerve fibres known to release adrenaline. Where the identity of the catecholamine is uncertain, catecholaminergic should be used.

(b) *Some other adjectives describing nerve fibre function* NANC is an acceptable abbreviation of non-adrenergic, non-cholinergic for peripheral efferent nerve fibres when the identity of the transmitter(s) is unknown other than the fact that neither (nor)-adrenaline nor acetylcholine is involved. It should be defined when introduced. NANCergic, e-NANC (or NANC-e) and i-NANC (or NANC-i) are not acceptable terms.

Glutamatergic, not glutaminergic, should be used to describe nerve fibres releasing glutamate. In referring to peptide-releasing nerve fibres (e.g. those that may release substance P or vasoactive intestinal peptide) the nomenclature to be used is peptidergic (X), e.g. peptidergic (SP), peptidergic (VIP), not SPergic, VIPergic.

The terms 5-hydroxytryptamine (5-HT) and 5-hydroxytryptaminergic (i.e. nerves releasing 5-hydroxytryptamine) are preferred to those of serotonin and serotoninergic. The term 5-HTergic is not acceptable, except to avoid frequent repetition of 5-hydroxytryptaminergic.

Likewise, the terms purinergic (ATP) and purinergic (adenosine) are preferred.

**6** *Terms used to describe agonist and antagonist action*

The following terms can be used without full definition. Where appropriate, other terms may be used but must be accompanied by a full definition.

*Terms used to describe affinity and potency*

(a) $EC_{50}$ The concentration of an agonist that produces 50% of the maximal response for that agonist *in vitro*. The agonist may be stimulatory or inhibitory. When $EC_{50}$ values are determined in the presence of other agonists or antagonists the concentration of the latter should be stated. Related terms, e.g. $EC_{25}$, are acceptable if accompanied by a full definition.

(b) $IC_{50}$ This term may be used in the following ways: (i) The concentration of antagonist that reduces the response to a sub-maximal concentration of agonist by 50%; the concentration of agonist should be stated. (ii) The concentration of competing agonist or antagonist that inhibits the binding of a radioligand by 50%; the concentration of radioligand should be stated.

(c) $ED_{50}$ This term may be used in the following ways: (i) The dose of an agonist or antagonist that produces 50% of the maximal possible effect of that agonist or antagonist *in vitro*. (ii) The dose of drug that produces the effect under investigation in 50% of the population.

(d) $K$ The dissociation equilibrium constant (mol $l^{-1}$) for ligand-receptor interactions. The reciprocal is called the affinity constant or association equilibrium constant. When necessary for clarity subscripts (letters or numerals, or a combination of both) may be added but these must be clearly explained when first used.

(e) $n_H$ The Hill coefficient.

(f) $pA_2$ The negative logarithm to base 10 of the concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit a given submaximal response. Note that the definition is empirical and does not pre-suppose the mechanism of antagonism. The $pA_2$ value can be determined from a Schild plot with unconstrained slopes, but only provides an estimate of the $pK_B$ if the antagonism has been shown to meet all of the criteria of competition.

(g) $pD_2$ The negative logarithm to base 10 of the $EC_{50}$.

(h) $pIC_{50}$ The negative logarithm to base 10 of the $IC_{50}$.

(i) $pK$ The negative logarithm to base 10 of $K$ (with or without supscripts as appropriate: see 6(d).

*Terms used to describe the mode of antagonism*

(a) *Competitive antagonism* Used to describe antagonists that bind reversibly to the agonist binding site.

(b) *Irreversible competitive antagonism* Used to describe antagonists that bind irreversibly to the agonist binding site.

(c) *Non-competitive* Used to describe antagonists that bind reversibly to a distinct (allotopic) site.

(d) *Irreversible non-competitive antagonism* Used to describe antagonists that bind irreversibly to a distinct (allotopic) site.

7 *Enzymes*

The IUBMB Enzyme Commission (EC) number and full name (Enzyme Nomenclature 1992, Academic Press, San Diego) must be quoted when first mentioned in text. Subsequently the accepted trivial name is used. Trivial names may be used in the title.

8 *Other nomenclature requirements*

(a) *Racemates* Authors must state unambiguously in the Methods section of papers which isomers were used, e.g. (+)- or (−)-propranolol, and must bring to the attention of the reader the composite character of drugs that are mixtures of stereoisomers. Furthermore, the implications of the composite nature of such drugs studied for the interpretation of the data measured and the conclusions drawn must be made explicit. Note that the terms d- or l- for dextro- and laevo-rotatory are now obsolete, and the prefixes (+)- or (−)- respectively should be used. Capital D and L refer to the absolute configurations and of course remain acceptable when appropriate.

(b) *Purines* This term should not be used as a synonym for purine nucleotides or nucleosides.

(c) *Eicosanoids* The system of nomenclature to be used for eicosanoids is that published in *Methods in Enzymology*, (1990), **187**, 1–9. This scheme incorporates recent changes in the style of abbreviation of hydroperoxy-, epoxy- and oxo-unsaturated fatty acids e.g. 12(S)-hydroperoxyeicosatetraenoic acid which was formerly abbreviated as 12(S)-HPETE now becomes 12(S)-HpETE. In manuscripts, the first use of the full chemical name of any eicosanoid should indicate double bond geometry when this is known.

(d) *Peptide nomenclature* The preferred style is capital letters to designate the first letter of the word. Otherwise, upper and lower case letters should be used (e.g. Enk-IR, enkephalin-like immunoreactivity). When numbers are used these should be placed after a hyphen on the same line as the abbreviation, e.g. ET-1.

(e) *Cell lines* Cell type, sources and originating species need to be defined.

(f) *Molecular biology* Abbreviations pertaining to molecular biological techniques need to be defined or presented in such a way that they can be recognised by the non-specialist e.g. the oligonucleotide sequence, TAGC.

(g) *Tension* Tension is force and should be calibrated in newtons (1 newton = 1kg $ms^{-1}$) or in kg weight, g weight, or mg weight etc. It should not be calibrated in units of mass (e.g. kg). (See Miller D.J. in *Trends Pharmacol. Sci.*, 1988, **9**, 124–5).

(h) *Ions* When referring to ions, the charge should be indicated. e.g. $Na^+$, $Ca^{2+}$, $2Na^+/Ca^{2+}$ exchange, etc.

(i) *Inhibitors of nitric oxide synthase* The most commonly used and currently accepted abbreviation for $N^G$-nitro-L-arginine and $N^G$-nitro-L-arginine methyl ester are L-NOARG and L-NAME. Unless alternative international agreement is reached, these will be the abbreviations accepted by the journal.

## PREPARATION OF MANUSCRIPTS

Authors are strongly recommended to read the full *Instructions to Authors* and *Nomenclature Guidelines for Authors* (*Br. J. Pharmacol.* 1995, **114,** 245–255) before submitting a manuscript for publication in the *British Journal of Pharmacology*. The manuscript and cover letter should be checked against the following list before mailing.

The original and one copy of the manuscript must be supplied. Manuscripts must be typed in double-line spacing on one side of A4 paper, in type not smaller than 12 characters per inch or 10 point. Both copies to include Tables and a set of labelled Figures. One set of Figures without numbers or letters is also to be included. The text to be arranged in the following subsections:

1. **Title**—To have no more than 150 characters on a separate page, which should also include a Short Title (50 characters maximum) and the name and address of the author for correspondence.
2. **Summary**—To be arranged in numbered paragraphs (Full Papers) or a single paragraph (Special Reports).
   —to include aims, principal results and conclusions.
   —to include Key words (10 maximum) at end of summary.
3. **Introduction**—To contain concise statements of the problem and the aims of the investigation.
4. **Methods**—To have brief but adequate account of the procedures; *full names of drugs* (*including those referred to by manufacturer's code*), sources of drugs and statistical tests to be stated.
5. **Results**—To have no repetition of data in Figures, Tables and text.
6. **Discussion**—Findings and conclusions to be placed in context of other relevant work.
   *NB* Simple repetition of results and unwarranted speculation are not acceptable.
7. **Acknowledgements**—Sources of support. Sources of drugs not widely available commercially.
8. **References**—All references in the text to be included in the Reference List and *vice versa*. References in alphabetical order with complete citations; Journals publishing 'in press' papers identified.
   *References to manuscripts submitted to other journals but not yet accepted are not allowed.*
9. **Tables**—Each on a separate page and prepared in accordance with current requirements of the Journal.
10. **Figures**—Both labelled and non-labelled Figures to be prepared in accordance with current requirements of the Journal (see *Instructions to Authors*, 1995, **114,** 245–251) and provided with Figure Number and Authors' names on back (*in pencil*).
    —each legend to be typed on a separate page and carrying keys to symbols.
    —keys to symbols and histograms must not appear on the figures themselves, but in the respective legends.
    —'box style' figures are not in keeping with the Journal style; line drawings etc must have only left-hand and bottom axes.
11. **Manuscripts**—To be accompanied by a declaration signed by each author that
    (a) results are original
    (b) approval of all persons concerned has been given to submit manuscripts for consideration (see also 12b)
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    (b) where appropriate, that *either* ethical approval has been given for investigation *or* Company or Institutional permission to publish work has been received.
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Failure to comply with *Instructions to Authors* may lead to substantial delays in processing, review and publication and may even jeopardize acceptance of the manuscript.

## NOMENCLATURE

Authors are reminded that accepted receptor and associated terminology is laid out in *Nomenclature Guidelines for Authors*, as published in the *British Journal of Pharmacology*, *Br. J. Pharmacol.*, 1995, **114,** 253–255.

## SPECIAL REPORTS

The purpose of *Special Reports* is to provide rapid publication for **new** and **important** results which the Editorial Board considers are likely to be of special pharmacological significance. *Special Reports* will have publication priority over all other material and so authors are asked to consider carefully the status of their work before submission.

In order to speed publication there is normally no revision allowed beyond very minor typographical or grammatical corrections. If significant revision is required, the Board may either invite rapid re-submission or, more probably, propose that it be re-written as a Full Paper and be re-submitted for consideration. In order to reduce delays, proofs of *Special Reports* will be sent to authors but **essential corrections must reach the Production Office within 48 hours of receipt.** Authors should ensure that their submitted material conforms exactly to the following requirements.

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