British Journal of Pharmacology

December 1992

Volume 107 Number 4 pages 903–1204

Br. J. Pharmacol. (1992), **107**, 905–907

SPECIAL REPORT

L-Arginine dilates rat pial arterioles by nitric oxide-dependent mechanisms and increases blood flow during focal cerebral ischaemia

Eiharu Morikawa, Sami Rosenblatt & [1]Michael A. Moskowitz

Stroke Research Laboratory, Neurosurgery and Neurology Service, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114, U.S.A.

L-Arginine ($\geqslant 30$ mg kg^{-1}, i.v.), but not D-arginine (300 mg kg^{-1}) administered 5 min after unilateral common carotid/middle cerebral artery occlusion increased regional cerebral blood flow (rCBF) within the dorsolateral ischaemic cortex in spontaneously hypertensive rats. L-Arginine (300 mg kg^{-1}) increased rCBF from 22 ± 2.7 to 33 ± 4% of baseline as measured by laser-Doppler flowmetry. This increase may explain the ability of L-arginine to reduce infarct size following focal cerebral ischaemia, as reported previously. The mechanism appears to be mediated by nitric oxide since topical L-NAME (1 μM), a nitric oxide synthase inhibitor, decreased pial arteriole calibre from 115 ± 2.2 to 106 ± 0.9% of baseline following L-arginine infusion (300 mg kg^{-1}).

Keywords: L-Arginine; nitric oxide; focal cerebral ischaemia; laser-Doppler flowmetry; vasodilatation; cranial window

Introduction Nitric oxide (NO) is synthesized from the amino acid L-arginine by the enzyme NO synthase (Palmer *et al.*, 1988). NO has been proposed as a mediator of endothelium-dependent vasodilatation and relaxes vascular smooth muscle through guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-dependent mechanisms (see Moncada *et al.*, 1991 for review).

Recently, we showed that L-arginine reduces infarct volume in two models of focal cerebral ischaemia (Morikawa *et al.*, 1992). To explain this effect, we hypothesized that L-arginine augments NO production and increases regional cerebral blood flow (rCBF) above the ischaemic threshold. In this paper, we examine the effects of L-arginine infusion on: (i) rCBF within ischaemic tissue using laser-Doppler flowmetry, and on (ii) normal pial vessels after NO synthase inhibition.

Methods *Laser-Doppler flowmetry* Thirty six male spontaneously hypertensive rats (SHR; 280–340 g; Charles River Labs, Wilmington, MA, U.S.A.) were subjected to common carotid artery (CCA)/middle cerebral artery (MCA) occlusion as described previously (Brint *et al.*, 1988). Briefly, anaesthesia was induced and maintained by halothane, 3 and 0.5% respectively, along with 70% nitrous oxide and the balance oxygen in ventilated animals. The MCA was occluded by a metallic clip (Zen clip, Ohwa Tsusho) just distal to the rhinal fissure within 1 min after CCA occlusion.

rCBF was monitored continuously (BPM 403A, TSI Inc.) as described (Koketsu *et al.*, 1992) through a craniotomy over the dorsolateral cortex (4–6 mm lateral, −2 mm to 1 mm rostral to bregma; the transitional zone from severe to mildly ischaemic in this model, Jacewicz *et al.* 1990).

Closed cranial window Twenty male Sprague Dawley (SD) rats (280–330 g; Charles River Labs) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1} i.p., plus 10 mg kg^{-1}, i.p. hourly), paralyzed (pancuronium bromide 0.5–1.0 mg, i.v.) and mechanically ventilated with O_2 supplemented room air; end-tidal P_{CO_2} was monitored continuously (Novametrix Medical Systems, Wallingford, CT). Pial vessels were visualized with an intravital microscope (200 × magnification; Leitz, Germany). A window was placed over the left parietal cortex. The space under the window was then filled with artificial CSF (Levasseur *et al.*, 1975) equilibrated at 37°C with a gas containing 10% O_2, 5% CO_2 and the balance nitrogen. Measurements were taken (VIA-100, Boeckler Instruments) after the image was transposed onto a video monitor (Dage MTI Inc., CCD-72 series, Michigan City, IN, U.S.A.).

Arterial blood pressure and blood gases were monitored and rectal temperature was maintained at 37°C in all experiments.

Chemicals L- or D-Arginine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in distilled water and adjusted to pH 7.0 with sodium hydroxide. N^G-nitro-L-arginine methyl ester (L-NAME; Sigma) was dissolved in artificial CSF immediately before use.

Results There were no significant differences in MAP, pHa, plasma glucose, P_{aO_2}, P_{aCO_2} or rectal temperature between treatment groups when rCBF or pial vessel diameter was monitored (data not shown).

CCA/MCA occlusion reduced rCBF by approximately 80% of baseline (Figure 1). L-Arginine, 30 and 300 mg kg^{-1} increased rCBF following occlusion whereas saline, 3 mg kg^{-1} L-arginine, or 300 mg kg^{-1} D-arginine did not. The findings do not appear to depend upon the choice of anaesthetic. When pentobarbitone (65 mg kg^{-1}) was used instead of halothane/nitrous oxide, L-arginine (300 mg kg^{-1}) also increased rCBF from 29 ± 6 to 44 ± 8% ($n = 4$, $P < 0.05$ by paired Student's t test).

L-Arginine (30 and 300 but not 3 mg kg^{-1}) increased pial vessel diameter in SD rats (Figure 2). Topical L-NAME (1 μM) significantly attenuated these responses. L-Arginine (30 mg kg^{-1}, i.v.) dilated pial arterioles when administered to SHR (113 ± 2.6% ($n = 4$); baseline diameter 43 ± 5.9 μm).

Discussion The observed changes in rCBF from below to approximately the ischaemic threshold for infarction in SHR

[1] Author for correspondence at: Stroke Research Laboratory, Massachusetts General Hospital, 32 Fruit Street, Boston, MA02114

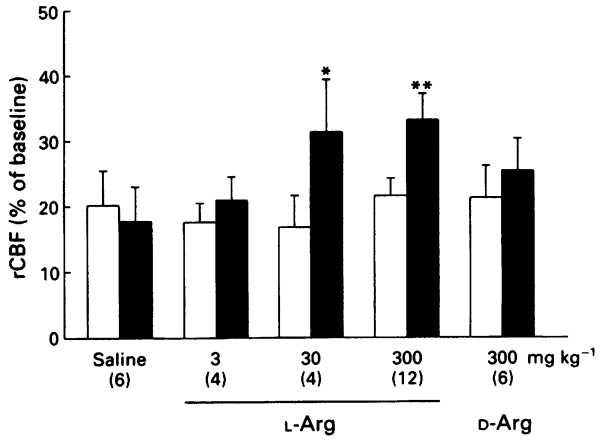

Figure 1 L-Arginine infusion (≥ 30 mg kg^{-1}, i.v.) but not D-arginine increased rCBF in the middle cerebral artery (MCA) region after combined common carotid artery (CCA)/MCA occlusion in SHR. After 15 min of stable rCBF recordings, the CCA/MCA were occluded as described and rCBF measured 2 and 5 min later (open columns). L-or D-Arginine or saline was then administered at a constant rate of 100 µl kg^{-1} min^{-1} for 10 min (Harvard infusion pump, Harvard Bioscience, South Natick, MA, U.S.A.) at the dosages indicated. Post-infusion rCBF, determined at 15 min intervals for the next 105 min, is expressed as the mean of these determinations (solid columns). Data are expressed as percentage of baseline rCBF prior to vessel occlusion (mean ± s.e.mean with number of animals in parentheses). rCBF 2 and 5 min after CCA/MCA occlusion was 20 ± 5.2% for the saline group, and did not differ between treatment groups. $*P<0.05$ and $**P<0.01$ as compared to pre-infusion rCBF, determined by paired Student's t test performed on percentage values.

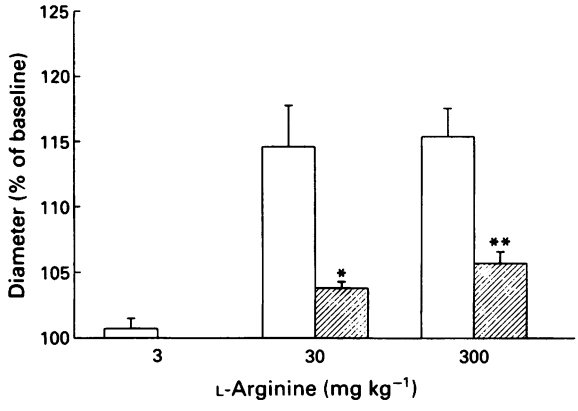

Figure 2 Dilatation of pial vessels following L-arginine infusion was significantly reduced by topical N^G-nitro-L-arginine methyl ester (L-NAME, 1 µM) in SD rats. Baseline diameters were measured after an equilibration period of 30 min through a closed cranial window. Pial vessel diameters (1–3 arterioles per animal) were measured 30 min following L-arginine infusion (3, 30, or 300 mg kg^{-1}, i.v. over 10 min; open columns). In some animals, L-NAME (1 µM) was applied topically 20 min before L-arginine infusion (hatched columns). Data are expressed as percentage of baseline diameter. L-NAME superfusion (1 µM) by itself did not change pial vessel diameter (100 ± 1.2%, $n = 7$). Baseline diameters (mean ± s.e.mean in µm) were 42 ± 2.7 [3 mg kg^{-1}, $n = 3$], 49 ± 3.7 [30 mg kg^{-1}, $n = 5$], and 40 ± 4.1 [300 mg kg^{-1}, $n = 5$] for L-arginine alone; 34 ± 7.0 [30 mg kg^{-1}, $n = 3$] and 40 ± 9.9 [300 mg kg^{-1}, $n = 4$] for L-arginine with L-NAME pretreatment. Error bars denote s.e.mean. $*P<0.05$ and $**P<0.01$, as compared to L-arginine infusion alone by unpaired Student's t test.

(Jacewicz *et al.*, 1992), appear sufficient to explain the previously reported decrease in infarct size following L-arginine infusion. The fact that pial arterioles dilated in response to L-arginine suggests that NO synthase (presumably within the endothelium or within innervating parasympathetic fibres, Nozaki *et al.*, 1992) is not saturated with substrate under resting conditions in the cerebral circulation, and that L-arginine-induced rCBF increases may not be unique to SHR. As indirect evidence, preliminary data indicate that L-arginine infusion also decreases infarct size in SD rats (unpublished observation).

The results described here may seem unexpected in view of published reports showing neurotoxic effects of NO (Dawson *et al.*, 1991) and cytoprotective effects of NO synthase inhibitors in stroke models (Nowicki *et al.*, 1991; Buisson *et al.*, 1992). However, methodological differences between our experiments and those of Nowicki *et al.* and Buisson *et al.* are noteworthy inasmuch as rodents used in their experiments were ventilating spontaneously. Hence, $PaCO_2$ values (not reported by them) were almost certainly high, and baseline blood flows correspondingly high. Alternatively, or in addition, the discrepant results may reflect differences between the effects of NO at the vessel wall versus brain parenchyma *per se* during cerebral ischaemia. These controversies notwithstanding, the findings described here raise the possibility that intravenous administration of L-arginine or other NO precursors may be useful for acutely increasing rCBF during ischaemic strokes in man.

These studies were supported by NINCDS No. NS10828 to the MGH Interdepartmental Stroke Program Project (MAM).

References

BRINT, S., JACEWICZ, M., KIESSLING, M., TANABE, J. & PULSINELLI, W.A. (1988). Focal brain ischemia in the rat: methods for reproducible neocorticol infarction using tandem occlusion of the distal middle cerebral and ipsilateral common carotid arteries. *J. Cereb. Blood Flow Metab.*, **8**, 474–485.

BUISSON, A., PLOTKINE, M. & BOULU, R.G. (1992). The neuroprotective effect of a nitric oxide inhibitor in a rat model of focal cerebral ischaemia. *Br. J. Pharmacol.*, **106**, 766–767.

DAWSON, V.L., DAWSON, T.M., LONDON, E.D., BREDT, D.S. & SNYDER, S.H. (1991). Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci, U.S.A.*, **88**, 6368–6371.

JACEWICZ, M., BRINT, S., TANABE, J., WANG, X. & PULSINELLI, W.A. (1990). Nimodipine pretreatment improves cerebral blood flow and reduces brain edema in conscious rats subjected to focal cerebral ischaemia. *J. Cereb. Blood Flow Metab.*, **10**, 903–913.

JACEWICZ, M., TANABE, J. & PULSINELLI, W.A. (1992). The CBF threshold and dynamics for focal cerebral infarction in spontaneously hypertensive rats. *J. Cereb. Blood Flow Metab.*, **12**, 359–370.

LEVASSEUR, J.E., WEI, E.P., RAPER, A.J., KONTOS, H.A. & PATTERSON, J.L. (1975). Detailed description of a cranial window technique for acute and chronic experiments. *Stroke*, **6**, 308–317.

KOKETSU, N., MOSKOWITZ, M.A., KONTOS, H.A., YOKOTO, M. & SHIMIZU, T. (1992). Chronic parasympathetic sectioning decreases regional cerebral blood flow during hemorrhagic hypotension and increases infarct size after middle cerebral artery occlusion in spontaneously hypertensive rats. *J. Cereb. Blood Flow Metab.*, **12**, 613–620.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MORIKAWA, E., HUANG, Z. & MOSKOWITZ, M.A. (1992). L-Arginine decreases infarct size caused by middle cerebral artery occlusion in spontaneously hypertensive rats. *Am. J. Physiol.*, (in press).

NOWICKI, J.P., DUVAL, D., POIGNET, H. & SCATTON, B. (1991). Nitric oxide mediates neuronal death after focal cerebral ischemia in the mouse. *Eur. J. Pharmacol.*, **204**, 339–340.

NOZAKI, K., MOSKOWITZ, M.A., MAYNARD, K.I., KOKETSU, N., DAWSON, T.M., BREDT, D.S. & SINDER, S.H. (1992). Possible origins and distribution of immunoreactive nitric oxide synthase – containing nerve fibers in rat and human cerebral arteries. *J. Cerebral Blood Flow Metab.*, (in press).

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

(Received August 6, 1992
Accepted September 2, 1992)

Br. J. Pharmacol. (1992), **107**, 908–909

SPECIAL REPORT

Protein kinase C-independent sensitization of contractile proteins to Ca^{2+} in α-toxin-permeabilized smooth muscle cells from the guinea-pig stomach

[1]Kazuhiko Oishi, *Mitsuo Mita, Teruaki Ono, *Takao Hashimoto & Masaatsu K. Uchida

Department of Molecular Pharmacology and *Department of Pharmacology, Meiji College of Pharmacy, 1-35-23 Nozawa, Setagaya-ku, Tokyo 154, Japan

Involvement of protein kinase C in receptor-operated Ca^{2+} sensitization of cell shortening was investigated by use of α-toxin-permeabilized smooth muscle cells from the fundus of the guinea-pig. Most of the isolated cells responded to 0.6 μM Ca^{2+} with a maximal shortening to approximately 65% of the resting cell length. Addition of acetylcholine (ACh) at a maximal concentration (10 μM) resulted in a marked decrease in the concentration of Ca^{2+} required to trigger a threshold response from 0.6 μM to 0.2 μM. The augmentation of Ca^{2+} sensitivity by ACh was not inhibited by specific protein kinase C inhibitors, calphostin C and K-252b at a concentration of 1 μM. These findings suggest that protein kinase C is not involved in the muscarinic receptor-operated augmentation of Ca^{2+} sensitivity.

Keywords: Ca^{2+} sensitivity; cell shortening; protein kinase C; smooth muscle cells; permeabilization; muscarinic receptor

Introduction We have recently shown the augmentation of Ca^{2+} sensitivity by stimulation of muscarinic receptors or guanosine 5'-triphosphate (GTP)-binding proteins in α-toxin-permeabilized single smooth muscle cells from the fundus of the guinea-pig stomach (Ono *et al.*, 1992). The involvement of protein kinase C in the increase of Ca^{2+} sensitivity has been proposed in studies on permeabilized smooth muscle tissues (Nishimura *et al.*, 1988; Itoh *et al.*, 1988). We, therefore, investigated the involvement of protein kinase C in receptor-operated Ca^{2+} sensitization of cell shortening in α-toxin-permeabilized single smooth muscle cells by use of specific protein kinase C inhibitors, calphostin C and K-252b.

Methods Cell isolation, measurement of cell shortening, and cell permeabilization were as described previously by Ono *et al.* (1992). Data shown in the figures are normalized by taking the baseline and the maximal shortening of each cell as 0 and 100%, respectively. Data are expressed as means ± s.e.means. Statistical significance was determined by Student's *t* test (paired). Calphostin C and K-252b were purchased from Kyowa Hakko Co. (Tokyo, Japan).

Results Most of the isolated cells responded to 0.6 μM Ca^{2+} with a maximal shortening (Figures 1a and 2a). Addition of acetylcholine (ACh) at a maximal concentration (10 μM) resulted in a marked decrease in the concentration of Ca^{2+} required to trigger a threshold response from 0.6 μM to 0.2 μM (Figures 1b and 2b). These results were consistent with previous observations (Ono *et al.*, 1992). When 1 μM calphostin C was present in the perfusion solution, ACh induced the sensitization to Ca^{2+} without any influence on the threshold concentration of Ca^{2+} (Figures 1a and 1b). The same finding was obtained with 1 μM K-252b (Figures 2a and 2b). These results suggest that protein kinase C is not involved in the muscarinic receptor-operated augmentation of Ca^{2+} sensitivity.

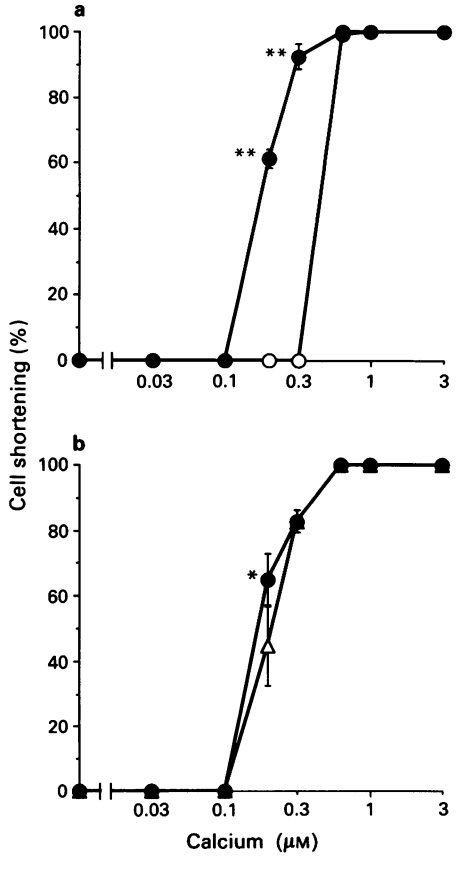

Figure 1 Effect of calphostin C on acetylcholine (ACh)-induced Ca^{2+} sensitization of cell shortening in α-toxin-permeabilized single smooth muscle cells. (a) The first and second shortenings were evoked by 10 nM to 3 μM Ca^{2+} in the absence (○) and presence (●) of 10 μM ACh plus 1 μM calphostin C, respectively. (b) The first and second shortenings were evoked by 10 nM to 3 μM Ca^{2+} plus 10 μM ACh in the absence (△) and presence (●) of 1 μM calphostin C, respectively. $n = 3$. $*P < 0.05$; $**P < 0.01$.

[1] Author for correspondence.

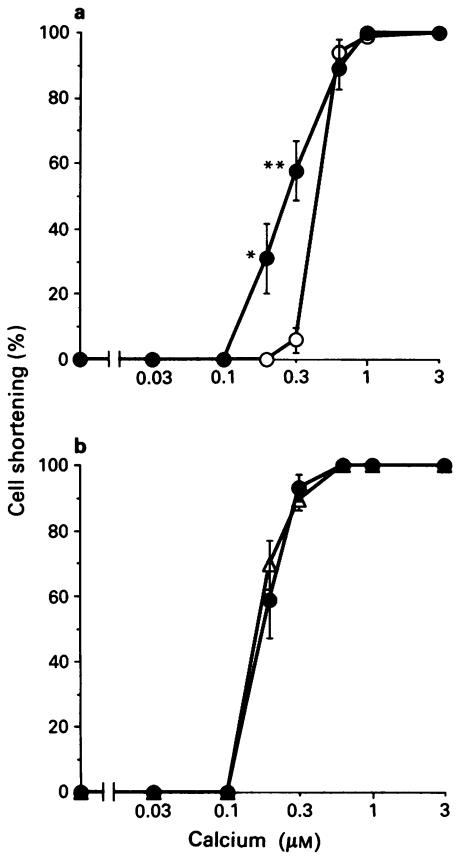

Figure 2 Effect of K-252b on acetylcholine (ACh)-induced Ca^{2+} sensitization of cell shortening in α-toxin-permeabilized single smooth muscle cells. (a) The first and second shortenings were evoked by 10 nM to 3 μM Ca^{2+} in the absence (○) and presence (●) of 10 μM ACh plus 1 μM K-252b, respectively. (b) The first and second shortenings were evoked by 10 nM to 3 μM Ca^{2+} plus 10 μM ACh in the absence (△) and presence (●) of 1 μM K-252b, respectively. $n = 3$. $^*P < 0.05$; $^{**}P < 0.01$.

Discussion In a previous paper, we have shown that GTP-binding proteins regulate the sensitivity of the contractile proteins to Ca^{2+} (Ono *et al.*, 1992). Two major superfamilies of GTP-binding proteins are present in mammalian tissues. One is the superfamily of heterotrimeric GTP-binding proteins which are now known to couple cell surface receptors to target enzymes such as phospholipase C (Freissmuth *et al.*, 1989). It has been shown that stimulation of various cell surface receptors including m1, m3, and m5 subtypes of muscarinic receptor leads to an activation of phospholipase C *via* a GTP-binding protein (Nathanson, 1987). We have shown here that specific protein kinase C inhibitors had no effect on the ACh-induced Ca^{2+} sensitization in the isolated single smooth muscle cell system. We previously showed that inositol 1,4,5-trisphosphate ($InsP_3$)-induced Ca^{2+} release is not involved in muscarinic receptor-operated shortening of the permeabilized cells (Ono *et al.*, 1992). Taken together, it is likely that heterotrimeric GTP-binding proteins which couple muscarinic receptors to phospholipase C are not involved in the augmentation of Ca^{2+} sensitivity.

In addition to such heterotrimeric GTP-binding proteins, the superfamily of monomeric GTP-binding proteins with molecular mass in the 20–30 kDa range has been implicated in the pathways of stimulus-response coupling. It has been shown that *rho* p21 families, which are thought to control cytoskeletal organization (Paterson *et al.*, 1990), are one of the most abundant small GTP-binding proteins present in bovine aortic smooth muscle (Kawahara *et al.*, 1990). Hirata *et al.* (1992) have clearly demonstrated that *rho* p21 is involved in the GTPγS-enhanced Ca^{2+} sensitivity of contraction in the rabbit mesenteric arterial smooth muscle and mentioned that *rho* p21 may be regulated by signal transduction pathways including protein kinase C-Ca^{2+} systems. Such a small GTP-binding protein could be another candidate for regulation of Ca^{2+} sensitivity, if located in the smooth muscle cells of the guinea-pig stomach. However, the possibility of regulation of *rho* p21 through protein kinase C system is ruled out by our present findings. Another novel pathway which regulates *rho* p21 remains to be clarified.

References

FREISSMUTH, M., CASEY, P.J. & GILMAN, A.G. (1989). G proteins control diverse pathways of transmembrane signaling. *FASEB J.*, **3**, 2125–2131.

HIRATA, K., KIKUCHI, A., SASAKI, T., KURODA, S., KAIBUCHI, K., MATSUURA, Y., SEKI, H., SAIDA, K. & TAKAI, Y. (1992). Involvement of *rho* p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. *J. Biol. Chem.*, **267**, 8719–8722.

ITOH, T., KUBOTA, Y. & KURIYAMA, H. (1988). Effects of a phorbol ester on acetylcholine-induced Ca^{2+} mobilization and contraction in the porcine coronary artery. *J. Physiol.*, **397**, 401–419.

KAWAHARA, Y., KAWATA, M., SUNAKO, M., ARAKI, S., KOIDE, M., TSUDA, T., FUKUZAKI, H. & TAKAI, Y. (1990). Identification of a major GTP-binding protein in bovine aortic smooth muscle cytosol as the *rho*A gene product. *Biochem. Biophys. Res. Commun.*, **170**, 673–683.

NATHANSON, N.M. (1987). Molecular properties of the muscarinic acetylcholine receptor. *Annu. Rev. Neurosci.*, **10**, 195–236.

NISHIMURA, J., KOLBER, M. & VAN BREEMEN, C. (1988). Norepinephrine and GTPγS increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. *Biochem. Biophys. Res. Commun.*, **157**, 677–683.

ONO, T., MITA, M., SUGA, O., HASHIMOTO, T., OISHI, K. & UCHIDA, M.K. (1992). Receptor-coupled shortening of α-toxin-permeabilized single smooth muscle cells from the guinea-pig stomach. *Br. J. Pharmacol.*, **106**, 539–543.

PATERSON, H.F., SELF, A.J., GARRETT, M.D., JUST, I., AKTORIES, K. & HALL, A. (1990). Microinjection of Recombinant $p21^{rho}$ induces rapid changes in cell morphology. *J. Cell Biol.*, **111**, 1001–1007.

(Received September 7, 1992
Accepted September 16, 1992)

Br. J. Pharmacol. (1992), **107**, 910–911

SPECIAL REPORT

The local intracoronary administration of methylene blue prevents the pronounced antiarrhythmic effect of ischaemic preconditioning

Agnes Vegh, Julius Gy Papp, Laszlo Szekeres & [1]*James Parratt

Department of Pharmacology, Albert Szent-Gyorgyi Medical University, Dom ter 12, Szeged, Hungary and *Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW

Short periods of coronary artery occlusion (2 × 5 min) markedly reduce the severity of arrhythmias and the changes in ST-segment elevation and in the degree of inhomogeneity of conduction during a subsequent 25 min occlusion of the left anterior descending coronary artery in anaesthetized dogs. These changes were completely reversed if methylene blue (5 mg min^{-1}) was infused into a side branch of the coronary artery throughout both the preconditioning and prolonged occlusions. These results suggest that the pronounced antiarrhythmic effects of preconditioning result from activation of guanylyl cyclase and result in increased levels of guanosine 3′:5′-cyclic monophosphate.

Keywords: Methylene blue; guanylyl cyclase; cyclic GMP; ischaemic preconditioning; ventricular arrhythmias; reperfusions; nitric oxide

Introduction When a prolonged occlusion of a coronary artery is preceded by either one or more shorter periods of occlusion of that same artery, both the degree of ischaemic damage (reviewed by Walker & Yellon, 1992) and the severity of life-threatening ventricular arrhythmias (Vegh *et al.*, 1990; 1992a) resulting from the prolonged occlusion are dramatically reduced. This phenomenon is known as preconditioning of the ischaemic myocardium. The mechanisms of this protection are unclear but one possibility is that the preconditioning stimulus 'primes' the heart to release 'endogenous myocardial protective substances' which may include adenosine (Liu *et al.*, 1991) and prostanoids (Vegh *et al.*, 1990). Recently, we have shown that the antiarrhythmic effects of preconditioning are attenuated by inhibition of the L-arginine/nitric oxide (NO) pathway (Vegh *et al.*, 1992b), suggesting that the generation of NO contributes to this protection. Since NO acts by stimulating soluble guanylyl cyclase and elevating guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels, another way of examining the possible role of NO in this protection is to inhibit the activation of this enzyme. We now describe experiments in which we have infused methylene blue (Martin *et al.*, 1985) directly into a small branch of the left coronary artery in anaesthetized dogs to examine whether this modified the protective (antiarrhythmic) effect of preconditioning.

Methods These have been described in detail elsewhere (Vegh *et al.*, 1990; 1991; 1992a,b). In brief, we used mongrel dogs with a weight in excess of 17 kg. They were anaesthetized with a mixture of chloralose and urethane (60 and 200 mg kg^{-1} respectively, given i.v.) and ventilated with room air. Following a left thoracotomy the anterior descending branch of the left coronary artery (LAD) was prepared for occlusion just proximal to the first main diagonal branch. We used a composite electrode to assess inhomogeneity of conduction and unipolar electrodes to measure epicardial ST-segment changes. A small branch of the LAD coronary artery immediately proximal to the proposed occlusion site (Vegh *et al.*, 1991) was catheterized for the local administration of methylene blue.

Forty four dogs were used in this study. Of these, 21 animals served as controls. In these, after stabilization following surgery, the LAD coronary artery was occluded for 25 min after which the ischaemic area was reperfused. Fourteen dogs were preconditioned as previously described (Vegh *et al.*, 1990; 1992a) by two 5 min coronary artery occlusions, with a 20 min reperfusion period between, followed 20 min after the second preconditioning occlusion by a prolonged (25 min) occlusion. The ischaemic area was then reperfused. A further group of 9 dogs were subjected to preconditioning as described above but methylene blue (MB; 10 mg ml^{-1}), infused at a rate of 0.5 ml min^{-1}, was infused throughout both preconditioning occlusions and throughout the prolonged occlusion. The protocol was to infuse MB for 5 or 10 min before each preconditioning occlusion, throughout the 5 min occlusion period and for 5 min after the start of reperfusion. MB was infused again for 5 min prior to the prolonged occlusion and throughout that occlusion period. The total doses administered were therefore 100 mg (for the first preconditioning occlusion), 75 mg (for the second preconditioning occlusion) and 150 mg for the prolonged occlusion.

Epicardial electrograms, the output from the composite electrode, systemic arterial and left ventricular (LV) pressures (Statham P23Dp transducers), and blood flow in the circumflex coronary artery (Statham SP 222 electromagnetic flow meter) were recorded on a Medicor R81 recorder. The assessment of ventricular arrhythmias during ischaemia and reperfusion, and the statistical tests used, were as outlined by Vegh *et al.* (1992a).

Results Local intracoronary administration of MB in the doses described above had no effect on arterial blood pressure (133 ± 9 mmHg systolic and 93 ± 5 mmHg diastolic before the infusions and 141 ± 10 mmHg and 93 ± 5 mmHg respectively at the end of the infusion period). Heart rate was also unchanged (164 ± 15 to 161 ± 11 beats min^{-1}). There were also no changes in LVEDP (7.2 ± 2.5 to 8.3 ± 0.3 mmHg), in diastolic coronary blood flow (46 ± 7 to 43 ± 7 ml min^{-1}) or in the output from the composite electrode (38 ± 6 to 38 ± 6 ms) after MB administration.

The effects on ventricular arrhythmias during the occlusion period and following reperfusion are shown in Figure 1. Preconditioning markedly reduced the total number of ventricular premature beats (VPBs), the episodes of ventricular tachycardia (VT), the number of animals exhibiting VT and

[1] Author for correspondence.

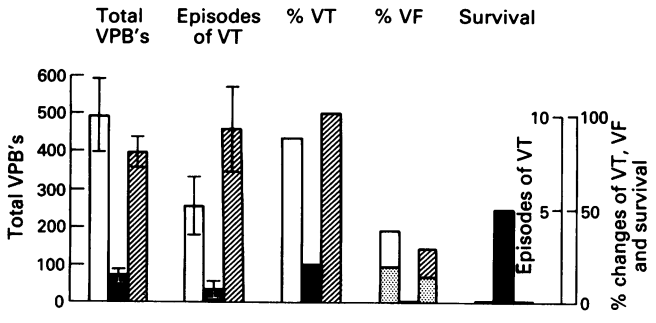

Figure 1 The total number of ventricular premature beats (VPB's), the number of episodes of ventricular tachycardia (VT), the incidences of VT and ventricular fibrillation (VF) and the survival from the combined-reperfusion insult in dogs subjected to a 25 min occlusion of the left anterior descending coronary artery. Preconditioning (solid columns, $n = 14$) markedly reduces the severity of these arrhythmias but this marked protection is not seen if preconditioning is carried out in the presence of methylene blue (hatched columns, $n = 7$). The incidence of VF is given both as total incidence throughout the 25 min occlusion period and during the first 5 min (stippled columns). Open columns = controls, $n = 21$.

ventricular fibrillation (VF) and the incidence of VF on reperfusion; 50% of these preconditioned animals survived the combined ischaemia-reperfusion insult. This marked protection afforded by preconditioning was completely reversed by MB (Figure 1) and there were no survivors from the combined ischaemia-reperfusion procedure.

The marked attenuation of ischaemia-induced changes in epicardial ST-segment elevation and in the degree of inhomogeneity of conduction within the ischaemic area (Vegh *et al.* 1992a; 1992b) were also reversed by MB. For example, ST-segment elevation at 1, 3, 5 and 10 min after the start of the prolonged occlusion in the control dogs was 5.2 ± 0.6, 13.4 ± 0.9, 13.6 ± 0.8 and 16.0 ± 1.0 mV respectively. In the preconditioned group the respective values at these times were 1.4 ± 0.4, 6.4 ± 0.7, 8.2 ± 0.8 and 10.0 ± 1.0 mV whereas in the preconditioned dogs given MB they were 2.1 ± 0.4, 8.3 ± 1.9, 12.5 ± 1.4 and 13.0 ± 1.5 mV. With inhomogeneity of conduction the changes at 1, 3 and 5 min following the start of occlusion were 20 ± 3, 80 ± 9 and 90 ± 10 ms in the controls, 0, 15 ± 4 and 36 ± 6 ms in the preconditioned group and 10 ± 7, 72 ± 10 and 77 ± 10 ms in the preconditioned dogs administered MB. The differences for each of these values between the preconditioned group and the controls were significant at each time at a level of at least $P < 0.05$ but there were no significant differences between the control dogs and those preconditioned dogs given MB.

Discussion The fact that when given throughout both the preconditioning periods and the prolonged occlusion, MB completely prevented the pronounced antiarrhythmic effects of preconditioning, suggests that it is the stimulation of soluble guanylyl cyclase that is responsible for this aspect of the cardioprotection afforded by preconditioning. It also indirectly supports the concept that nitric oxide is involved in this protection (Vegh *et al.*, 1992b). The abolition of the antiarrhythmic effect of preconditioning by MB cannot be explained by haemodynamic changes or by alterations in coronary blood flow. MB, in the doses used, was devoid of effects on blood pressure, heart rate, coronary blood flow and myocardial contractility, at least as assessed for measurements for LVEDP and LV$dP/dt$, neither of which was changed by the compound.

We suggest that this study throws light on the mechanisms of the pronounced antiarrhythmic effect of preconditioning. One possibility under consideration is that the ischaemia associated with the brief preconditioning occlusions results in the release of some activator of guanylyl cyclase such as nitric oxide. Certainly some NO 'donors' such as molsidomine and nitroglycerin are active against early ischaemia-induced ventricular arrhythmias. The 'priming' procedure of preconditioning might result in increased levels of cyclic GMP such that these were elevated before the onset of the prolonged occlusion. There is already evidence, albeit in a different model, for an antiarrhythmic effect of lipid soluble analogues of cyclic GMP which are also more resistant to breakdown by phosphodiesterase. Billman (1990) found, in conscious dogs with a left ventricular free wall infarct subjected to a combination of exercise and ischaemia (left circumflex artery occlusion), that the incidence of VF was drastically reduced following the intravenous administration of either 8-bromo cyclic GMP or dibutyryl cyclic GMP (from 17/17 in the control dogs to 1/14). The mechanisms of such a myocardial effect of elevated cyclic GMP are unclear but possibilities include a reduction in calcium influx and/or of intracellular calcium release or activation of sarcolemmal potassium channels (reviewed recently by Henderson *et al.*, 1992). It would be important to examine directly how preconditioning alters cyclic GMP, and in which cells, and to determine whether selective inhibition of the phosphodiesterase enzyme responsible for its breakdown is, like preconditioning, a powerful antiarrhythmic procedure.

This work was supported by the Wellcome Trust (travel and subsistence grant) and by the Hungarian Academy of Sciences. We thank Rozsa Bite for superb technical assistance.

References

BILLMAN, G.E. (1990). Effect of carbachol and cyclic GMP on susceptibility to ventricular fibrillation. *FASEB J.*, **4**, 1668–1673.

HENDERSON, A.H., LEWIS, M.J., SHAH, A.M. & SMITH, J.A. (1992). Endothelium, endocardium, and cardiac contraction. *Cardiovasc. Res.*, **26**, 305–308.

LIU, G.S., THORNTON, J., VAN WINKLE, D.M., STANLEY, A.W.H., OLSSON, R.A. & DOWNEY, J.M. (1991). Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart. *Circulation*, **84**, 350–356.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by haemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

VEGH, A., SZEKERES, L. & PARRATT, J.R. (1990). Protective effects of preconditioning of the ischaemic myocardium involve cyclooxygenase products. *Cardiovasc. Res.*, **12**, 1020–1022.

VEGH, A., SZEKERES, L. & PARRATT, J.R. (1991). Local intracoronary infusions of bradykinin profoundly reduce the severity of ischaemia-induced arrhythmias in anaesthetised dogs. *Br. J. Pharmacol.*, **104**, 294–295.

VEGH, A., KOMORI, S., SZEKERES, L. & PARRATT, J.R. (1992a). Antiarrhythmic effects of preconditioning in anaesthetised dogs and rats. *Cardiovasc. Res.*, **26**, 487–495.

VEGH, A., SZEKERES, L. & PARRATT, J.R. (1992b). Preconditioning of the ischaemic myocardium; involvement of the L-arginine nitric oxide pathway. *Br. J. Pharmacol.*, **107**, (in press).

WALKER, D.M. & YELLON, D.M. (1992). Ischaemic preconditioning: from mechanisms to exploitation. *Cardiovasc. Res.*, **26**, 734–739.

(Received September 10, 1992
Accepted September 24, 1992)

Br. J. Pharmacol. (1992), **107**, 912–918

Discrimination between ET_A- and ET_B-receptor-mediated effects of endothelin-1 and [$Ala^{1,3,11,15}$]endothelin-1 by BQ-123 in the anaesthetized rat

[1]Marc Bigaud & John T. Pelton

Marion Merrell Dow Research Institute, 16 rue d'Ankara, 67084 Strasbourg, France

1 The influence of BQ-123 (a selective ET_A-receptor antagonist) on the haemodynamic response elicited by endothelin-1 (ET-1) and [$Ala^{1,3,11,15}$]ET-1 (a selective ET_B-receptor agonist) was studied in anaesthetized rats instrumented with ultrasonic Doppler flow probes on the carotid, coeliac, mesenteric, renal and iliac arteries.

2 BQ-123 alone (1.6 μmol kg^{-1}, i.v.) induced a decrease in femoral mean arterial pressure (AP), accompanied by a systemic vasodilatation. The response was maximal after 3 min and then returned slowly to baseline. None of these effects was observed after a 0.016 μmol kg^{-1} dose of BQ-123.

3 ET-1 (1 nmol kg^{-1}, i.v.) induced a biphasic response characterized by a transient initial decrease in AP accompanied by regional vasodilatation (mainly in the carotid and iliac beds) and by immediate mesenteric and renal vasoconstrictions. This was followed, within 1 min, by a marked and prolonged increase in AP accompanied by systemic vasoconstriction. Pretreatment with BQ-123 (1.6 μmol kg^{-1}, i.v., 8 min before ET-1) increased and prolonged the vasodilator effect of ET-1 (mainly in the carotid, coeliac, mesenteric and iliac beds) and reduced its systemic vasoconstrictor effects with marked regional differences (the coeliac, mesenteric and renal beds being poorly affected).

4 [$Ala^{1,3,11,15}$]ET-1 (3 nmol kg^{-1}, i.v.) induced an initial and marked decrease in AP accompanied by regional vasodilatation (mainly in the carotid, coeliac and iliac beds) and by mesenteric and renal vasoconstrictions. This was followed, within 5 min, by a small increase in AP and systemic vasoconstriction. All these effects were dose-dependent. Pretreatment with BQ-123 (1.6 μmol kg^{-1}; 8 min before ET-1) did not modify the early effect of [$Ala^{1,3,11,15}$]ET-1, but abolished its secondary vasoconstrictor effect except in the mesenteric bed.

5 This study demonstrates that pretreatment with BQ-123 not only reduced a large part of the sustained vasoconstrictor activity of ET-1, suggesting the involvement of ET_A-receptors, but also enhanced the early vasodilator activity of ET-1 revealing a functional antagonism between the two effects. The vasodilator effect of [$Ala^{1,3,11,15}$]ET-1 was not affected by BQ-123 and ET-1 induced a similar vasodilatation, that was potentiated by BQ-123, suggesting the involvement of ET_B-receptors in this vasodilator response. Marked regional differences were however observed which might be partly related to different levels of functional antagonism between ET_B- and ET_A-mediated effects, but differences in receptor types, or subtypes, cannot be excluded, mainly in the mesenteric and renals beds.

Keywords: Haemodynamics; endothelin-1; [$Ala^{1,3,11,15}$]endothelin-1; BQ-123; endothelin antagonist; anaesthetized rats

Introduction

Endothelin-1 (ET-1) has been shown to induce a biphasic haemodynamic response characterized by a marked initial and transient systemic vasodilatation followed by a potent and sustained systemic vasoconstriction (Wright & Fozard, 1988; Yanagisawa *et al.*, 1988; Gardiner *et al.*, 1989; 1990; Le Monnier De Gouville *et al.*, 1990b). This dual activity of ET-1 (vasodilator and vasoconstrictor) is now well established but, despite numerous studies, the mechanisms involved are not well understood (Masaki *et al.*, 1991).

Two types of endothelin receptors have been identified and cloned. One type, termed ET_A (Arai *et al.*, 1990) has a higher affinity for ET-1 compared to the other members of the endothelin family. The other, termed ET_B (Sakurai *et al.*, 1990) has a similar affinity for all members of the endothelin family. Both receptor types are widely distributed in non-cardiovascular and cardiovascular tissues (Masaki *et al.*, 1991), with vascular smooth muscle cells expressing the ET_A type (Arai *et al.*, 1990; Lin *et al.*, 1991) and the vascular endothelial cells expressing the ET_B type (Sakurai *et al.*, 1990; Sakamoto *et al.*, 1991). The functions of these two distinct endothelin receptors remain to be elucidated. However, because of their interesting localization within the vascular wall, it has been suggested that the ET_A type might be responsible for the direct vasoconstrictor activity of ET-1 and ET_B type for its vasodilator activity through the release of endothelium-derived mediators (Vane, 1990; Masaki, 1991). Such a suggestion was, until recently, difficult to verify because of the lack of specific endothelin receptor antagonists.

Recently, a cyclic pentapeptide, isolated from a bacterial fermentation broth and named BE-18257B (*cyclo*[D-Glu-L-Ala-D-*allo*-Ile-L-Leu-D-Trp]), was described as a weak but selective antagonist of ET_A-receptors (Ihara *et al.*, 1991). A significant inhibition of ET-1-induced pressor responses in conscious rats was also observed with a relatively high dose of BE-18257B (50 mg kg^{-1}, i.p. 1 h before administration of ET-1 i.v.). A more water soluble analogue of this compound, BQ-123 (*cyclo*[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]), has been described as an even more potent and selective ET_A-receptor antagonist, with an IC_{50} in binding experiments, of 7.3 nM for ET_A-receptors, in porcine aortic smooth muscle cells, and a 2,500 fold selectivity for ET_A relative to ET_B-receptors in porcine cerebellum (Ihara *et al.*, 1992; Nakamichi *et al.*, 1992). BQ-123 appears to be a competitive antagonist of ET-1-induced contractions of isolated porcine coronary artery ($pA_2 = 7.4$), although it is a non-competitive antagonist

[1] Author for correspondence.

at ET_A-receptors of SK-N-MC human neuroblastoma cells (Hiley *et al.*, 1992). Furthermore, at a dose of 1 mg kg^{-1}, i.v., BQ-123 antagonized ET-1-induced hypertension in the conscious rat (Ihara *et al.*, 1992).

The main objective of the present work was to discriminate between the ET_A-receptor and ET_B-receptor-mediated effects of ET-1 on blood pressure and regional blood flows of anaesthetized rats, by pretreating the animals with the ET_A-receptor antagonist, BQ-123. The effects of BQ-123 on the haemodynamic responses induced by ET-1 were also compared to the effects induced by [Ala^{1,3,11,15}]ET-1, a linear ET-1 analogue, described as an ET_B-selective agonist (Hiley *et al.*, 1990; Pelton & Miller, 1991; Saeki *et al.*, 1991) with an IC_{50}, in binding experiments, of 0.33 nM for ET_B-receptors in porcine cerebellum and a 6,700 fold selectivity for ET_B relative to ET_A-receptors in vascular smooth muscle cells (Nakamichi *et al.*, 1992).

Part of this work was communicated to the December 1991 Meeting of the British Pharmacological Society (Bigaud, 1992).

Methods

Male, Sprague-Dawley rats (300–400 g) were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p., supplemented as required) and their trachea cannulated to allow artifical ventilation (10 ml kg^{-1}, 60 strokes min^{-1}). Catheters were inserted into the right femoral artery, for mean arterial blood pressure and heart rate measurements, and the right femoral vein for i.v. injections. Miniaturized pulsed Doppler probes (Haywood *et al.*, 1981) were placed around the right carotid, coeliac, superior mesenteric, left renal and iliac arteries. The incisions were then sutured and the preparation allowed to stabilize for 1 h.

Phasic and mean Doppler shift signals, regarded as a good index of blood velocity (flow) (Haywood *et al.*, 1981), were measured with a VF-1 Pulsed Doppler Flowmeter from Crystal Biotech (U.S.A.). Percentage changes in regional vascular conductances were calculated from mean regional Doppler shift signals divided by the mean systemic AP (Gardiner *et al.*, 1990). The haemodynamic parameters were monitored continuously with a TA2000 Gould stripchart recorded coupled to a data-acquisition system (Dataflow from Crystal Biotech, U.S.A.).

In one group of rats, ET-1 (nmol kg^{-1}) was administered as an i.v. bolus injection (0.01 ml 100g^{-1}, flushed with 0.5 ml saline). This dose has been previously considered as just submaximal (Fozard & Part, 1992) and, in preliminary experiments for the present study, it was observed that 1 nmol kg^{-1} was the maximal tolerated dose of ET-1 and that 3 nmol kg^{-1} was lethal in 50% of the animals. Two other groups of rats were randomly pretreated, 8 min before i.v. injection of ET-1 (1 nmol kg^{-1}), with 1 mg kg^{-1} (1.6 μmol kg^{-1} as i.v. bolus injections) of BQ-123, a dose previously shown to antagonize the pressor effect of ET-1 (Ihara *et al.*, 1992), or with a 100 times lower dose (0.016 μmol kg^{-1}, as i.v. bolus injections) which was expected to be without any influence and used as a control. In a fourth group, 0.1, 0.3, 1, 3 or 10 nmol kg^{-1} of [Ala^{1,3,11,15}]ET-1 were randomly administered as i.v. bolus injections (0.01 ml 100 g^{-1}, flushed with 0.5 ml saline) in order to construct a dose-response curve. The dose of 3 nmol kg^{-1} of [Ala^{1,3,11,15}]ET-1, which elicited maximal depressor and pressor responses, was administered to a fifth group of rats pretreated with 1 mg kg^{-1} of BQ-123 (1.6 μmol kg^{-1}, as i.v. bolus injections 8 min before i.v. injection of [Ala^{1,3,11,15}]ET-1). For each experiment, parameters were recorded for 30 min following ET-1 administration. To avoid tachyphylaxis (Le Monnier de Gouville *et al.*, 1990a), each rat received only one dose of ET-1 or [Ala ^{1,3,11,15}]ET-1. In preliminary experiments, it was observed that prior injections of saline (0.01 ml 100 g^{-1} followed by 0.5 ml) had no influence on the haemodynamic parameters measured, nor on the responses induced by ET-1 and [Ala^{1,3,11,15}]ET-1.

Peptides

Human/porcine ET-1 was purchased from Peptide Institute (Osaka, Japan) and dissolved in distilled water to give 10^{-4} M stock solutions. The exact concentrations of these solutions were checked by absorbance spectrophotometry at 280 nm using an extinction coefficient of 7245 M^{-1} cm^{-1}. The stock solutions were then aliquoted, lyophilized and stored at − 20°C. The peptide was redissolved and diluted in saline on the day of the experiment. [Ala^{1,3,11,15}]ET-1 was purchased from Neosystem Laboratory (France), and solutions were made daily in saline. The exact concentrations of these solutions were checked by absorbance spectrophotometry at 280 nm using an extinction coefficient of 6890 M^{-1} cm^{-1}. The compound BQ-123 was synthesised at Marion Merrell Dow Strasbourg by standard solid phase peptide synthesis techniques (Merrifield, 1963; Atkinson & Pelton, 1992), stored at − 20°C and diluted in saline before each experiment. The exact concentration of BQ-123 in solution was checked by absorbance spectrophotometry at 280 nm with an extinction coefficient of 5600 M^{-1} cm^{-1}.

Statistical analysis

All data are expressed as mean values ± s.e.mean of n individual determinations. Student's t test for paired comparisons was used to evaluate the statistical significance between basal values for the haemodynamic parameters, measured before and after BQ-123 (Table 1). Changes (%) relative to baseline were analysed by Friedman two-way analysis of variance (programme SYSTAT). Student's t test for unpaired comparisons was used to evaluate the statistical significance between group means. P values of less than 0.05 were taken as significant.

Results

The haemodynamic parameters measured in the different experimental groups, one hour post-surgery, did not differ significantly between the groups (Table 1).

Haemodynamic changes induced by BQ-123 in anaesthetized rats

Intravenous injections of 0.016 μmol kg^{-1} of BQ-123 induced no significant haemodynamic changes (Figure 1, Table 1). However, injections of 1.6 μmol kg^{-1} of BQ-123 induced a decrease in femoral mean arterial pressure accompanied by increases in vascular conductances in all the beds studied (Figure 1), with no significant change in heart rate. Maximal depressor response (− 24.9 ± 4.3%) occurred 3 to 4 min after injection and, in all the beds studied, vascular conductance increased to a similar extent (38.6 ± 8.8% in the carotid, 30.3 ± 3.1 in the coeliac, 34.5 ± 4.1% in the mesenteric, 47.8 ± 11.1% in the renal and 61.9 ± 14.9% in the iliac bed) with, however, a possible tendency for the coeliac and mesenteric beds to be less affected than the others. Eight min after injection of 1.6 μmol kg^{-1} of BQ-123, mean arterial pressure was still significantly depressed (− 13 ± 5%; $P < 0.05$) and renal and iliac vascular conductances were still significantly elevated (36 ± 11% and 38 ± 17%, respectively; $P < 0.05$), whereas carotid, coeliac and mesenteric vascular conductances had returned to their initial values (Table 1).

Influence of BQ-123 on haemodynamic changes induced by endothelin-1

In control animals, 1 nmol kg^{-1}, i.v. of ET-1 induced the well-described biphasic response characterized by an immed-

Table 1 Basal values of femoral mean arterial pressure (AP), heart rate (HR), and vascular conductances measured in the carotid (Ca), coeliac (Coe), mesenteric (M), renal (R) and iliac (I) beds of anaesthetized rats, 1 hour post-surgery

Group:	1	2	3	4	5
n	7	6	6	6	4
AP (mmHg)	98 ± 4	109 ± 4	109 ± 3	110 ± 4	109 ± 5
		108 ± 7	100 ± 3*		97 ± 7*
HR (b min^{-1})	289 ± 12	315 ± 22	274 ± 11	281 ± 6	294 ± 4
		304 ± 15	270 ± 11		272 ± 10
Vascular conductances (kHz mmHg^{-1}):					
Ca	0.026 ± 0.004	0.020 ± 0.003	0.027 ± 0.009	0.034 ± 0.004	0.027 ± 0.005
		0.024 ± 0.009	0.027 ± 0.005		0.030 ± 0.005
Coe	0.039 ± 0.005	0.056 ± 0.008	0.053 ± 0.009	0.058 ± 0.005	0.039 ± 0.007
		0.059 ± 0.008	0.058 ± 0.008		0.042 ± 0.006
M	0.055 ± 0.010	0.060 ± 0.007	0.052 ± 0.007	0.050 ± 0.006	0.055 ± 0.006
		0.071 ± 0.007	0.063 ± 0.009		0.060 ± 0.008
R	0.047 ± 0.008	0.055 ± 0.004	0.039 ± 0.011	0.048 ± 0.005	0.037 ± 0.003
		0.065 ± 0.011	0.054 ± 0.007*		0.049 ± 0.007*
I	0.022 ± 0.004	0.025 ± 0.002	0.023 ± 0.005	0.020 ± 0.004	0.028 ± 0.005
		0.029 ± 0.013	0.034 ± 0.011*		0.037 ± 0.009*

For the groups concerned, the values measured 8 min after the administration of BQ-123 are given on a second line. Group 1 received 1 nmol kg^{-1} endothelin-1 (ET-1); group 2 received 1 nmol kg^{-1} ET-1, 8 min after 0.016 μmol kg^{-1} BQ-123; group 3 received 1 nmol kg^{-1} ET-1, 8 min after 1.6 μmol kg^{-1} BQ-123; group 4 received 3 nmol kg^{-1} [Ala^{1,3,11,15}]ET-1; group 5 received 3 nmol kg^{-1} [Ala^{1,3,11,15}]ET-1 8 min after 1 6 μmol kg^{-1} BQ-123. Vascular conductances are expressed as Doppler shift signals divided by the mean AP.

*$P<0.05$ compared to post-surgery measurement.

iate and transient decrease in mean arterial pressure which reached a maximum within 30 s and disappeared after 1 min. The peak depressor response was concomitant with significant increases in regional vascular conductances, i.e. vasodilatation, in the carotid and iliac beds, whereas the coeliac and mesenteric beds were not significantly affected

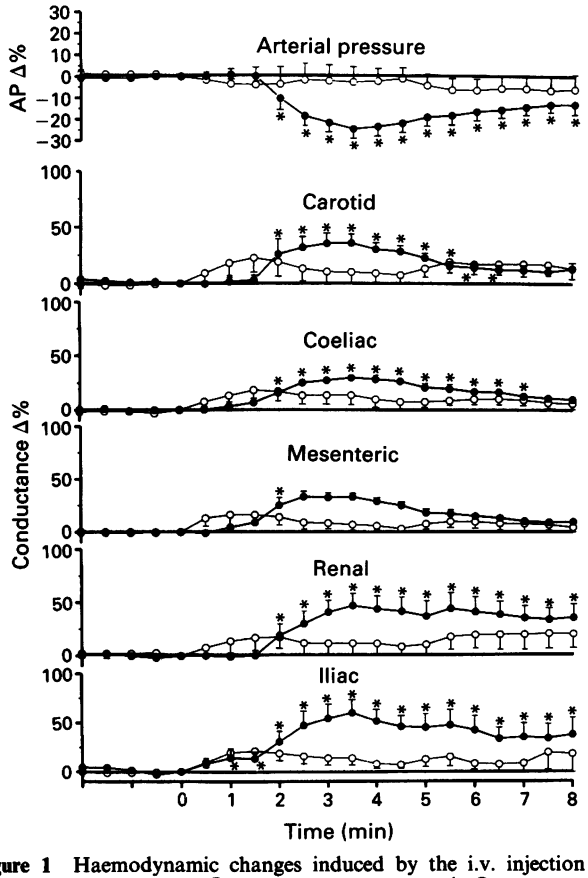

Figure 1 Haemodynamic changes induced by the i.v. injection of BQ123 0.016 μmol kg^{-1} (○; $n = 6$) or 1.6 μmol kg^{-1} (●; $n = 10$) into anaesthetized rats. Results are expressed as % changes from base line values. Vertical bars are s.e.mean. *$P<0.05$ compared to base lines.

and the renal conductance was already significantly reduced (Figure 2). After the first min of the response, the depressor effect and the regional vasodilatation disappeared and were replaced by an intense and prolonged hypertension, reaching a maximum within 2 min, accompanied by a systemic vasoconstriction as the vascular conductances dropped in all the beds, particularly in the renal bed (Figure 2). Heart rate was not affected during the first 2 min of the response, then tended to decrease by a maximum of − 20 ± 6% after 20 min as the pressure remained elevated (data not illustrated).

No significant change in the cardiovascular response elicited by ET-1 was observed after pretreatment with 0.016 μmol kg^{-1} of BQ-123 (data not illustrated). After pretreatment with 1.6 μmol kg^{-1} of BQ-123, the response induced by ET-1 (1 nmol kg^{-1}) was markedly modified (Figure 2). The early depressor response was prolonged (from 1 to 3 min) and enhanced by about 2 fold (from − 24.2 ± 7.7 to − 43.0 ± 3.4%; $P<0.01$). The early vasodilator response was also prolonged (Figure 2) and the ET-1-induced increase in vascular conductance was augmented by about 2 fold in the carotid bed (from 98.9 ± 41.6% to 218.5 ± 29.9%; $P<0.05$) and by between 4 to 5 fold in the coeliac (from 10.1 ± 15.9% to 47.0 ± 12.5%; $P<0.01$), mesenteric (from 15.9 ± 20.7% to 79.1 ± 21.3%; $P<0.05$) and iliac beds (from 56.1 ± 21.9% to 240.5 ± 25.6%; $P<0.01$). In the presence of BQ-123 (1.6 μmol kg^{-1}), the ET-1-induced secondary pressor response was significantly diminished by about half (from 41.2 ± 4.7% to 27.1 ± 5.9%; $P<0.05$) and its duration was shortened; indeed, it disappeared almost completely after 25 min. The systemic vasoconstriction was also reduced with, however, striking regional differences. The carotid and iliac ET-1-induced vasoconstrictors were the most sensitive to BQ-123 since in both beds the decreases in vascular conductances were reduced by a little less than half (from − 71.8 ± 3.4% to − 42.7 ± 3.5% and − 72.3 ± 5.8% to − 41.4 ± 8.4% respectively; $P<0.05$) and disappeared within 30 min. The ET-1-induced mesenteric vasoconstriction was more resistant to BQ-123 since its maximum was reduced by less than 20% (from − 71.7 ± 4.0% to − 58.4 ± 2.9%; $P<0.05$) and persisted 30 min after ET-1 injection. Finally, the duration and the intensity of the coeliac and renal vasoconstrictions were not significantly affected by the pretreatment with BQ-123 (from − 44.4 ± 11.9% to − 58.5 ± 5.1% and from − 90.6 ± 6.4% to − 96.8 ± 1.7% respectively) (Figure 2).

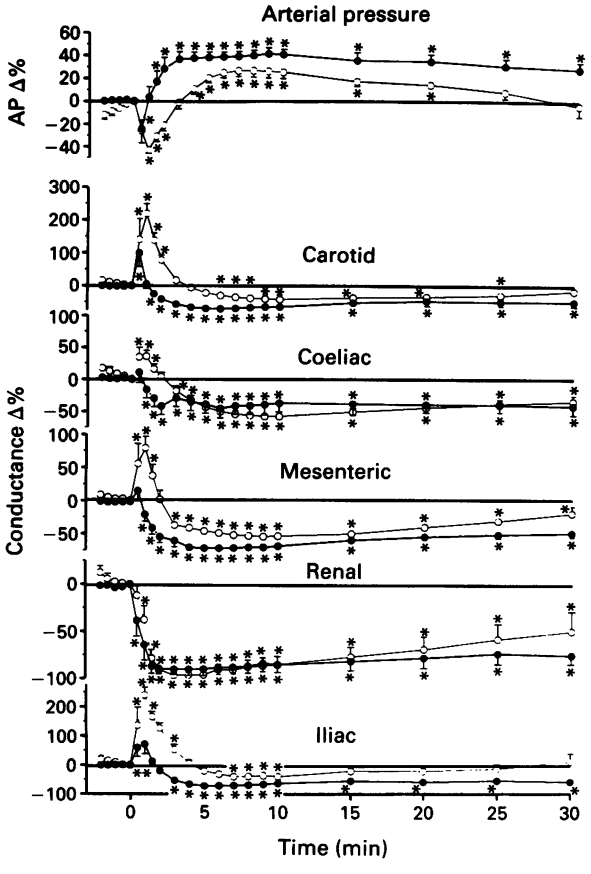

Figure 2 Haemodynamic changes induced by the i.v. injection of 1 nmol kg^{-1} endothelin-1 (ET-1) into control anaesthetized rats (●) and after pretreatment with 1.6 μmol kg^{-1} BQ123 (○). Results are expressed as % changes from base line values. Vertical bars are s.e.mean. *$P<0.05$ compared to base lines. Statistics for comparisons between responses obtained under the two conditions are given in the text.

Haemodynamic changes induced by [Ala^{1,3,11,15}]ET-1 in the anaesthetized rat

A representative example of the cardiovascular response elicited by 3 nmol kg^{-1} of [Ala^{1,3,11,15}]ET-1 is shown in Figure 3. [Ala^{1,3,11,15}]ET-1 induced an immediate depressor effect accompanied simultaneously by marked, but transient, increases in carotid, coeliac and iliac blood flows and by prominent decreases in mesenteric and renal blood flows. This early response lasted for at least 3 min before declining gradually and, between 15 and 20 min after [Ala^{1,3,11,15}]ET-1 injection, being replaced by a small pressor response accompanied by reduced blood flow in all the beds studied (Figure 3). The average response induced by 3 nmol kg^{-1} of [Ala ^{1,3,11,15}]ET-1 is shown in Figure 5. The early depressor response reached a maximum within the first min and was concomitant with simultaneous pronounced vasodilatation in the carotid, coeliac and iliac beds and vasoconstriction in the mesenteric and renal beds. The secondary pressor response, mild compared to that induced by ET-1, was maximal after 15 min and was accompanied by a small systemic vasoconstriction, most prominent in the mesenteric and renal beds. There was, however, no significant effect in the ilial bed. Both phases of the response were dose-dependent (Figure 4) and no significant change in HR was observed with any dose of [Ala^{1,3,11,15}]ET-1 (data not illustrated). A maximal depressor effect was obtained with 3 nmol kg^{-1}. At this dose, the early response observed in the iliac and renal beds was also maximal and the early responses observed in the carotid, coeliac and mesenteric beds were not significantly different from those obtained with 1 nmol kg^{-1}. Furthermore, 3 nmol kg^{-1} of [Ala^{1,3,11,15}]ET-1 also induced a maximal secondary pressor/vasoconstrictor response. However, whatever the dose used [Ala^{1,3,11,15}]ET-1 never induced a significant secondary iliac vasoconstriction (Figure 4).

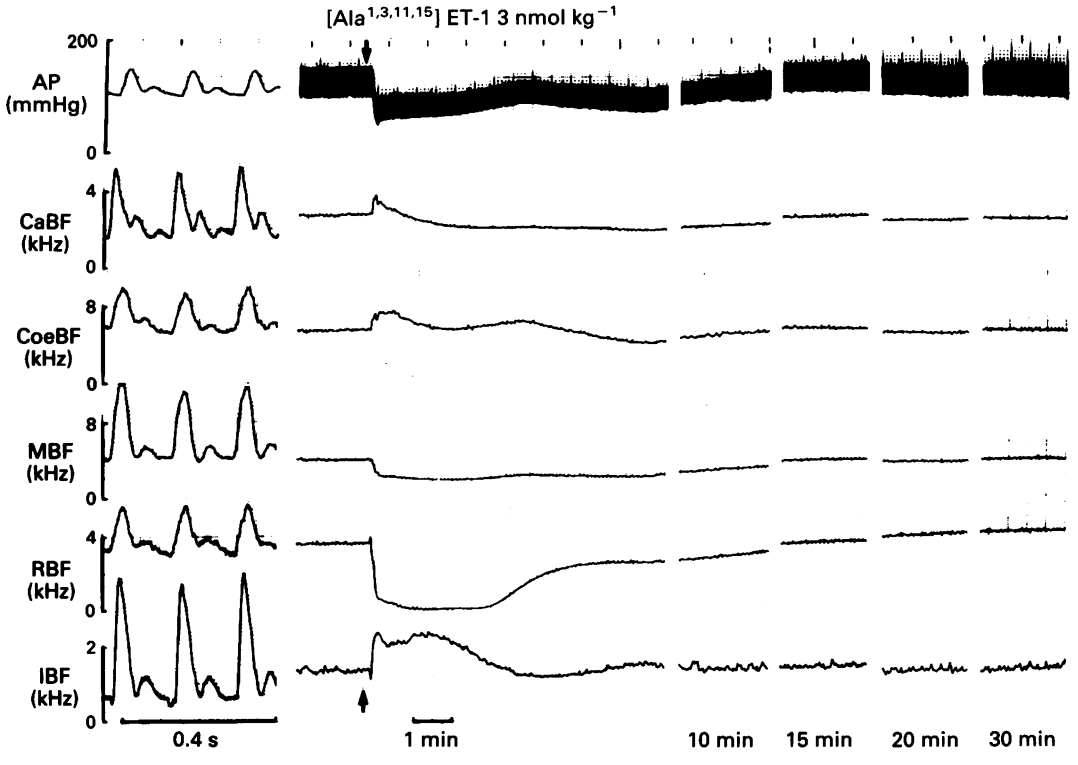

Figure 3 Representative example of the haemodynamic changes in arterial pressure (AP) and regional blood flows (BF), measured in the carotid (Ca), coeliac (Coe), mesenteric (M), renal (R) and iliac (I) arteries, induced by the i.v. injection of 3 nmol kg^{-1} [Ala^{1,3,11,15}]ET-1 into an anaesthetized rat.

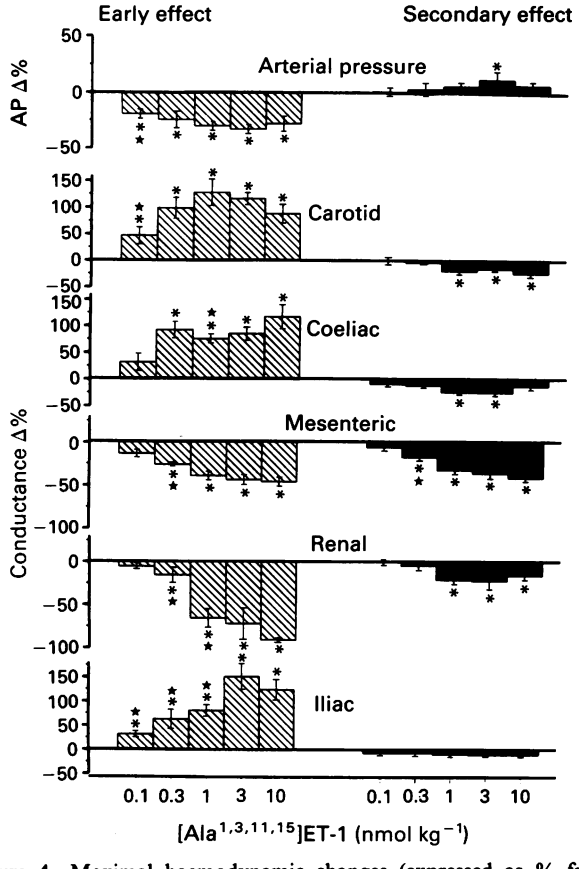

Figure 4 Maximal haemodynamic changes (expressed as % from baseline values) observed after injections of [Ala^{1,3,11,15}]ET-1 (0.1, 0.3, 1, 3 and 10 nmol kg^{-1}) into anaesthetized rats. The maximal early effect usually occurred within the 2 first min of the response, whereas the maximal secondary effect occurred around the 10th min. *$P<0.05$ compared to base lines: ★$P<0.05$ compared to the maximal response.

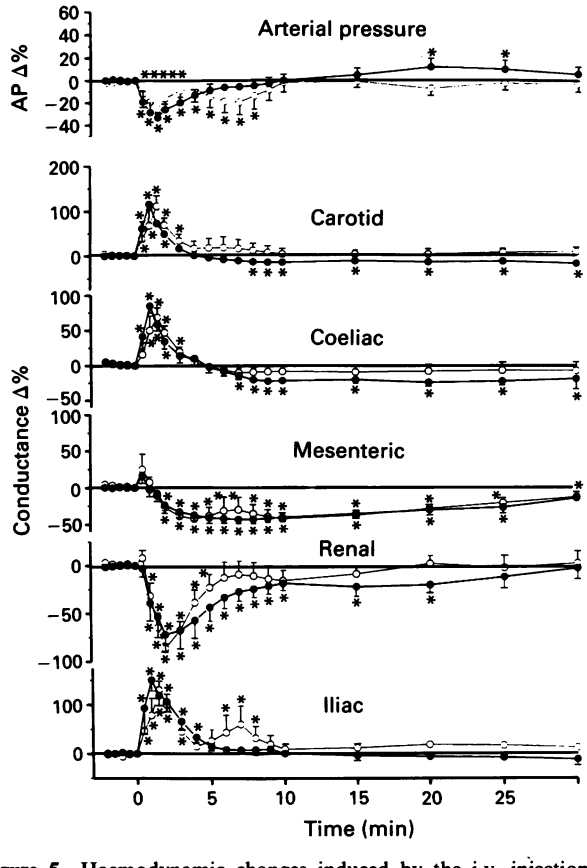

Figure 5 Haemodynamic changes induced by the i.v. injection of 3 nmol kg^{-1} [Ala^{1,3,11,15}]ET-1 in control anaesthetized rats (●) and after pretreatment with 1.6 μmol kg^{-1} BQ123 (○). Results are expressed as % changes from base line values. Vertical bars are s.e.mean. *$P<0.05$ compared to base lines. Statistics for comparisons between responses obtained under the two conditions are given in the text.

Influence of BQ-123 on haemodynamic changes induced by [Ala^{1,3,11,15}]ET-1

The early depressor response elicited by [Ala^{1,3,11,15}]ET-1 was not significantly affected in its amplitude (from $-33.1 \pm 4.1\%$ to $-34.8 \pm 1.1\%$) by pretreatment with 1.6 μmol kg^{-1} of BQ-123, neither were the early vasodilatations in the carotid (from $115.9 \pm 11.3\%$ to $111.9 \pm 26.7\%$), coeliac (from $90.4 \pm 10.6\%$ to $83.0 \pm 9.4\%$) and iliac (from $150.6 \pm 26.7\%$ to $130.3 \pm 22.9\%$) beds, nor the early vasoconstrictions in the mesenteric (from $-43.0 \pm 5.9\%$ to $-42.6 \pm 6.1\%$) and renal (from $-72.0 \pm 18.2\%$ to $-86.2 \pm 4.8\%$) beds. However, the duration of the carotid and iliac vasodilatation tended to be prolonged to almost 5 min, whereas the duration of the renal vasoconstriction tended to be diminished (Figure 5). The secondary pressor activity of [Ala^{1,3,11,15}]ET-1 was abolished (from $15.7 \pm 4.2\%$ to $3.6 \pm 7.1\%$; $P<0.05$) and the secondary vasoconstrictor activity was markedly altered with marked regional differences: the carotid, coeliac and renal vasoconstrictions were abolished (from $-15.6 \pm 1.0\%$ to $0.2 \pm 3.8\%$, from $-24.9 \pm 5.6\%$ to $-10.6 \pm 6.2\%$ and from $-23.5 \pm 6.1\%$ to $4.6 \pm 7.4\%$, respectively; $P<0.05$), whereas the mesenteric vasoconstriction was not significantly affected (from $-32.6 \pm 5.3\%$ to $-42.9 \pm 6.1\%$) (Figure 5).

Discussion

The cyclic pentapeptide BQ-123 has been described as a highly selective antagonist of ET_A-receptors compared to ET_B-receptors (Ihara *et al.*, 1992; Nakamichi *et al.*, 1992), inhibiting the ET-1-mediated pressor effect in the conscious rat without altering the ET-1-induced depressor effect. The present study confirms that pretreatment of anaesthetized rats with 1.6 μmol kg^{-1} (1 mg kg^{-1}) of BQ-123 effectively reduces the sustained pressor and systemic vasoconstrictor effects of ET-1, thus supporting the concept that ET_A-receptors are responsible for the direct sustained vasoconstrictor activity of ET-1 (Masaki, 1991; Vane, 1991). There are, however, well-documented regional differences in the vasoconstrictor effects of ET-1, the renal and mesenteric beds being particularly sensitive to the peptide (Wright & Fozard, 1988; Gardiner *et al.*, 1989; Han *et al.*, 1989; Le Monnier de Gouville *et al.*, 1990b). The present study describes similar regional differences which are emphasized after pretreatment with BQ-123. ET-1-induced vasoconstriction in the renal, mesenteric and coeliac beds were little affected by 1.6 μmol kg^{-1} of BQ-123 compared to the carotid and iliac beds and such differences may reflect different densities of ET_A-receptors in the different regions. The splenic artery (fed by the coeliac artery) and the renal artery have been shown to be particularly rich in ET_A-receptors (Hemsén *et al.*, 1991). It is thus possible that, in these particular beds, the submaximal pressor dose of ET-1 used (1 nmol kg^{-1}) might in fact be supramaximal and the apparent resistance to blockade by BQ-123 might simply be a reflection of the use of a suboptimal blocking dose. The use of higher doses of BQ-123, which was technically impossible in this study, will certainly clarify this point. Nevertheless, we cannot exclude a possible coexistence in the coeliac, renal or mesenteric vascular beds of another endothelin-receptor, or subtype of ET_A-receptor,

not antagonized by BQ-123 and also involved in the ET-1-mediated vasoconstriction.

One of the major findings of the present study is that the pretreatment of the animals with BQ-123 not only reduces the pressor effect of ET-1 but also markedly accentuates its primary depressor activity. This observation is not in agreement with early reports concerning the infuence of this ET_A-receptor antagonist on ET-1-mediated depressor effects in conscious rats (Ihara *et al.*, 1991; 1992). The fact that the depressor activity of ET-1 was more marked in the conscious state than after anaesthesia may explain such a divergence. The increase of ET-1 mediated depressor effects in the presence of BQ-123 was concomitant with a large increase in ET-1-mediated vasodilatation in all beds, except in the renal bed where ET-1 had no vasodilator activity. This enhancement of vasodilatation seems likely to reflect the fact that the haemodynamic effect of ET-1 is the result of a functional antagonism between its two opposite activities. It is clear that the ET_A-receptor-mediated component provides the majority of the haemodynamic response elicited by a relatively high dose (1 nmol kg^{-1}) of ET-1, in the anaesthetized rat, and that its influence masks the real amplitude of the vasodilator component, which can however be seen in the presence of BQ-123. On the other hand, this enhanced vasodilator component may well interfere with the development of the ET_A-mediated vasoconstriction already antagonized by BQ-123. The mechanism underlying the vasodilator activity of ET-1 remains a matter of debate (for review see Masaki *et al.*, 1991; Vane, 1991; Fozard & Part, 1992), but could be due to the stimulation of ET_B-receptors, perhaps accompanied by the release of vasodilator mediators such as endothelium-derived relaxing factor (EDRF) and prostacyclin. Thus, the existence of regional differences in ET_A- and ET_B-receptor densities would generate different regional equilibria between these two functionally antagonist activities and contribute to the regional differences previously described.

A linear analogue of ET-1, [Ala^{1,3,11,15}]ET-1, which has been described as a highly selective ET_B-receptor agonist (Saeki *et al.*, 1991), elicited a prominent, dose-dependent, early depressor response, followed by a mild secondary pressor effect in the anaesthetized rat (Douglas & Hiley, 1991; present study) accompanied by a weak regional vasoconstriction which was markedly reduced by pretreatment with BQ-123. This observation demonstrates that [Ala^{1,3,11,15}]ET-1 can induce a relatively weak ET_A-mediated secondary vasoconstriction *in vivo*, although with some regional differences since, as observed with ET-1, the sustained mesenteric vasoconstriction elicited by [Ala^{1,3,11,15}]ET-1 was not reduced by BQ-123 at the dose of 1.6 μmol kg^{-1}. Higher doses of BQ-123 would be necessary to interpret fully such regional differences. However, they seem unlikely to be the result of a differential displacement of the equilibrium between the regional vasoconstrictor and vasodilator components since BQ-123 did not modify significantly the vasodilator component induced by [Ala^{1,3,11,15}]ET-1. The existence, within the mesenteric bed, of another type of vasoconstrictor receptor, or perhaps an ET_A-receptor subtype with a lower affinity for BQ-123 cannot be excluded.

Assuming that, in the presence of BQ-123, the haemodynamic responses elicited by [Ala^{1,3,11,15}]ET-1, with the exception of the effect observed in the mesenteric bed, are strictly ET_B-mediated, then [Ala^{1,3,11,15}]ET-1 revealed a marked and relatively prolonged early depressor activity accompanied simultaneously by a large vasodilatation in the carotid, coeliac and iliac beds, and by a transient but intense renal vasoconstriction. Such observations would suggest that both depressor/vasodilator and renal vasoconstrictor activities of [Ala^{1,3,11,15}]ET-1 are mediated by ET_B-receptors. Alternatively, ET_A-receptor subtypes, stimulated by [Ala^{1,3,11,15}]ET-1 but insensitive to BQ-123 (at the dose of 1.6 μmol kg^{-1}), might exist in the renal bed, and if ET_B-receptors are present in this bed, functionally antagonize a possible ET_B-mediated renal vasodilatation. Whatever the case, the early responses induced by [Ala^{1,3,11,15}]ET_1 and ET-1, in the presence of BQ-123, were similar, suggesting that both agonists stimulated the same receptors, that the early vasodilator activity of ET-1 is ET_B-receptor-mediated and that the same mechanism is responsible for the early renal vasoconstriction induced by both agonists. However, the fact that the vasodilatation elicited by [Ala^{1,3,11,15}]ET-1 was always smaller than that induced by ET-1 may suggest that [Ala^{1,3,11,15}]ET-1 is a partial agonist at ET_B-receptors compared to ET-1. This may explain why, unlike ET-1, [Ala^{1,3,11,15}]ET-1 was unable to induce a mesenteric vasodilatation in the presence of BQ-123.

BQ-123 alone induced a transient, dose-dependent, depressor effect, accompanied by vasodilatation of all beds studied. This observation is not in agreement with the work reported by Ihara *et al.* (1992) which described no significant haemodynamic effects of BQ-123 in the conscious rat. We cannot explain such a discrepancy. However, since the affinity of BQ-123 for ET_B-receptors has been described as 2500 times lower than for ET_A-receptors (Ihara *et al.*, 1992; Nakamichi *et al.*, 1992) and since BQ-123 did not induce similar regional differences to those induced by [Ala^{1,3,11,15}]ET-1, BQ-123-induced systemic vasodilatation seems unlikely to be due to the stimulation of ET_B-receptors. The inhibition of a putative basal ET_A-receptor-mediated ET-1 tone is one possible mechanism.

The present study was an attempt to discriminate between ET_A- and ET_B-receptor-mediated haemodynamic effects of ET-1. The results indicate that the early ET-1 mediated vasodilatation can be mainly ascribed to the stimulation of ET_B-receptors and the sustained ET-1-mediated vasoconstriction to the stimulation of ET_A-receptors. Regional differences in the effects of ET-1 and [Ala^{1,3,11,15}]ET-1 are described, which might be partly related to an equilibrium existing between the functional antagonism resulting from stimulation of the two receptor types and dependent on the density in ET_A- and ET_B-receptors present within the different vascular beds. However, part of the mesenteric and renal vasoconstrictions could not be simply described as either ET_A- or ET_B-receptor-mediated. The existence of an as yet unknown ET receptor type, or subtype, cannot be excluded.

We are grateful to Drs R.C. Miller and J. Huggins for their critical comments on the manuscript.

References

ARAI, S., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, **348**, 730–732.

ATKINSON, R.A. & PELTON, J.T. (1992). Conformational study of cyclo[D-Trp-D-Asp-Pro-D-Val-Leu], an endothelin-A receptor-selective antagonist. *FEBS Lett.*, **296**, 1–6.

BIGAUD, M. (1992). Simultaneous regional vasodilator and vasoconstrictor effects of endothelin ET_B receptor stimulation in anaesthetized rats. *Br. J. Pharmacol.*, **105**, 298P.

DOUGLAS, S.A. & HILEY, C.R. (1991). Endothelium-dependent mesenteric vasorelaxant effects and systemic actions of endothelin (16–21) and other endothelin-related peptides in the rat. *Br. J. Pharmacol.*, **104**, 311–320.

FOZARD, J.R. & PART, M.L. (1992). The role of nitric oxide in the regional vasodilator effects of endothelin-1 in the rat. *Br. J. Pharmacol.*, **105**, 744–750.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1989). Regional hemodynamic effects of endothelin-1 in conscious, unrestrained, wistar rats. *J. Cardiovasc. Pharmacol.*, **13**(suppl. 5), S202–S204.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1990). Regional haemodynamic of endothelin-1 and endothelin-3 in conscious Long Evans and Brattlboro rats. *Br. J. Pharmacol.*, **99**, 107–112.

HAN, S.P., TRAPANI, A.J., FOX, K.F., WESTFALL, T.C. & KNUEPFER, M.M. (1989). Effects of endothelin on regional haemodynamics in conscious rats. *Eur. J. Pharmacol.*, **159**, 303–305.

HAYWOOD, J.R., SHAFFER, R.A., FASTENOW, C., FINK, G.D. & BRODY, M.J. (1981). Regional blood flow measurement with pulsed Doppler flowmeter in conscious rat. *Am. J. Physiol.*, **241**, H273–H278.

HEMSÉN, A., LARSSON, O. & LUNDBERG, J.M. (1991). Characteristics of endothelin A and B binding sites and their vascular effects in pig peripheral tissues. *Eur. J. Pharmacol.*, **208**, 313–322.

HILEY, C.R., COWLEY, D.J., PELTON, J.T. & HARGREAVES, A.C. (1992). BQ-123, cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu), is a non-competitive antagonist of the actions of endothelin-1 in SK-N-MC human neuroblastoma cells. *Biochem. Biophys. Res. Commun.*, **184**, 504–510.

HILEY, C.R., JONES, C.R., PELTON, J.T. & MILLER, R.C. (1990). Binding of [^{125}I]-endothelin-1 to rat cerebellar homogenates and its interactions with some analogues. *Br. J. Pharmacol.*, **101**, 319–324.

IHARA, M., FUKURODA, T., SAEKI, T., NISHIKIBE, M., KOJIRI, K., SUDA, H. & YANO, M. (1991). An endothelin receptor (ET_A) antagonist isolated from *Streptomyces misakiensis*. *Biochem. Biophys. Res. Commun.*, **178**, 132–137.

IHARA, M., NOGUCHI, K., SAEKI, T., FUKURODA, T., TSUCHIDA, S., KIMURA, S., FUKAMI, T., ISHIKAWA, K., NISHIKIBE, M. & YANO, M. (1992). Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.*, **50**, 247–255.

LE MONNIER DE GOUVILLE, A.C., LIPPTON, H., COHEN, G., CAVERO, I. & HYMAN, A. (1990a). Vasodilator activity of endothelin-1 and endothelin-3: rapid development of cross-tachyphylaxis and dependence on the rate of endothelin administration. *J. Pharmacol. Exp. Ther.*, **254**, 1024–1028.

LE MONNIER DE GOUVILLE, A.C., MONDOT, S., LIPPTON, H., HYMAN, A. & CAVERO, I. (1990b). Hemodynamic and pharmacological evaluation of the vasodilator and vasoconstrictor activity of endothelin-1 in rats. *J. Pharmacol. Exp. Ther.*, **252**, 73–84.

LIN, H.Y., KAJI, E.H., WINKEL, G.K., IVES, H.E. & LODISH, H.F. (1991). Cloning and functional expression of a vascular smooth muscle endothelin 1 receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 3185–3189.

MASAKI, I. (1991). Tissue specificity of the endothelin-induced responses. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 7), S1–S4.

MASAKI, T., KIMURA, S., YANAGISAWA, M. & GOTO, K. (1991). Molecular and cellular mechanism of endothelin regulation. *Circulation*, **84**, 1457–1468.

MERRIFIELD, R.B. (1963). Solid phase peptide synthesis. 1 The synthesis of a tetrapeptide. *J. Am. Chem. Soc.*, **85**, 2149–2154.

NAKAMICHI, K., IHARA, M., KOBAYASHI, M., SAEKI, T., ISHIKAWA, K. & YANO, M. (1992). Different distribution of endothelin receptor subtypes in pulmonary tissues revealed by the novel selective ligands BQ-123 and [Ala^{1,3,11,15}]ET-1. *Biochem. Biophys. Res. Commun.*, **182**, 144–150.

PELTON, J.T. & MILLER, R.C. (1991). The role of disulphide bonds in endothelin-1. *J. Pharmacol.*, **43**, 43–45.

SAEKI, T., IHARA, M., FUKURODA, T., YAMAGIWA, M. & YANO, M. (1991). [Ala^{1,3,11,15}]endothelin-1 analogs with ET_B agonistic activity. *Biochem. Biophys. Res. Commun.*, **179**, 286–292.

SAKAMOTO, A., YANAGISAWA, M., SAKURAI, T., TAKUWA, Y., YANAGISAWA, H. & MASAKI, T. (1991). Cloning and functional expression of human cDNA for the ET_B endothelin receptor. *Biochem. Biophys. Res. Commun.*, **178**, 656–663.

SAKURAI, T., YANAGISAWA, M., TAKUWAT, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348**, 732–735.

VANE, J. (1990). Endothelins come home to roost. *Nature*, **348**, 673.

WRIGHT, C.E. & FOZARD, J.R. (1988). Regional vasodilation is prominent feature of the hemodynamic response to endothelin in anaesthetized spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **155**, 202–203.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

(Received March 24, 1992
Revised July 20, 1992
Accepted July 24, 1992)

Br. J. Pharmacol. (1992), **107**, 919–923

Histamine H_3 receptors modulate the release of [^{3}H]-acetylcholine from slices of rat entorhinal cortex: evidence for the possible existence of H_3 receptor subtypes

[1]J. Clapham & G.J. Kilpatrick

Department of Neuropharmacology, Glaxo Group Research, Park Road, Ware, Herts, SG12 0DP

1 The effect of agents which interact with the histamine H_3 receptor on potassium-stimulated tritium release from slices of rat entorhinal cortex preloaded with [^{3}H]-choline is described. We have examined the effects of the selective H_3 receptor agonist, (**R**)-α-methylhistamine (RAMH), and a number of H_3 receptor antagonists, including the selective compound thioperamide, on the potassium-stimulated release of tritium.

2 In the presence of mepyramine and ranitidine, RAMH (0.01–10 μM) inhibited potassium-stimulated tritium release in a concentration-dependent manner, $EC_{50} = 0.11$ μM. The maximum inhibition was approximately 50%.

3 Thioperamide displaced the RAMH concentration-response curve to the right yielding a pK_B value of 8.4. There was no change in the maximum response to RAMH.

4 Other H_3 receptor antagonists, including impromidine and burimamide, also caused rightwards displacement of the linear portion of the RAMH concentration-response curve. However, phenylbutanoylhistamine and betahistine, which are reported to be relatively potent H_3 receptor antagonists, showed very low affinity.

5 Thioperamide (0.001–1 μM) alone enhanced the potassium-stimulated release of tritium in a concentration-dependent manner. Maximum effects were observed at 0.1–1 μM thioperamide, enhancing release by approximately 20%.

6 Results are discussed in terms of the regulatory role of H_3 receptors on acetylcholine release and the possible existence of H_3 receptor subtypes.

Keywords: Acetylcholine release; entorhinal cortex; histamine H_3 receptors

Introduction

The existence of a third histamine receptor pharmacologically distinct from both the H_1 and H_2 receptor was suggested by Arrang and colleagues in 1983. This histamine receptor, termed H_3, was originally characterized as a presynaptic autoreceptor modulating histamine synthesis and release in slices of rat cerebral cortex. With the more recent discovery of a selective agonist, (**R**)-α-methylhistamine (RAMH), and a selective antagonist, thioperamide, for this receptor (Arrang *et al.*, 1987), other workers have reported that H_3 receptors also function as presynaptic inhibitory heteroreceptors controlling the release of noradrenaline (Schlicker *et al.*, 1989) and 5-hydroxytryptamine (Schlicker *et al.*, 1988) in the central nervous system. These receptors also appear to modulate the release of non-adrenergic non-cholinergic transmitters (Ichinose & Barnes, 1989; Taylor & Kilpatrick, 1992) and cholinergic transmission (Trzeciakowski, 1987; Ichinose *et al.*, 1989) in the periphery. Indeed, it has recently been demonstrated that activation of the H_3 receptor inhibits the release of tritium, presumably [^{3}H]-acetylcholine, from the guinea-pig isolated longitudinal muscle myenteric plexus preincubated with [^{3}H]-choline (Poli *et al.*, 1991).

We now describe the development and preliminary characterization of an *in vitro* model to investigate H_3 receptor-mediated modulation of acetylcholine release in the central nervous system. Parts of this study have been presented, in abstract form, to the British Pharmacological Society (Clapham & Kilpatrick, 1992).

[1] Author for correspondence.

Methods

Preparation of slices of rat entorhinal cortex

Male Lister Hooded rats (250–300 g, Glaxo bred) were stunned and decapitated. The brains were removed and cooled on ice. The entorhinal cortex was dissected from each brain (10 rats per experiment, each providing two entorhinal cortices) and placed on dry filter paper. The tissue was chopped transversely through the cortical layers with a McIlwain chopper to produce 0.3 mm slices. The pooled cut tissue was placed in a 25 ml sterilin tube containing oxygenated (95% O_2/5% CO_2) Krebs solution. The slices were then separated by shaking and washed (3 × 20 ml) in Krebs solution. The Krebs solution was replaced by Krebs solution containing 39.75 mM KCl (NaCl was decreased to 83 mM to maintain tonicity) at 37°C for 20 min. This depolarization procedure was included to deplete endogenous neurotransmitters and enhance the subsequent uptake of [^{3}H]-choline.

After washing thoroughly with fresh Krebs solution (4 × 20 ml) the slices were incubated at 37°C for 40 min in 5 ml Krebs containing [^{3}H]-choline chloride (0.1 μM; 87 Ci mmol^{-1}, New England Nuclear). The slices were then washed with fresh Krebs solution (4 × 20 ml) in order to remove excess radioactivity. Approximately 100 μl of settled slices were placed in each of 20 superfusion chambers which were then superfused with Krebs solution at 37°C (flow rate 0.5 ml min^{-1}) containing 1 μM hemicholinium for 60 min.

Experimental design

After a 60 min equilibration period, samples of superfusate were collected every 4 min. Two stimulation methods were examined. Samples were either depolarized at 12 min (S_1) and

48 min (S_2) and 20 samples per chamber collected; or slices were depolarized once only at 12 min (S_1) and 10 samples were collected. Depolarization was achieved by changing the superfusing solution to Krebs buffer containing 20 mM KCl for 4 min (tonicity was maintained by reducing the NaCl concentration to 103 mM).

When the double stimulation method was used, drugs were included in the perfusate 20 min before the S_2 stimulation to the end of the experiment. Under these conditions the potassium-evoked tritium release was calcium-dependent (results not shown).

With the single stimulation method, antagonists were present in the perfusate from the start of the 60 min equilibration period onwards and agonists were incluced 20 min before the S_1 stimulation to the end of the experiment. Antagonist effects were quantified on their ability either to displace the full RAMH concentration-response curve (CRC) to the right or to shift a 2-point RAMH CRC (0.1 and 1 μM) in a similar fashion.

Quantification of tritium released

Acetylcholine release was estimated from the outflow of tritium from the tissue. This was measured by liquid scintillation spectrometry (5 min count time/sample), 10 ml of Emulsifier Safe scintillation fluid (Packard) being added to each 4 min sample of superfusate. At the end of the experiment the tissue slices from each chamber were solubilized in 1 ml of Soluene-100 (Packard) and the tritium was determined. The disintegrations per min for each 4 min collection period were converted to fractional release by dividing by the total amount of radioactivity present in the tissue at the start of that 4 min collection period. The stimulated release was calculated as the difference between potassium-evoked and basal release.

Data analysis

The results are expressed as either the mean S_2/S_1 ratio or the mean fraction released by S_1 ± standard error of the mean of n determinations. These data are then expressed as a percentage inhibition or stimulation of the Krebs control response. Concentration-response curve data were analysed by the programme 'ALLFIT' to yield pD_2 values. Antagonist pK_B values were estimated from the equation:

$$pK_B = \log (\text{concentration ratio} - 1) - \log(\text{concentration of antagonist})$$

Statistical analyses were performed by use of the unpaired t test.

Drugs and solutions

The composition of Krebs-Henseleit medium used (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.75, KH_2PO_4 1.2, $CaCl_2$ 1.25, $MgSO_4.7H_2O$ 1.2 and glucose 11.

Drugs were dissolved in water and diluted in Krebs-Henseleit medium.

Gifts of burimamide and impromidine trihydrochloride (SmithKline Beecham) and mepyramine maleate (Rhone Poulenc) are gratefully acknowledged. Betahistine dihydrochloride was purchased from Aldrich Chemical Company Ltd. (U.K.) and hemicholinium-3 from Sigma Chemical Company Ltd. (U.K.). Thioperamide, (R)-α-methylhistamine hydrochloride, ranitidine hydrochloride and phenylbutanoylhistamine were synthesized by the Chemistry Research Department, Glaxo Group Research.

Results

Potassium-evoked release of tritium from slices of rat entorhinal cortex preloaded with [^{3}H]-choline

The inclusion of potassium chloride (K^+; 20 mM) in the perfusate (S_1) for 4 min resulted in a release of tritium (Figure 1). The fraction released by S_1 stimulation was routinely between 0.05 and 0.07. A second exposure to potassium (S_2) resulted in a similar release of tritium. Using this 'double stimulation method' control S_2/S_1 ratios were routinely between 0.8 and 1.2 (Figure 2).

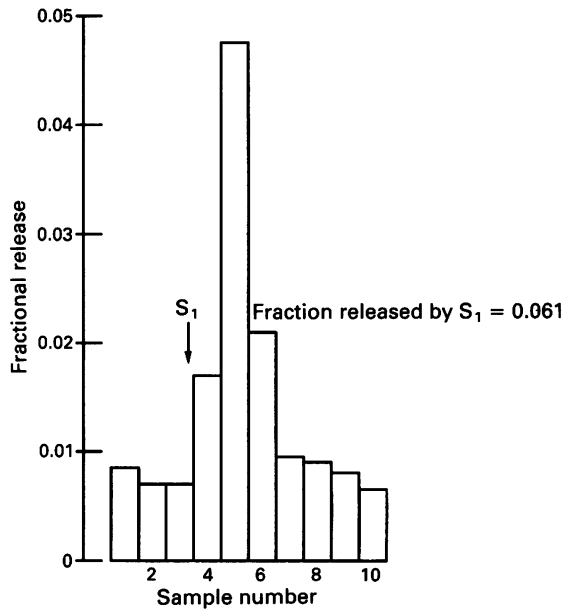

Figure 1 Data from a typical experiment using slices of rat entorhinal cortex showing the fractional release of tritium induced by potassium (20 mM) applied at S_1 for 4 min.

Effect of (R)-α-methylhistamine (RAMH) assessed using two different stimulation methods

Addition of RAMH (0.3, 1 and 3 μM) to the perfusate 20 min before S_2 resulted in a concentration-dependent inhibition of tritium release from the rat entorhinal cortex on the second stimulation (Table 1). The highest concentration of RAMH tested (3 μM) induced an inhibition of approximately 30%.

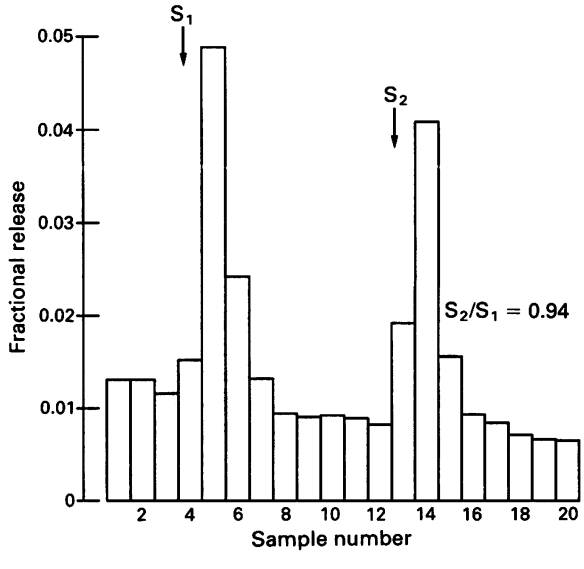

Figure 2 Data from a typical experiment using slices of rat entorhinal cortex showing the fractional release of tritium induced by potassium (20 mM) applied at S_1 and S_2 for 4 min.

Table 1 Effect of (R)-α-methylhistamine (RAMH) on tritium release from slices of rat entorhinal cortex by the double potassium stimulation method

Treatment (μM)	*Evoked tritium release* S_2/S_1 ratio	(n)	*% Inhibition of control*
K^+ control	0.92 ± 0.02	(11)	
RAMH (0.3)	0.79 ± 0.03*	(6)	14.1
RAMH (1.0)	0.70 ± 0.03*	(15)	23.9
RAMH (3.0)	0.62 ± 0.01*	(5)	32.6

Values shown are the mean ± s.e.mean of data taken from 4 experiments (n = number of superfusion chambers per treatment) *$P<0.005$ vs control

In a single experiment, addition of RAMH (3 μM) to the perfusate 20 min before S_1 resulted in an inhibition of approximately 40% (results not shown) i.e. greater than was obtained with the S_2/S_1 system. When the two stimulation methods were compared directly in the same experiment, RAMH (3 μM) inhibited K^+-evoked tritium release from the entorhinal cortex by approximately 22% by the S_2/S_1 method and by 44% by the S_1 only method (Figure 3). In subsequent experiments, therefore, the single stimulation method was employed.

Effect of mepyramine and ranitidine on RAMH-induced inhibition of tritium release

Inclusion of mepyramine (3 μM) and ranitidine (10 μM) in the perfusing Krebs solution had no significant effect on either basal tritium release or K^+-evoked tritium release. In the absence of mepyramine and ranitidine the mean control (K^+) fraction released was 0.067 ± 0.005 ($n = 4$ experiments). When the H_1 and H_2 antagonists were included the fraction released was 0.071 ± 0.004 ($n = 5$ experiments). The apparent pD_2 value of RAMH in the absence of mepyramine and ranitidine was 5.9 (Hill slope = 0.64); this was increased to 7.1 (Hill slope = 0.88) when mepyramine and ranitidine were included (Figure 4). There did not appear to be any change in the maximum inhibitory response to RAMH. The effect of the inclusion of either mepyramine (3 μM), ranitidine (10 μM)

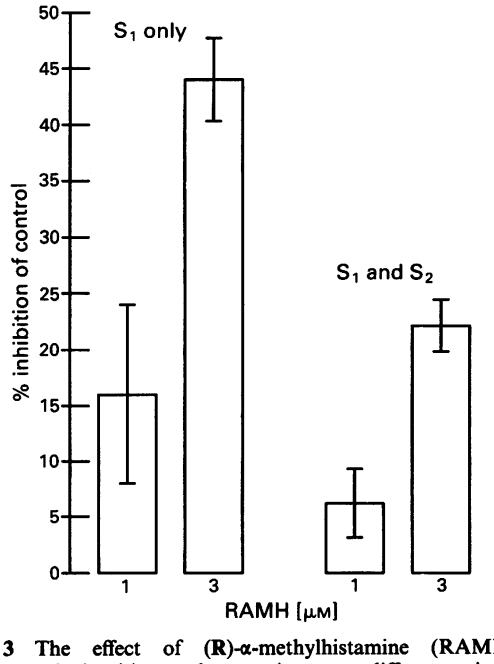

Figure 3 The effect of (R)-α-methylhistamine (RAMH) on potassium-evoked tritium release using two different stimulation methods. Values shown are the mean ± s.e.mean (vertical bars) of data taken from 3–4 superfusion chambers per treatment.

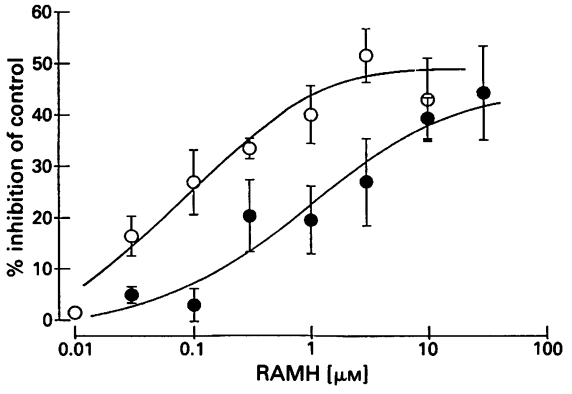

Figure 4 The effects of the H_1 receptor antagonist, mepyramine and the H_2 receptor antagonist, ranitidine on (R)-α-methylhistamine (RAMH)-induced inhibition of tritium release. RAMH (●) $pD_2 = 5.9$, RAMH in the presence of mepyramine (3 μM) and ranitidine (10 μM) (○), $pD_2 = 7.1$. Values shown are the mean ± s.e.mean (vertical bars) of data taken from 4 experiments, 3–4 superfusion chambers per treatment.

or a combination of the two antagonists was examined. At 0.3 μM RAMH alone induced an inhibition of 10.8%. This effect was increased to 17.6% in the presence of mepyramine and to 21.6% in the presence of ranitidine. However, when both antagonists were present, the greatest inhibitory effect of RAMH (0.3 μM) was observed, 29.7%. Both antagonists were therefore included in subsequent experiments.

Effect of thioperamide on RAMH-induced inhibition of tritium release

Thioperamide (0.03 μM) displaced the RAMH concentration-response curve (CRC) to the right in a parallel fashion with no decrease in the maximum inhibition. A pK_B value of 8.4 was calculated (Figure 5; RAMH Hill slope = 0.43, RAMH in the presence of thioperamide Hill slope = 0.35). Thioperamide (0.03 μM) alone appeared to enhance tritium release in 3 out of the 5 experiments; control fraction released = 0.052 ± 0.003 ($n = 10$ chambers), thioperamide fraction released = 0.062 ± 0.009 ($n = 8$ chambers). However this effect did not reach the level of statistical significance.

Effect of thioperamide on potassium-evoked tritium release

Inclusion of thioperamide (1 μM) in the perfusate 60 min before S_1, induced an increase in K^+-evoked tritium release of 23.0 ± 6.7% ($n = 3$ experiments). When a concentration-response curve was constructed for thioperamide (0.001–1 μM) in a separate series of experiments, maximum stimulatory effects of approximately 20% were observed between 0.1 and 1 μM (Figure 6). The half maximal effect of thioperamide was achieved at 6.0 ± 2.9 nM ($pD_2 = 8.2$).

Determination of the effects of H_3 receptor antagonists using a 2-point RAMH CRC

Five H_3 receptor antagonists were tested at a single concentration expected to displace a linear portion of the RAMH CRC approximately 10 fold. Apparent parallel rightwards displacements of the RAMH CRC were obtained with thioperamide, burimamide, impromidine and betahistine (compounds listed in order of potency); however phenybutanoylhistamine had no effect. Concentrations tested together with calculated pK_B values are presented in Table 2.

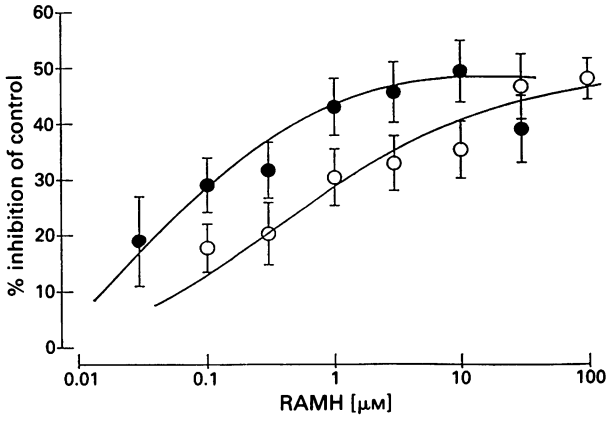

Figure 5 The effect of the H_3 receptor antagonist, thioperamide on (R)-α-methylhistamine (RAMH)-induced inhibition of tritium release. RAMH (●), RAMH in the presence of thioperamide (0.03 μM) (○), thioperamide $pK_B = 8.4$. Values shown are the mean ± s.e.mean (vertical bars) of data taken from 5 experiments, 3–4 superfusion chambers per treatment.

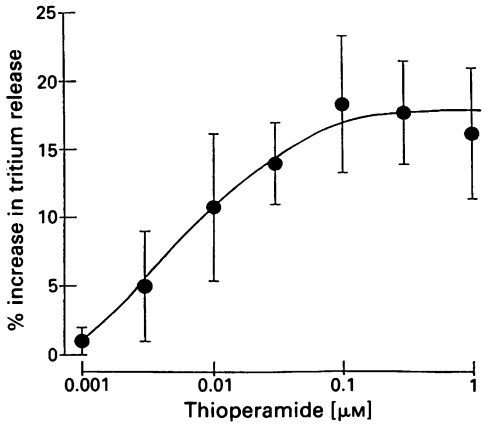

Figure 6 The effect of the H_3 receptor antagonist, thioperamide on potassium (20 mM)-evoked tritium release. Values shown are the mean ± s.e.mean (vertical bars) of data taken from 3–6 experiments.

Table 2 Apparent affinity values of H_3 receptor antagonists for antagonism of (R)-α-methylhistamine (RAMH)-induced inhibition of tritium release

Antagonist	*Concentration tested (μM)*	*Approximate pK_B value*	*Number of experiments*
Thioperamide	0.03	9.0 ± 0.1	3
Impromidine	3	6.3 ± 0.2	3
Burimamide	3	7.1 ± 0.4	3
Phenylbutanoyl-histamine	10	<5	2
Betahistine	100	4.5 ± 0.3	3

Values shown are the mean ± s.e.mean

Discussion

In this paper we describe a preliminary characterization of the effects of the selective H_3 receptor agonist, RAMH, and a number of H_3 receptor antagonists including the selective compound, thioperamide, on the calcium-dependent, potassium-stimulated release of tritium from slices of rat entorhinal cortex preloaded with [^{3}H]-choline. Barnes *et al.* (1989) have previously demonstrated that, using a similar methodology but including an acetylcholinesterase inhibitor in the perfusing Krebs medium, the basal tritium release is predominantly [^{3}H]-choline, but the release stimulated by potassium is [^{3}H]-acetylcholine and the levels of [^{3}H]-choline are too small to be defined.

Initial observations revealed that, when using the double potassium stimulation method and calculating an S_2/S_1 ratio, RAMH (3 μM) inhibited tritium release by some 30%. However, the largest effects of RAMH, inhibiting release by approximately 50% at 3–10 μM, were observed when a single potassium stimulation was applied to the tissue slices. We have no good explanation for this at present. The slope of the RAMH inhibition curve appeared to be shallow. This may have been due to experimental variability. Alternative explanations include the involvement of more than one receptor and further studies are clearly required.

The maximal inhibitory effect of RAMH is similar to that observed in the histamine release assay (Arrang *et al.*, 1987) in which RAMH is reported to inhibit potassium-evoked [^{3}H]-histamine release from slices of rat cerebral cortex preloaded with [^{3}H]-histidine by up to 60%. However, in the noradrenaline (Schlicker *et al.*, 1989) and 5-hydroxytryptamine (Schlicker *et al.*, 1988) release assays, the inhibitory effects of RAMH are considerably less, maximum inhibition being approximately 25%.

RAMH is a selective H_3 receptor agonist, but it is likely that H_1 and H_2 receptors are involved in its regulation of [^{3}H]-acetylcholine release. Thus, when experiments were performed in the presence of mepyramine and ranitidine, the potency of RAMH increased approximately 10 fold. The control potassium stimulated release of tritium, however, was unaffected by the inclusion of these antagonists. Preliminary studies in which ranitidine and mepyramine were studied separately (results not shown) indicate that both H_1 and H_2 receptors were involved in the response to RAMH and the effects of H_1 and H_2 receptor blockade were additive. The precise mechanisms involved are unknown, but one can postulate that H_1 and H_2 receptor stimulation may enhance acetylcholine release. In histamine release assays (Arrang *et al.*, 1983) the inhibitory action of exogenous histamine is unaffected by the presence of mepyramine and cimetidine. In addition, the pD_2 value for RAMH is 8.4 (Arrang *et al.*, 1987), over 10 fold greater than that observed in the acetylcholine release assay. The difference may be explained by a lower receptor reserve or less efficient coupling in the [^{3}H]-acetylcholine release assay.

Confirmation that the inhibitory effects of RAMH on acetylcholine release are mediated through the H_3 receptor come from antagonist studies. Thioperamide antagonized the effect of RAMH yielding a pK_B value of 8.4, or 9.0 as calculated with a 2 point curve. These values are in agreement with its antagonist potency in other *in vitro* preparations of the H_3 receptor (e.g. $pK_i = 9.0$, Arrang *et al.*, 1987; $pK_B = 9.0$, Hew *et al.*, 1990; $pK_B = 8.1$, Taylor & Kilpatrick, 1992). Similarly, burimamide ($pK_B = 7.1$) and impromidine ($pK_B = 6.3$) antagonized the effects of RAMH with potency values close to those reported in other *in vitro* assays of the H_3 receptor ($pK_i = 7.2$ and 7.2 respectively, Arrang *et al.*, 1983; $pK_B = 6.8$ and 6.6 respectively, Taylor & Kilpatrick, 1992). However, phenylbutanoylhistamine (PBH; 10^{-5} M)

Table 3 Apparent affinity values of phenylbutanoylhistamine (PBH) and betahistine in four *in vitro* models of the H_3 receptor

H_3 antagonist	*[^{3}H]-ACh release pK_B*	*[^{3}H]-HA release pK_i*	*[^{3}H]-RAMH binding pK_i*	*GPI (NANC) pK_B*
PBH	<5	7.1[a]	6.8[c]	5.6[d]
Betahistine	4.5	5.2[b]	6.2[c]	≤4[d]

RAMH = R-α-methylhistamine; PBH = phenylbutanoylhistamine. References: (a) Timmerman (1990); (b) Arrang *et al.* (1985); (c) Kilpatrick & Michel (1991); (d) Taylor & Kilpatrick (1992)

was without antagonist effect and betahistine was very weak, $pK_B = 4.5$. This is of interest to the developing discussion on the existence of H_3 receptor subtypes. PBH and betahistine are relatively potent inhibitors of the H_3 receptor controlling [^{3}H]-histamine release and inhibiting [^{3}H]-RAMH binding (see Table 3), yet are weak in other H_3 receptor-containing preparations such as non-adrenergic non-cholinergic (NANC)-mediated contractions of the guinea-pig isolated ileum. Clearly, the [^{3}H]-acetylcholine release assay fits in with these latter preparations.

Besides inhibiting the effects of RAMH, thioperamide alone enhanced potassium-evoked tritium release indicating that there may be some endogenous histamine tone. This effect was concentration-dependent and the maximum enhancement was approximately 20%. We may be underestimating this effect since the tissue prestimulation before loading with [^{3}H]-choline would deplete endogenous histamine levels. A similar effect of thioperamide may also occur *in vivo* since a recent report reveals that, when administered peripherally to rats, it increases acetylcholine release as measured by microdialysis (Mochizuki *et al.*, 1991).

In conclusion, the selective H_3 receptor agonist, RAMH, inhibited the release of tritium from slices of rat entorhinal cortex preloaded with [^{3}H]-choline. Antagonists for the H_3 receptor, including the selective compound thioperamide, inhibited the effect of RAMH. These antagonist studies also provide evidence for the postulated existence of subtypes of the H_3 receptor, although these observations need to be investigated further before reaching firm conclusions. Thioperamide alone enhanced release in its own right. This latter effect indicates that there may be an inhibitory H_3 receptor tone on acetylcholine release.

We are very grateful to Miss S. Cook for typing this manuscript.

References

ARRANG, J.M., GARBARG, M., LANCELOT, J.C., LECOMTE, J.M., POLLARD, H., ROBBA, M., SCHUNACK, W. & SCHWARTZ, J.C. (1987). Highly-potent and selective ligands for histamine H_3-receptors. *Nature*, **327**, 117–123.

ARRANG, J.M., GARBARG, M., QUACH, T.J., DAM TRUNG TUONG, T., YERAMIAN, E. & SCHWARTZ, J.C. (1985). Actions of betahistine at histamine receptors in the brain. *Eur. J. Pharmacol.*, **111**, 73–84.

ARRANG, J.M., GARBARG, M. & SCHWARTZ, J.C. (1983). Auto-inhibition of brain histamine release mediated by a novel class (H_3) of histamine receptor. *Nature*, **302**, 832–837.

BARNES, J.M., BARNES, N.M., COSTALL, B., HOROVITZ, Z.P. & NAYLOR, R.J. (1989). Angiotensin II inhibits the release of [^{3}H]-acetylcholine from rat entorhinal cortex *in vitro*. *Brain Res.*, **491**, 136–143.

CLAPHAM, J. & KILPATRICK, G.J. (1992). Histamine H_3 receptor-mediated modulation of [^{3}H]-acetylcholine release from slices of rat entorhinal cortex. *Br. J. Pharmacol.*, **105**, 42P.

HEW, R.W.S., HODGKINSON, C.R. & HILL, S.J. (1990). Characterization of histamine H_3-receptors in guinea-pig ileum with H_3-selective ligands. *Br. J. Pharmacol.*, **101**, 621–624.

ICHINOSE, M. & BARNES, P.J. (1989). Histamine H_3-receptors modulate nonadrenergic noncholinergic neural bronchoconstriction in guinea-pig *in vivo*. *Eur. J. Pharmacol.*, **174**, 49–55.

ICHINOSE, M., STRETTON, C.D., SCHWARTZ, J.C. & BARNES, P.J. (1989). Histamine H_3-receptors inhibit cholinergic neurotransmission in guinea-pig airways. *Br. J. Pharmacol.*, **337**, 588–590.

KILPATRICK, G.J. & MICHEL, A.D. (1991). Characterisation of the binding of the histamine H_3 receptor agonist [^{3}H]-(R)-alpha methyl histamine to homogenates of rat and guinea-pig cortex. *Agents Actions*, **33**, 69–75.

MOCHIZUKI, T., YAMATODANI, A., OKAKURA, K., HORII, A. & WADA, H. (1991). Histaminergic modulation of hippocampal acetylcholine release. *Soc. Neurosci.*, **17**, part 1, p169.8.

POLI, E., STARK, H. & BERTACCINI, G. (1991). Histamine H_3 receptor activation inhibits acetylcholine release from the guinea-pig myenteric plexus. *Agents Actions*, **33**, 167–169.

SCHLICKER, E., BETZ, R. & GOTHERT, M. (1988). Histamine H_3 receptor-mediated inhibition of serotonin release in the rat brain cortex. *Naunyn-Schmiedebergs. Arch. Pharmacol.*, **337**, 588–590.

SCHLICKER, E., FINK, K., HINTERTHANER, M. & GOTHERT, M. (1989). Inhibition of noradrenaline release in the rat brain via presynaptic H_3 receptors. *Naunyn-Schmiedebergs. Arch. Pharmacol.*, **340**, 633–638.

TAYLOR, S.J. & KILPATRICK, G.J. (1992). Characterization of histamine H_3 receptors controlling non-adrenergic non-cholinergic contractions of the guinea-pig isolated ileum. *Br. J. Pharmacol.*, **105**, 667–674.

TIMMERMAN, H. (1990). Histamine H_3 ligands: Just pharmacological tools or potential therapeutic agents? *J. Med. Chem.*, **33**, 4–11.

TRZECIAKOWSKI, J.P. (1987). Inhibition of guinea-pig ileum contractions mediated by a class of histamine receptor resembling the H_3 subtype. *J. Pharmacol. Exp. Ther.*, **243**, 874–880.

(Received May 26, 1992
Revised July 20, 1992
Accepted July 24, 1992)

Br. J. Pharmacol. (1992), **107**, 924–931

Noradrenaline modulates smooth muscle activity of the isolated intravesical ureter of the pig through different types of adrenoceptors

[1]M. Hernández, D. Prieto, U. Simonsen, L. Rivera, M.V. Barahona & A. García-Sacristán

Departamento de Fisiología, Facultad de Veterinaria, Universidad Complutense, 28040-Madrid, Spain

1 We have studied the effects of α- and β-adrenoceptor agonists and antagonists on both phasic peristaltic activity and basal tone of the isolated intravesical ureter of the pig by means of isometric techniques *in vitro*.

2 Spontaneous phasic activity was exhibited by 21% of pig intravesical ureter preparations manifested as rhythmic contractions with average frequency and amplitude of 2.54 ± 0.18 min^{-1} and 1.48 ± 0.16 g ($n = 31$), respectively.

3 Adrenaline, noradrenaline and phenylephrine induced concentration-dependent increases in both phasic activity and basal tone of ureteral preparations, all three agonists being more potent in modifying ureteral phasic activity than baseline tone. B-HT 920, B-HT 933 and clonidine had no significant effect.

4 Phentolamine (10^{-9}– 10^{-7} M) and prazosin (3×10^{-11}– 3×10^{-8} M) significantly inhibited increases in both frequency of phasic activity and baseline tone induced by a submaximal dose of noradrenaline. Rauwolscine (10^{-9}– 10^{-7} M) affected only the tone evoked by noradrenaline and higher concentrations of this antagonist were needed to block phasic activity.

5 Pretreatment of ureteral strips with the β-adrenoceptor antagonist, propranolol (10^{-6} M), significantly increased the maximum contraction evoked by noradrenaline. After incubation with phentolamine (10^{-6}M), noradrenaline (10^{-7}–10^{-6} M) decreased phasic activity induced by prostaglandin $F_{2\alpha}$ (10^{-5} M). Isoprenaline and salbutamol also abolished $PGF_{2\alpha}$-induced phasic activity. Pafenolol (10^{-6} M) and butoxamine (10^{-6} M) blocked the inhibitory effect of noradrenaline, isoprenaline, and salbutamol on $PGF_{2\alpha}$-induced phasic activity.

6 These results suggest that noradrenaline may modulate both phasic peristaltic activity and basal tone of pig intravesical ureter through both α- and β-adrenoceptors.

Keywords: Porcine intravesical ureter; α-adrenoceptors; β-adrenoceptors; phasic activity; tone

Introduction

Transport of urine from pelvis to bladder is due to spontaneous peristaltic contractions of the ureter initiated by pacemaker activity at the renal pelvis and sustained, in part, by the local release of prostaglandins (Notley, 1970; Al-Ugaily *et al.*, 1986; Thulesius *et al.*, 1986). Peristalsis, which represents the phasic contraction of the ureteral smooth muscle is most likely myogenic, the autonomic nervous system playing a modulating role (Schulman, 1985).

Blok *et al.* (1985) observed that the ureterovesical junction develops a peristalic activity which promotes active urine bolus transport from ureter to bladder and also reduces outflow resistance of this functional unit during the peristaltic contraction. Moreover, Morita *et al.* (1987) suggested that autonomic nerves may affect the rate of urine transport through the canine ureter by modulating not only peristaltic frequency but also urine bolus volume.

Morphological studies have demonstrated that the density of the nerve supply increases progressively from the top end of the ureter towards the bladder, the pelvic segment being more richly innervated than the lumbar, suggesting a higher nervous control of the intravesical ureter (Hannappel & Golenhofen, 1974). In addition, several studies have shown by means of histochemical techniques, that there is a rich network of adrenergic nerve fibres at the distal ureter and ureterovesical junction (El-Badawi & Schenk, 1971; Schulman *et al.*, 1973; Schulman, 1985; Prieto *et al.*, 1989; 1990) and numerous ganglion cells that constitute the ureterovesical ganglion complex which could play an important role in the coordination of the ureter and bladder activity at the ureterovesical junction (Schulman, 1985).

Since *in vitro* studies at the distal ureter (Labadía *et al.*, 1987) and ureterovesical junction (Benedito *et al.*, 1990; Rivera *et al.*, 1992a,b) are scarce, the aim of the present study was to characterize the postjunctional adrenoceptors involved in the responses of the porcine intravesical ureter to sympathetic neurotransmitters by use of selective α- and β-adrenoceptor agonists and antagonists.

Methods

Adult pigs of either sex with no lesions in their urinary tract were selected from the local slaughterhouse. Urinary bladders with attached ureters were removed immediately after the animals were killed and kept in chilled physiological saline solution (PSS) at 4°C. The adjacent connective and fatty tissues were removed with care and longitudinal preparations (4–6 mm long and 2–3 mm wide) of the intravesical ureter were isolated from the bladder by dissection. The strips were suspended vertically in 30 ml organ baths containing PSS maintained at 37°C and gassed with 95% oxygen and 5% carbon dioxide, pH = 7.4. The distal end of the preparations was attached to a metal hook and the other end connected to isometric transducers (Grass FT 03) with the signal continuously recorded on a polygraph (Grass 79E). Passive tension of 2 g was applied to the preparations and they were allowed to equilibrate for 60 min.

Experimental procedure

The contractile capacity of the preparations was challenged by exposing the preparations to 120 mM potassium-rich physiological saline solution (K^+PSS). Induced phasic

[1] Author for correspondence.

activity described by frequency (number of contractions min^{-1}) and amplitude (g) of rhythmic contractions, and increases in basal tone (g) were examined by addition of isolated increasing concentrations of noradrenaline, adrenaline, the α_1-selective agonist, phenylephrine and the α_2-selective agonists, B-HT 920, B-HT 933 and clonidine. Noradrenaline concentration-response curves were generated in the presence of cocaine (10^{-6} M), corticosterone (10^{-6} M) to block neuronal and extraneuronal uptake, respectively, and propranolol (10^{-6} M) to block the β-adrenoceptors.

Due to the development of a strong tachyphylaxis of the tissue to the agonists, two consecutive concentration-response curves could not be constructed in the same preparation. Since the response to single submaximal concentrations of noradrenaline (10^{-5} M) was reproducible after repetitive exposures, it was used to determine the effect of the non-selective α-adrenoceptor antagonist, phentolamine, the α_1-adrenoceptor antagonist, prazosin and the α_2-adrenoceptor antagonist, rauwolscine. The preparations were incubated with the antagonist for 20 min before noradrenaline was added. Control preparations without antagonist incubation were run parallel to correct for tissue fatigue and time-induced changes. When studying relaxation, the preparations were exposed to a single dose (10^{-5} M) of prostaglandin $F_{2\alpha}$ and after obtaining rhythmic phasic activity, the relaxing action of the non-selective β-adrenoceptor agonist, isoprenaline, or the β_2-adrenoceptor agonist, salbutamol, was determined. Finally, experiments were performed with preparations incubated either with the non-selective β-adrenoceptor antagonist, propranolol, the β_1-adrenoceptor antagonist, pafenolol, or the β_2-adrenoceptor antagonist, butoxamine, and the protocol for the study of relaxation responses as described above was repeated.

Drugs and solutions

The composition of physiological saline solution (PSS) was (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 24.9, glucose 11, KH_2PO_4 1.2, EDTA (ethylene diamine tetra-acetic acid) 0.027. The K^+PSS was PSS with KCl exchanged for NaCl on equimolar basis. Stock solutions were prepared daily in distilled water.

The following drugs were used: (−)-adrenaline HCl, (±)-noradrenaline HCl (Serva, Germany), B-HT 920 (5-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazol-(4,5-d)-azepin-dihydrochloride), B-HT 933 (2-amino-6-ethyl-4,5,7,8-tetrahydro-6H-oxazol-(4,5-d)-azepin-dihydrochloride), isoprenaline HCl (Boehringer Ingelheim, Germany), butoxamine HCl (Burroughs Wellcome, U.K.), clonidine HCl, cocaine HCl, corticosterone, pafenolol HCl, phentolamine HCl, phenylephrine HCl and propranolol HCl (Sigma, U.S.A.), prazosin HCl (Pfizer, U.K.), prostaglandin $F_{2\alpha}$ (Upjohn, U.S.A.), rauwolscine (Roth, Germany) and salbutamol (Glaxo, U.S.A.).

Adrenaline was prepared in 0.25 N HCl and further diluted in twice distilled water. Prazosin was dissolved in warm water (50°C) at pH 4–5 with constant agitation. Prostaglandin $F_{2\alpha}$ was dissolved in benzylic alcohol. The other drugs were dissolved in twice distilled water containing ascorbic acid (10^{-3} M). Previous experiments showed that the solvents had no effect on the preparations.

Calculations

For each concentration-response curve, the concentration required to give half-maximal response (EC_{50}) was determined by computerized iteration, fitting the responses and logarithmic concentrations to the Hill equation (Graph Pad software 3.0, San Diego, Calif., U.S.A.). Sensitivities to drugs are expressed in terms of pD_2 values, where $pD_2 = -\log EC_{50}$, the EC_{50} being the agonist concentration needed to produce 50% of the maximal response. pIC_{50} values for antagonists were calculated as the negative logarithm of the antagonist concentration required to inhibit 50% of the response induced by a single dose (10^{-5} M) of the agonist (Skärby & Larsson, 1987).

Statistics

The results are expressed as mean ± s.e.mean. Statistical differences were calculated by Student's *t* test and one-way analysis of variance (ANOVA) with an *a posteriori* Bonferroni test (Wallestein *et al.*, 1980). Differences were considered significant with a probability level of $P<0.05$.

Results

Spontaneous activity

Of 148 isolated intravesical ureteral preparations of the pig, 31 (21%) showed spontaneous phasic contractions with a frequency of $2.54 \pm 0.18\ min^{-1}$ ($n = 31$) and amplitude of 1.48 ± 0.16 g ($n = 31$) (Figure 1). The spontaneous contractions persisted on average for 5 h after mounting the preparations.

Responses to α-adrenoceptor agonists and antagonists

Adrenaline (Ad), noradrenaline (NA), and phenylephrine (Phe) induced concentration-dependent increases in both frequency of phasic activity and basal tone of porcine intravesical ureter preparations (Figures 2 and 3). However, B-HT 920, B-HT 933 and clonidine had only sporadic or no effect even after precontraction with either 40–80 mM potassium, 10^{-5} M $PGF_{2\alpha}$ or 10^{-5} M phenylephrine.

Concentration-response curves constructed by adding individual doses of noradrenaline could not be repeated for the same preparations due to the development of strong tachyphylaxis to the agonists. Thus, in the first concentration-response curve, the pD_2 and E_{max} values were 5.22 ± 0.04 and 1.33 ± 0.08 ($n = 8$) respectively, while in a second curve the pD_2 and E_{max} values were 5.08 ± 0.03 and 0.97 ± 0.06 ($P<0.05$ and $P<0.01$, respectively, paired *t* test). However, both contractions and increases in frequency induced by a single submaximal dose (10^{-5} M) of noradrenaline were reproducible after repetitive exposures. The frequency and tone developed by a single dose (10^{-5} M) of noradrenaline in a first exposure were 21.25 ± 1.72 con-

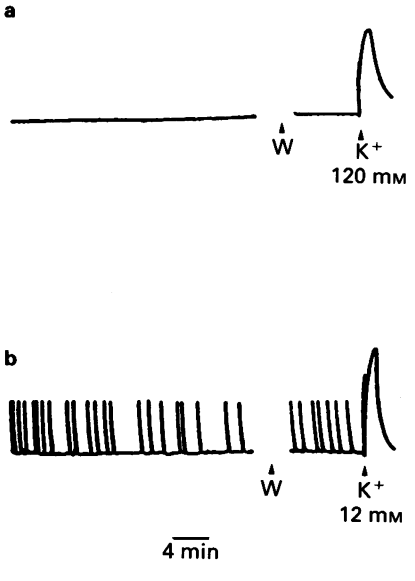

Figure 1 Typical isometric recordings of isolated intravesical ureter of the pig without (a) and with (b) spontaneous phasic activity, but both responding to K^+-PSS.

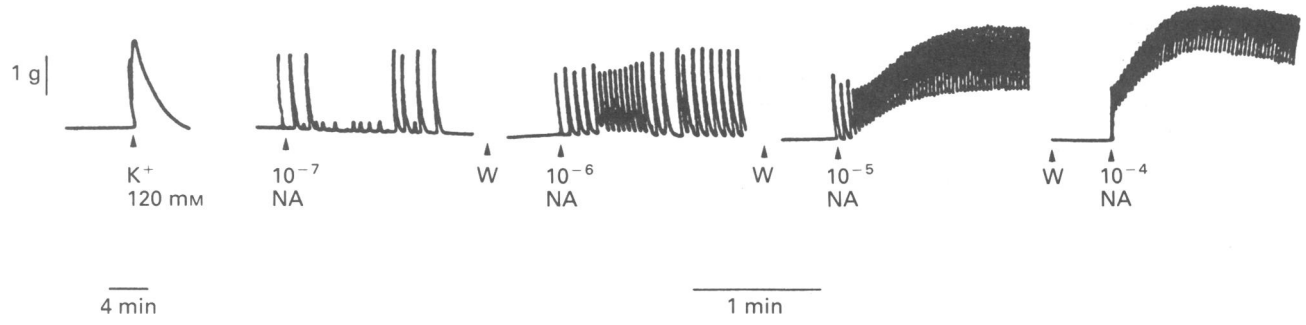

Figure 2 Traces showing the response of intravesical ureteral preparations of the pig to 120 mM K^+-PSS and increasing concentrations of noradrenaline (NA, 10^{-7}–10^{-4} M) added in single doses with washout (W) between each response. Numbers indicate molar concentration in the bath.

tractions min^{-1} and 0.92 ± 0.07 g respectively, compared to 20.56 ± 1.64 contractions min^{-1} and 0.94 ± 0.08 g, respectively, in a fifth exposure ($n = 16$).

Table 1 shows pD_2 and E_{max} values for the different adrenoceptor agonists, for both phasic activity and tone. The agonists were equipotent in inducing tone and phasic activity since there was no significant difference amongst the pD_2 values: Ad = NA = Phe. However, the maximal response (E_{max}) in ureteral basal tone evoked by adrenaline was significantly larger than E_{max} to noradrenaline or phenylephrine: Ad > NA = Phe. The maximum effect, E_{max}, in phasic activity to adrenaline was larger than that to phenylephrine, while E_{max} induced by noradrenaline was not significantly different from those induced by adrenaline or phenylephrine (Table 1). All three agonists were more effective in inducing phasic activity than in increasing basal tone of porcine intravesical ureter preparations (Table 1, Figure 3).

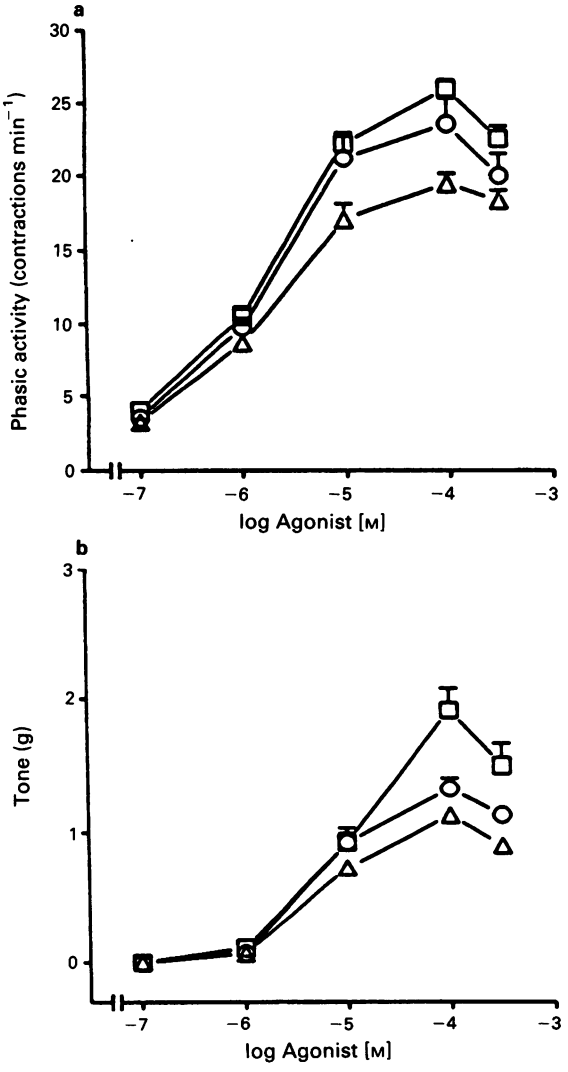

Figure 3 Concentration-response curves of porcine intravesical ureteral preparations to increasing single doses of noradrenaline (○), adrenaline (□) and phenylephrine (△). (a) Phasic activity, and (b) tone of the preparations. Each point represents mean (± s.e.mean, vertical bar) of 8 preparations. Results are expressed as absolute values.

Pretreatment of ureteral strips with the non-selective β-adrenoceptor antagonist, propranolol (10^{-6} M), significantly enhanced the maximum increase in basal tone induced by noradrenaline ($P < 0.05$) without affecting sensitivity. Moreover, cocaine (10^{-6} M) significantly ($P < 0.01$) increased the pD_2 value for phasic activity of the noradrenaline concentration-response curve, but did not affect the E_{max} (Table 2, Figure 4). Incubation with corticosterone (10^{-6} M), which blocks noradrenaline extraneuronal uptake, did not alter the response to 10^{-5} M noradrenaline; the tone was 1.03 ± 0.34 g and the phasic activity 13.25 ± 1.57 contractions min^{-1} before, and tone 1.23 ± 0.37 g and phasic activity 12.88 ± 1.63 contractions min^{-1} after incubation with corticosterone ($n = 6$).

Phentolamine (10^{-9}–10^{-7} M) significantly inhibited both tone ($P < 0.001$) and phasic activity ($P < 0.001$) induced by a submaximal dose of noradrenaline (Table 3, Figure 5). The α_1-adrenoceptor antagonist, prazosin (3×10^{-11}–3×10^{-8} M), caused a significant inhibition ($P < 0.001$) of increases in tone induced by noradrenaline, whereas higher concentrations of antagonist were needed to block the noradrenaline-induced phasic activity (Table 3, Figure 6). Likewise, prazosin significantly ($P < 0.001$) affected both tone ($pIC_{50} = 9.72 \pm 0.09$) and phasic activity ($pIC_{50} = 9.87 \pm 0.06$) evoked by phenylephrine (10^{-5} M) (Figure 7).

The α_2-selective adrenoceptor antagonist, rauwolscine, inhibited increments in basal tone evoked by noradrenaline at concentrations as small as 3×10^{-9} M ($P < 0.01$), but had no effect on phasic activity (Table 3, Figure 8). Tonic contractions to noradrenaline of the porcine intravesical ureter were invariably blocked by α-adrenoceptor antagonists, whereas higher concentrations of antagonists were needed to block the noradrenaline-induced phasic activity (Table 3).

Responses to β-adrenoceptor agonists and antagonists

Figure 9 shows the effects of β-adrenoceptor agonists and antagonists on intravesical ureteral preparations of the pig: 10^{-5} M $PGF_{2\alpha}$ induced phasic activity with a frequency of 3.12 ± 0.31 contractions min^{-1} and an amplitude of 1.69 ± 0.24 g ($n = 12$). Cumulative additions of isoprenaline did not affect the phasic activity induced by $PGF_{2\alpha}$ until reaching concentrations of 10^{-8} M, which totally abolished the phasic activity. The effect of isoprenaline was reversed by 10^{-6} M propranolol. Both pafenolol (10^{-6} M) and butoxamine (10^{-6} M) reversed the inhibitory effect of 10^{-8} M isoprenaline on the $PGF_{2\alpha}$-induced phasic activity, although higher con-

Table 1 Effect of α-adrenoceptor agonists on porcine intravesical ureter

		Tone		*Phasic activity*	
Agonist	n	pD_2	E_{max}	pD_2	E_{max}
Noradrenaline	8	5.22 ± 0.04	1.33 ± 0.08	5.82 ± 0.06*	23.6 ± 0.17
Adrenaline	8	5.05 ± 0.05	1.91 ± 0.17[a]	5.81 ± 0.05*	26.0 ± 0.6
Phenylephrine	8	5.20 ± 0.05	1.13 ± 0.06[b]	5.77 ± 0.07*	19.5 ± 0.7[b]

n, number of ureters. $pD_2 = -\log EC_{50}$; EC_{50} is the effective concentration which induces 50% of the maximal response. E_{max} is the maximum effect in case of ureteral tonus (g) or phasic activity (number contractions min^{-1}). Results are expressed in absolute values as means ± s.e.mean. [a]Significantly different parameter compared to noradrenaline ($P<0.05$, *a posteriori*, Bonferroni). [b]Significantly different parameter compared to adrenaline ($P<0.05$, *a posteriori*, Bonferroni). *Significantly different comparing pD_2 of phasic activity to tone of the agonist response in the same preparation ($P<0.05$, paired *t* test).

Table 2 Effects of cocaine and propranolol on the noradrenaline-induced responses in the porcine intravesical ureter

		Tone		*Phasic activity*	
Antagonist	n	pD_2	E_{max}	pD_2	E_{max}
Control	8	5.31 ± 0.03	1.40 ± 0.08	5.91 ± 0.06	23.3 ± 1.8
Cocaine	6	5.50 ± 0.09	1.50 ± 0.15	6.52 ± 0.15[a]	23.8 ± 2.0
Propranolol	6	5.18 ± 0.10	2.40 ± 0.25[a,b]	5.93 ± 0.06[b]	30.6 ± 1.6
Cocaine + propranolol	6	5.37 ± 0.10	2.06 ± 0.08	5.95 ± 0.02[b]	27.0 ± 1.3

n, number of ureters. $pD_2 = -\log EC_{50}$; EC_{50} being effective concentration to produce 50% of the maximal response. E_{max} is the maximal effect of noradrenaline on basal ureteral tonus (g) or phasic activity (number contractions min^{-1}). Results are expressed in absolute values as means ± s.e.mean. [a]Significantly different parameter compared to control ($P<0.05$, *a posteriori*, Bonferroni). [b]Significantly different parameter compared to cocaine ($P<0.05$, *a posteriori*, Bonferroni).

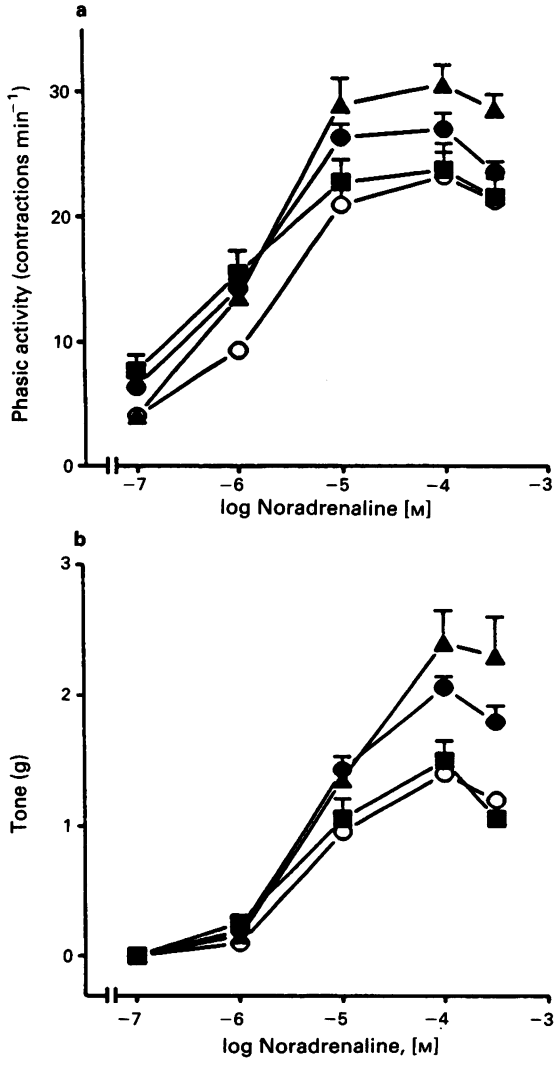

Table 3 Effects of α-adrenoceptor antagonists on tone and phasic activity induced by noradrenaline (10^{-5} M) in porcine intravesical ureter

		Tone	*Phasic activity*
Antagonist	n	pIC_{50}	pIC_{50}
Phentolamine	6	8.43 ± 0.08	7.65 ± 0.57
Prazosin	6	9.57 ± 0.08	8.17 ± 0.06
Rauwolscine	6	8.22 ± 0.20	-

n, number of ureters. pIC_{50} defined as the negative logarithm of the antagonist concentration that causes a 50% inhibition of the contraction induced by noradrenaline (10^{-5} M). Results are expressed in absolute values as means ± s.e.mean.

centrations of isoprenaline were needed to inhibit the phasic activity induced by $PGF_{2\alpha}$ when incubating with pafenolol than when incubating with butoxamine. Moreover, the selective β_2-adrenoceptor agonist, salbutamol (10^{-7} M) abolished the phasic activity induced by $PGF_{2\alpha}$ and this effect was reversed by prior incubation with butoxamine (10^{-6} M).

Discussion

The present *in vitro* study indicates low spontaneous activity in the intravesical ureter and suggests that adrenergic neurotransmitters play an important role in the regulation of ureteral smooth muscle activity. These findings are supported

Figure 4 Concentration-response curves of intravesical ureter of the pig to noradrenaline alone (○) and in the presence of cocaine (■), propranolol (▲), and cocaine and propranolol (●). (a) Phasic activity, and (b) tone of the preparations. Results are means (± s.e.mean, vertical bar) of 6–8 preparations and expressed as absolute values.

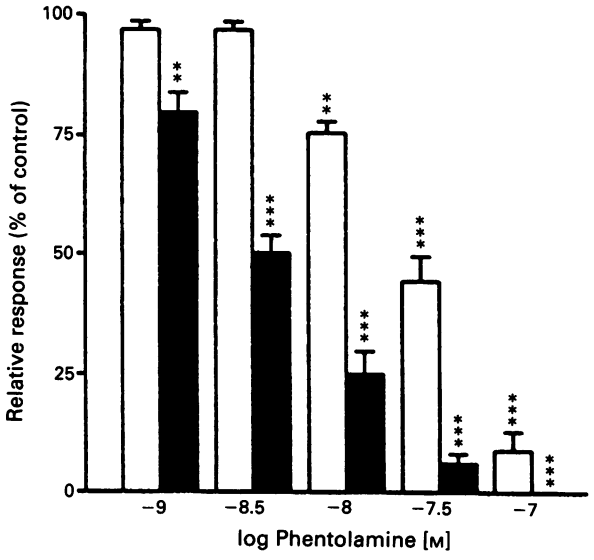

Figure 5 Effects of phentolamine on phasic activity (open columns) and tone (solid columns) induced by noradrenaline (10^{-5} M). Each column represents mean (± s.e.mean, vertical bars) of 6 strips. Results show the relative response to noradrenaline after incubation with increasing concentrations of phentolamine.
*$P<0.05$; **$P<0.01$; ***$P<0.001$ (indicate the response is significant compared to control in absolute values, paired t test).

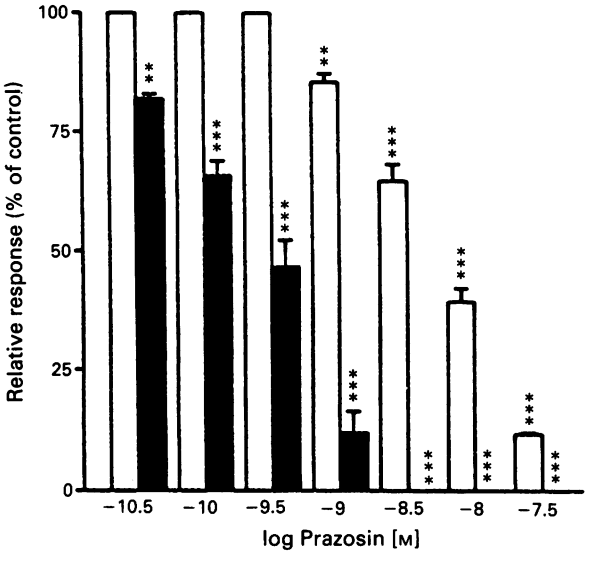

Figure 6 Effects of prazosin on phasic activity (open columns) and tone (solid columns) induced by noradrenaline (10^{-5} M). Each column represents mean (± s.e.mean, vertical bars) of 6 tissues. Results show the relative response to noradrenaline after incubation with increasing concentrations of prazosin.
*$P<0.05$; **$P<0.01$; ***$P<0.001$ (indicate the response is significant compared to control in absolute values, paired t test).

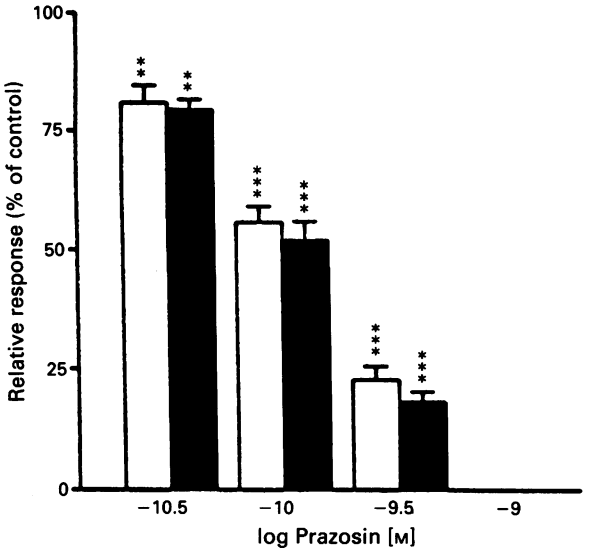

Figure 7 Effect of prazosin on phasic activity (open columns) and tone (solid columns) induced by phenylephrine (10^{-5} M). Each column represents mean (± s.e.mean, vertical bars) of 8 tissues. Results show the relative response to phenylephrine after incubation with increasing concentrations of prazosin.
*$P<0.05$; **$P<0.01$; ***$P<0.001$ (indicate the response is significant compared to control in absolute values, paired t test).

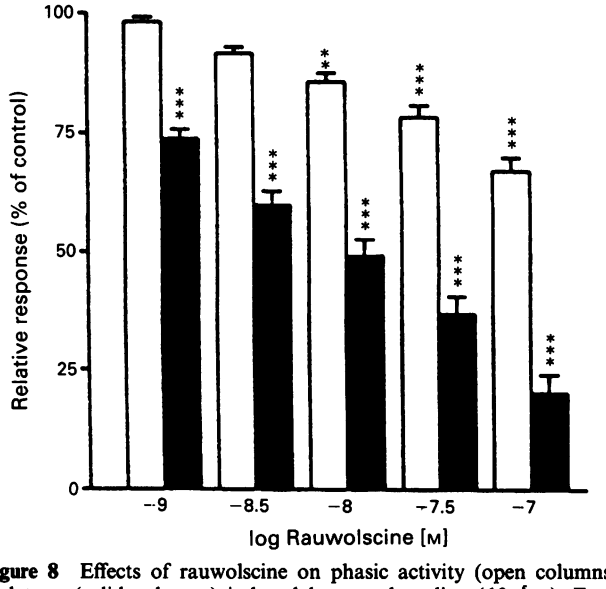

Figure 8 Effects of rauwolscine on phasic activity (open columns) and tone (solid columns) induced by noradrenaline (10^{-5} M). Each column represents mean (± s.e.mean, vertical bars) of 6 experiments. Results show the relative response to noradrenaline after incubation with increasing concentrations of rauwolscine.
*$P<0.05$; **$P<0.01$; ***$P<0.001$ (indicate the response is significant compared to control in absolute values, paired t test).

by the presence of several subpopulations of adrenoceptors probably with different functions. α_1-Adrenoceptors increase both phasic activity and basal tone of the intravesical ureter, while the α_2-adrenoceptors mainly affect the tonus of the ureteral wall. Furthermore, our results show that induced phasic contractile activity is abolished by β-adrenoceptor agonists acting through both subtypes of β-adrenoceptors.

The ureterovesical junction (UVJ) plays an important role in both the active transport of urine bolus from ureter to bladder and the prevention of vesicoureteral reflux during bladder filling (El-Badawi & Schenk, 1971; Blok *et al.*, 1985). Two types of pressure waves in the transport of urine through the ureterovesical junction have been described by use of urodynamic techniques. Firstly, fast pressure waves, which represent ureter peristaltic activity and discharge urine bolus into the bladder and secondly, slow pressure waves due to detrusor activity upon urine transport through the UVJ (Blok *et al.*, 1985). The activity of UVJ as a whole preparation *in vitro* has been characterized (Benedito *et al.*, 1991; Rivera *et al.*, 1992a,b), but to our knowledge this is the first *in vitro* study describing the activity and influence of autonomic drugs on the isolated ureteral component of the UVJ.

Ureteral peristalsis is responsible for transport of the urine

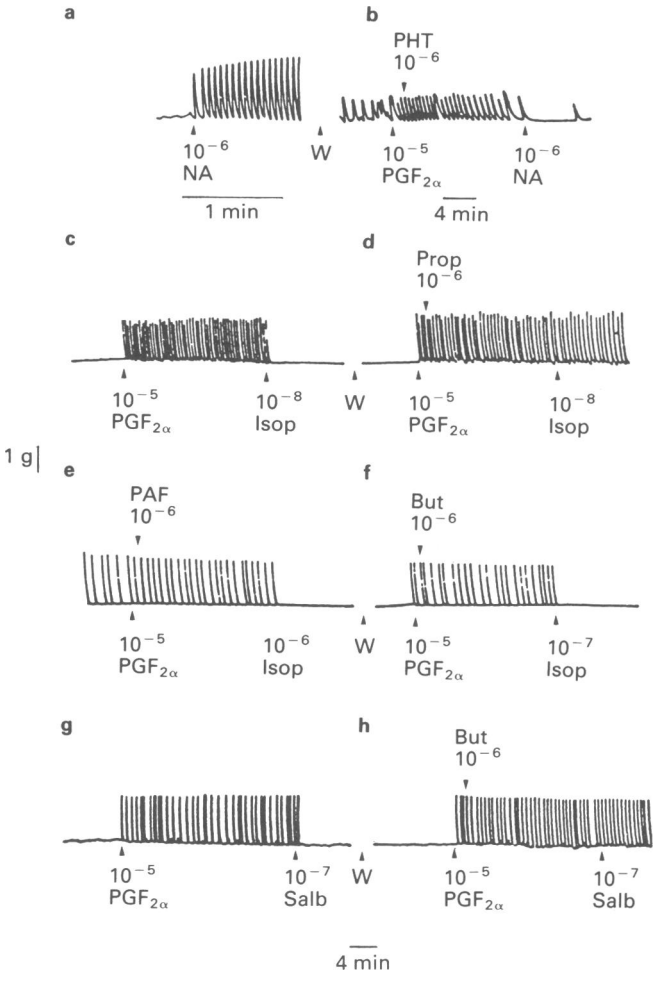

Figure 9 Effect of noradrenaline (NA, 10^{-6} M) in absence (a) and presence (b) of phentolamine (10^{-6} M) (PHT) on phasic activity induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-5} M). The inhibitory effect of isoprenaline (Isop) on phasic activity induced by prostaglandin $F_{2\alpha}$(10^{-5} M) (c). Effects of propranolol (Prop) (d), pafenolol (Paf) (e) and butoxamine (But) (f) on relaxant response of isoprenaline in porcine intravesical ureter. Action of salbutamol (Salb) in absence (g) and presence (h) of butoxamine on pig isolated intravesical ureteral preparations.

bolus from kidney to bladder. This peristalsis is controlled by a pacemaker located in the intrarenal extension of the ureter, calix (Thulesius *et al.*, 1987). In our study, 21% of the intravesical ureteral preparations shows spontaneous phasic contractions with an intermittent motility pattern. Phasic activity might be the consequence of the electrical spikes generated by the renal pacemaker (Mostwing, 1986; Thulesius *et al.*, 1986). Thus, Thulesius *et al.* (1986) found spontaneous contractions in all preparations from intrarenal extension of the ureter, while the spontaneous activity in segments of the dog middle ureter was 49% (Yano *et al.*, 1984). Therefore, the rare spontaneous activity found in our preparations might be ascribed to the distance from the intravesical ureteral segments to the renal pacemaker. Furthermore, there is a higher autonomic innervation with a marked influence at the distal parts of the ureter (Schulman, 1985; Prieto *et al.*, 1989; 1990).

Cocaine caused a significant leftwards shift of the frequency concentration-response curve for noradrenaline in the pig intravesical ureter, inducing earlier appearance of phasic activity, results which agree with those found in the mesenteric artery, where cocaine increased the sensitivity to noradrenaline without affecting the maximum contractile response (Högestätt & Andersson, 1984). According to Langer (1981), our observations therefore suggest that, after cocaine incubation, a higher fraction of the agonist becomes available for the activation of α-adrenoceptors. Since the main role of cocaine is to inhibit the neuronal uptake of noradrenaline, these results reinforce the role of sympathetic nerves in the modulation of ureteral smooth muscle activity.

Several pressure studies in the canine ureter have shown that autonomic nerves affect the rate of urine transport through the ureter by modulating not only ureteral peristaltic frequency but also urine bolus volume. Noradrenaline causes an increase in ureteral peristalsis, an elevation in intraureteral baseline and contractile pressure, and a decrease in a bolus volume, with a resulting decrease in the rate of fluid transport. Moreover, noradrenaline produced a marked increase in ureteral resistance at both low and high flow rates (Morita *et al.*, 1986; 1987).

In our study, adrenaline and noradrenaline evoked dose-dependent contractions of pig intravesical ureter, which suggests that α- predominate over β-adrenoceptor. This is consistent with previous investigations in horse ureter (Labadía *et al.*, 1987) and sheep ureterovesical junction (Rivera *et al.*, 1992a). However, Yano *et al.* (1984) found that noradrenaline and adrenaline produced biphasic changes in the spontaneous contractions of dog isolated middle ureters. At low concentrations, they induced increments in frequency and tone, while at high concentrations, they attenuated both parameters. Thus, they considered that at lower concentrations both noradrenaline and adrenaline would stimulate contractile properties of ureteral smooth muscle through activation of α-adrenoceptors and at higher concentrations they would attenuate them through activation of β-adrenoceptors. These discrepancies between the latter and our results could be due to interspecies differences and, in particular, to regional heterogeneity in the reactivity of ureteral segments to adrenergic stimuli.

In our work, the fact that propranolol increased the maximum effect on tone induced by noradrenaline but not the frequency of phasic activity, could indicate a possible modulatory role of the β-adrenoceptors in the regulation of the ureteral wall tonus.

Due to the development of strong tachyphylaxis to the adrenoceptor agonists and the impossibility of constructing two consecutive concentration-response curves with single doses in the same preparation of porcine ureteral tissue, the α-adrenoceptor characterization in the present study has been based on a comparison of the potency and maximum effects of both agonists and antagonists, following the theoretical model proposed by Skärby & Larsson (1987) for single concentrations of agonists.

Phenylephrine was equipotent with noradrenaline, whereas the selective α_2-agonist, B-HT 920 failed to elicit contractile activity, which suggests a predominance of α_1-subtype adrenoceptors, results similar to those obtained by García-Pascual *et al.* (1991) in the lamb preprostatic urethra.

Phentolamine inhibited the contractile effects on both frequency and tone induced by noradrenaline, which is consistent with functional studies in the dog ureter (Morita *et al.*, 1987) where phentolamine produced a 67% prolongation of the ureteral discharge interval, an 84% increase in bolus volume and an 18% increase in the rate of fluid transport. Moreover, Yano *et al.* (1984) showed that phentolamine reversed the increased responses induced by noradrenaline to the inhibitory ones in dog isolated middle ureters.

The selective block of α_1-adrenoceptors with prazosin produced inhibition of the stimulatory effects of noradrenaline and phenylephrine. The pIC_{50} value (8.2) for prazosin on the noradrenaline-induced phasic activity could be compared to the pA_2 values for the antagonist found in other tissues of the lower urinary tract, such as rabbit trigone, urethral and prostate smooth muscle (Honda *et al.*, 1985), and lamb urethra (García-Pascual *et al.*, 1991). On the other hand, we had earlier reported a lower pA_2 value for prazosin in the sheep ureterovesical junction (Rivera *et al.*, 1992a), which

could be ascribed either to the presence of detrusor muscle in this preparation or to species differences.

Rauwolscine showed less potent inhibitory action than prazosin. In fact, this α_2-selective antagonist only blocked the tone without affecting the phasic activity induced by a submaximal dose of noradrenaline. However, B-HT 920, B-HT 933 and clonidine failed to elicit contractions, despite the initial depolarization induced by low concentrations of potassium, prostaglandin $F_{2\alpha}$ and phenylephrine, which have been found to induce or enhance B-HT 920 contractions in human saphenous vein (Harker *et al.*, 1990) and in cow oviductal artery (Costa *et al.*, 1992). Since the range of concentrations of rauwolscine used in our study is supposed to be selective for α_2-adrenoceptors (Andersson *et al.*, 1984), we cannot discard the presence of α_2-adrenoceptors in porcine ureteral tissue which could be possibly involved in the maintenance of ureteral wall tonus as proposed by Ford *et al.* (1989) in vascular smooth muscle.

On the other hand, noradrenaline had an inhibitory effect on the phasic activity induced by prostaglandin $F_{2\alpha}$ after previous blockade of α-adrenoceptors with phentolamine, which suggests the presence of a population of β-adrenoceptors, which had already been manifested by the increased noradrenaline contractile response after blockade with propranolol. Moreover, isoprenaline caused a total inhibition of phasic rhythmic activity induced by prostaglandin $F_{2\alpha}$ and by prior administration of propranolol, the relaxant response of isoprenaline was abolished. This is consistent with functional *in vivo* studies in the canine ureter, where isoprenaline caused a decrease in ureteral peristalsis frequency and a fall in ureteral wall tonus and contractile pressure or it completely abolished peristalsis and bolus formation. Moreover isoprenaline significantly decreased ureteral resistance at both low and high flow rates. These changes were accompanied by an increase in the rate of fluid transport (Kondo *et al.*, 1985; Morita *et al.*, 1987). Furthermore, Yano *et al.* (1984) confirmed this relaxant response of isoprenaline on canine isolated ureteral preparations. These data agree with those found in other structures of urogenital tract such as oviduct, where the stimulation of β-adrenoceptors facilitates the transport of the ovum or embryon (Isla *et al.*, 1989). However, Bloch *et al.* (1984) showed that isoprenaline had no effect on guinea-pig isolated ureter previously contracted with $BaCl_2$. They concluded that the ureter may have β-adrenoceptors but they probably have little physiological significance. This variability could be due to species differences or the use of different agonists for precontraction of the preparations. The inhibitory effect of isoprenaline on ureteral function and studies of the cyclase-phosphodiesterase system provide strong evidence in favour of a β-adrenoceptor inhibitory mechanism in the ureter.

The inhibitory effect of noradrenaline on phasic activity of pig intravesical ureter could be considered to be mediated by both β_1- and β_2-adrenoceptors, because of the inhibition by pafenolol and butoxamine of the isoprenaline relaxant responses, results similar to those found in horse ureter (Labadía *et al.*, 1987) and sheep ureterovesical junction (Rivera *et al.*, 1992a).

Our data lead us to conclude that noradrenaline increases both phasic activity and basal tone of pig intravesical through a population of α_1-adrenoceptors, although the presence of α_2-adrenoceptors possibly involved in the maintenance of ureteral wall tonus cannot be excluded. Moreover, noradrenaline contractile responses seem to be modulated through a population of β-adrenoceptors which also cause inhibition of ureteral phasic activity through both β_1- and β_2-adrenoceptors.

This work was supported by project PM 88-0035 (DGYCIT). The authors thank Boehringer Ingelheim-Karl Thomae Company for gift of B-HT 920 and B-HT 933 and Madrid Municipal Slaughterhouse for donated ureters. They also wish to thank Miss Julia M. Barton for correcting the manuscript.

References

AL-UGAILY, L., THULESIUS, O. & ANGELO-KHATTAR, M. (1986). New evidence for prostaglandin induced motility of the ureter. *Scand. J. Urol. Nephrol.*, **20**, 225–229.

ANDERSSON, K.-E., LARSSON, B. & SJOGREN, C. (1984). Characterization of the α-adrenoceptors in the female rabbit urethra. *Br. J. Pharmacol.*, **81**, 293–300.

BENEDITO, S., PRIETO, D., RIVERA, L., COSTA, G. & GARCIA-SACRISTAN, A. (1991). Mechanisms implicated in the histamine response of the sheep ureterovesical junction. *J. Urol.*, **146**, 184–187.

BLOCH, R., DECKER, N. & KOSTAKOPOULOS, A. (1984). Effects of theophylline and isoproterenol on the activity of the isolated guinea-pig ureter. *Urol. Int.*, **39**, 308–311.

BLOK, C., VENROOIJ, G.E.P.M. VAN & COOLSAET, B.L.R.A. (1985). Dynamics of the ureterovesical junction: a qualitative analysis of the ureterovesical pressure profile in the pig. *J. Urol.*, **134**, 818–824.

COSTA, G., ISLA, M., GARCIA-PASCUAL, A., JIMENEZ, E., RECIO, P., LABADIA, A. & GARCIA-SACRISTAN, A. (1992). Characterization of postsynapic α-adrenoceptors in the arteries supplying the oviduct. *Br. J. Pharmacol.*, **105**, 381–387.

EL-BADAWI, A. & SCHENK, E.A. (1971). A new theory of the innervation of the urinary bladder musculature. II The innervation of the ureterovesical junction. *J. Urol. (Balt.)*, **105**, 368–371.

FORD, S.P., FARLEY, D.B. & VAN ORDEN, D.E. (1989). Role of postsynaptic α_2-adrenergic receptors (AR) in mediating uterine arterial tone in pigs. *Society for Gynecology Investigation, San Diego C.A.*, Abstr. 127.

GARCIA-PASCUAL, A., COSTA, G., ISLA, M. & GARCIA-SACRISTAN, A. (1991). Characterization of α-adrenoceptors in the preprostatic urethra of sexually immature male lambs. *Eur. J. Pharmacol.*, **203**, 259–265.

HANNAPPEL, J. & GOLENHOFEN, K. (1974). The effect of catecholamines on ureteral peristalsis in different species (dog, guinea-pig and rat). *Pflügers Arch.*, **350**, 55–68.

HARKER, C.T., OUSLEY, P.J., HARRIS, E.J., EDWARDS, J.M., TAYLOR, LL.M. & PORTER, J.M. (1990). The effects of cooling on human saphenous vein reactivity to adrenergic agonists. *J. Vasc. Surg.*, **12**, 45–49.

HOGESTATT, E.D. & ANDERSSON, K.-E. (1984). On the postjunctional α-adrenoceptors in rat cerebral and mesenteric arteries. *J. Auton. Pharmacol.*, **4**, 161–173.

HONDA, K., MIYATA-OSAWA, A. & TAKENAKA, T. (1985). α_1-Adrenoceptor subtype mediating contraction of the smooth muscle in the lower urinary tract and prostate of rabbits. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **330**, 16–21.

ISLA, M., COSTA, G., GARCIA-PASCUAL, A., TRIGUERO, D. & GARCIA-SACRISTAN, A. (1989). Intrinsic spontaneous activity and β-adrenoceptor-mediated tubal dilatation affect ovum transport in the oviduct of the cow. *J. Reprod. Fert.*, **85**, 79–87.

KONDO, S., MORITA, T., SAEKI, H. & TSUCHIDA, S. (1985). Effects of autonomic drugs on in vivo recording of electromyograms of canine renal pelvis and ureter. *Urol. Int.*, **40**, 260–266.

LABADIA, A., RIVERA, L., COSTA, G. & GARCIA-SACRISTAN, A. (1987). Alpha and beta-adrenergic receptors in the horse ureter. *Rev. Esp. Fisiol.*, **43**, 421–426.

LANGER, S.Z. (1981). Presynaptic regulation of the release of catecholamines. *Pharmacol. Rev.*, **32**, 337–362.

MORITA, T., WADA, I., SAEKI, H., TSUCHIDA, S. & WEISS, R.M. (1987). Ureteral urine transport: changes in bolus volume, peristaltic frequency, intraluminal pressure and volume of flow resulting from autonomic drugs. *J. Urol.*, **137**, 132–135.

MORITA, T., WHEELER, M.A. & WEISS, R.M. (1986). Effects of noradrenaline, isoproterenol and acetylcholine on ureteral resistance. *J. Urol.*, **135**, 1296–1298.

MOSTWING, J.L. (1986). Electrical and mechanical aspects of bladder contractility. *D. Phil. Thesis, Oxford.*

NOTLEY, R.G. (1970). The musculature of the human ureter. *Br. J. Urol.*, **42**, 724–727.

PRIETO, D., BENEDITO, S., RIVERA, L., HERNANDEZ, M. & GARCIA-SACRISTAN, A. (1990). Autonomic innervation of the equine urinary bladder. *Anat. Histol. Embryol.*, **19**, 276–285.

PRIETO, D., BENEDITO, S., RODRIGO, J., MARTINEZ-MURILLO, R. & GARCIA-SACRISTAN, A. (1989). Distribution and density of neuropeptide Y-immunoreactive nerve fibers and cells in the horse urinary bladder. *J. Auton. Nerv. Syst.*, **27**, 173–180.

RIVERA, L., HERNANDEZ, M., BENEDITO, S., PRIETO, D. & GARCIA-SACRISTAN, A. (1992a). Mediation of contraction and relaxation by alpha- and beta-adrenoceptors in the ureterovesical junction of the sheep. *Res. Vet. Sci.*, **52**, 57–61.

RIVERA, L., HERNANDEZ, M., BENEDITO, S., PRIETO, D. & GARCIA-SACRISTAN, A. (1992b). Mediation of contraction by cholinergic muscarinic receptors in the ureterovesical junction. *J. Auton. Pharmacol.*, **12**, 175–182.

SCHULMAN, C.C. (1985). Innervation of the ureter: a histochemical and ultrastructural study. In *Urodynamics, Upper and Lower Urinary Tract*, II. ed. Lutzeyer, W. & Hannappel, J. pp. 292–361. Berlin: Springer-Verlag.

SCHULMAN, C.C., DUARTE-ESCALANTE, O., BOYARSKY, S. & GREGOIR, W. (1973). New concepts of ureterovesical innervation. *J. Urol. (Baltimore)*, **109**, 381–384.

SKARBY, T.V. & LARSSON, B. (1987). Theoretical and functional studies on α_1 and α_2-adrenoceptors: and examination using the Schild plot. *J. Auton. Pharmacol.*, **7**, 185–198.

THULESIUS, O., ANGELO-KHATTAR, M. & ALI, M. (1987). The effect of prostaglandin synthesis inhibition on motility of the sheep ureter. *Acta Physiol. Scand.*, **131**, 51–54.

THULESIUS, O., UGAILY-THULESIUS, L. & ANGELO-KHATTAR, M. (1986). Generation and transmission of ovine ureteral contractions with special reference to prostaglandins. *Acta Physiol. Scand.*, **127**, 485–490.

WALLESTEIN, S., ZUCKER, G.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47**, 1–9.

YANO, S., UEDA, S., IKEGAMI, K., MUTOH, S. & SAKANASHI, M. (1984). Actions of some autonomic drugs on spontaneous contractions of isolated dog ureter. *Urol. Int.*, **39**, 100–104.

(Received February 26, 1992
Revised June 1, 1992
Accepted July 27, 1992)

Br. J. Pharmacol. (1992), **107**, 932–938

Modulation of barrier function of bovine aortic and pulmonary artery endothelial cells: dissociation from cytosolic calcium content

[1]Kevin W. Buchan & [2]William Martin

Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

1 Barrier function and cytosolic free calcium content $[Ca^{2+}]_i$ was measured in monolayers of bovine pulmonary artery endothelial cells (BPAEC) and bovine aortic endothelial cells (BAEC).

2 Thrombin (1 u ml^{-1}) increased albumin transfer across monolayers of BPAEC but not BAEC, yet induced biphasic increases in $[Ca^{2+}]_i$ in both endothelial cell types, consisting of a rapid, initial phasic component which decayed to a lower, more sustained plateau phase.

3 4β-Phorbol 12-myristate 13-acetate (PMA; 0.3–3000 nM) increased albumin transfer across monolayers of BPAEC and BAEC, but had no effect on basal levels of $[Ca^{2+}]_i$ in either endothelial cell type.

4 Treatment of BPAEC and BAEC with forskolin (30 μM), an activator of adenylate cyclase, had no effect on resting transfer of albumin, but inhibited that stimulated by PMA (600 nM). It also inhibited the thrombin (1 u ml^{-1})-induced increase in albumin transfer across monolayers of BPAEC, but enhanced the plateau phase of the associated increase in $[Ca^{2+}]_i$.

5 Treatment of BPAEC and BAEC with either atriopeptin II (100 nM), an activator of particulate guanylate cyclase, or 8 bromo cyclic GMP (30 μM) had no effect on resting or PMA (600 nM)-stimulated transfer of albumin. Both agents did, however, inhibit the thrombin (1 u ml^{-1})-induced increase in albumin transfer across monolayers of BPAEC, but had no effect on the associated increase in $[Ca^{2+}]_i$.

6 These data suggest a dissociation between the ability of agents that increase or decrease albumin transfer and their effects on $[Ca^{2+}]_i$. Consequently, activation of protein kinase C may be the major stimulus for trans-endothelial transfer of macromolecular solutes. Endothelial barrier function is enhanced by elevation of either cyclic AMP or cyclic GMP content. Cyclic AMP appears to act by inhibiting the actions of protein kinase C, while cyclic GMP may act to inhibit a key step proximal to activation of this enzyme.

Keywords: Endothelium; vascular permeability, cytosolic calcium; fura-2; cyclic AMP; cyclic GMP; protein kinase C; phorbol esters; thrombin; atrial natriuretic factor

Introduction

The vascular endothelium is the interface between the blood and the interstitium and fulfils the essential function of regulating the exchange of fluid, solutes and cells between these two compartments. This barrier function is subject to dynamic regulation, and is modulated by many factors *in vivo*. For example, increased transfer is stimulated by trans-endothelial endocytosis (Palade, 1960), or, following stimulation by inflammatory mediators such as histamine and bradykinin at post-capillary venules, by endothelial cell contraction and consequent formation of inter-endothelial gaps (Majno & Palade, 1961; Svensjö *et al.*, 1979). Conversely, barrier function can be enhanced by β-adrenoceptor agonists, a property utilised to limit vascular leakage induced by inflammatory mediators (Marciniak *et al.*, 1978; Svensjö *et al.*, 1979).

Important new insights into the mechanisms regulating inflammatory oedema have been gained by the development of endothelial cell culture systems. For example, histamine and thrombin have been shown to increase macromolecular transfer across endothelial monolayers obtained from human umbilical vein (Rotrosen & Gallin, 1986; Killackey *et al.*, 1986) and bovine pulmonary artery (Minnear *et al.*, 1989; Lum *et al.*, 1989).

The precise nature of the effector pathways linking receptor occupation to increases in macromolecular transfer are not, however, fully elucidated. It has been proposed that elevation of cytosolic calcium is the primary trigger on the basis that histamine-induced increases in macromolecular transfer and calcium mobilisation occur over a similar concentration-range (Rotrosen & Gallin, 1986). Furthermore, the calcium ionophore, A23187, induces macromolecular transfer across endothelial monolayers (Shasby *et al.*, 1985; Gudgeon & Martin, 1989) and thrombin-induced transfer is inhibited following inhibition of calcium influx by lanthanum, or buffering of intracellular calcium with quin-2 (Lum *et al.*, 1989). Calcium may not be the only trigger, however, since phorbol esters are known to induce endothelial contraction (Antonov *et al.*, 1986; Grigorian & Ryan, 1987) and macromolecular transfer (Gudgeon & Martin, 1989; Lynch *et al.*, 1990). These actions of phorbol esters are probably mediated by stimulation of protein kinase C since they are mimicked by synthetic diacylglycerols but not by inactive phorbol esters and are blocked by H7, an inhibitor of protein kinase C.

The ability of β-adrenoceptor agonists to enhance barrier function *in vivo* has also been demonstrated in endothelial monolayers cultured from human umbilical vein, bovine pulmonary artery and bovine and porcine aorta (Gudgeon & Martin, 1989; Minnear *et al.*, 1989; Martin & Luck, 1991; Langeler & Van Hinsbergh, 1991). Enhancement of barrier function probably results from elevation of endothelial cyclic AMP content since it is mimicked by other stimulants of adenylate cyclase (Stelzner *et al.*, 1989; Yamada *et al.*, 1990; Langeler & Van Hinsbergh, 1991), namely, forskolin, cholera toxin and iloprost, a stable analogue of prostacyclin. It is

[1] Present address: Glaxo Group Research, Park Road, Ware, Herts, SG12 0DP.
[2] Author for correspondence.

also enhanced by membrane permeant analogues of adenosine 3':5'-cyclic monophosphate (cyclic AMP), and by theophylline and isobutylmethylxanthine, which inhibit phosphodiesterase (Casnocha *et al.*, 1989; Gudgeon & Martin, 1989; Stelzner *et al.*, 1989; Yamada *et al.*, 1990). Enhancement of barrier function has also been reported following treatment with membrane permeant analogues of guanosine 3':5'-cyclic monophosphate (cyclic GMP) or elevation of endothelial cyclic GMP content by atrial natriuretic factors and sodium nitroprusside, which stimulate particulate and soluble guanylate cyclase, respectively (Yamada *et al.*, 1990; Lofton *et al.*, 1991).

The aim of this study was to determine if elevation of cyclic AMP or cyclic GMP content inhibits agonist-stimulated macromolecular transfer across monolayers of endothelial cells cultured from bovine pulmonary artery and aorta by blocking either calcium mobilisation or the stimulation of protein kinase C. A preliminary account of these findings has already been published (Buchan & Martin, 1991a).

Methods

Isolation of bovine aortic and pulmonary artery endothelial cells

Bovine aortic endothelial cells (BAEC) were isolated as described previously (Buchan & Martin, 1991b). Briefly, bovine thoracic aortae were removed shortly after death and flushed with sterile saline containing benzyl penicillin (100 units ml^{-1}) and streptomycin (100 $\mu g\ ml^{-1}$). Following ligation of the proximal end and cannulation of the distal end with an adaptor connected to a syringe, 60 ml of the same saline solution was infused into the lumen, and the aorta was transported back to the laboratory, where all subsequent procedures were carried out in a laminar flow hood. Following ligation of the intercostal arteries, 20 ml of a sterile collagenase solution (0.1%; Type II; Sigma: in Dulbecco's modification of Eagle's Medium (DMEM)) was infused into the lumen and the vessel incubated for 25 min at 37°C in an atmosphere of 5% CO_2 in air. The vessel was then gently massaged and the endothelial cells harvested by centrifugation (200 *g*; 4 min; 10°C) and resuspended in complete culture medium (DMEM containing 10% foetal calf serum, 10% newborn calf serum, 4 mM glutamine, 200 units ml^{-1} benzyl penicillin and 200 $\mu g\ ml^{-1}$ streptomycin). Following a second centrifugation, the cell pellet was resuspended in 50 ml of complete culture medium and seeded into 3 separate 80 cm^2 culture flasks (Gibco). The cells were grown in an atmosphere of 5% CO_2 in air, and typically reached confluence within 4–6 days.

Bovine pulmonary artery endothelial cells (BPAEC) were isolated by a similar method to that outlined above for BAEC. The pulmonary artery was removed, flushed with sterile saline and the proximal end and one of the two distal branches were ligated. The other distal branch was cannulated with an adaptor connected to a syringe and 20 ml of the sterile saline solution was infused into the vessel. At the laboratory, 10 ml of sterile collagenase solution (0.1% in DMEM) was infused into the lumen and the vessel incubated and the cells harvested as indicated above for BAEC. The cells were grown in culture similarly to BAEC except that thymidine (10 μM) was added to enhance growth (Laskey *et al.*, 1990).

Tissue culture materials were obtained from Gibco (Paisley, U.K.) unless otherwise indicated.

Measurement of endothelial barrier function

Upon reaching confluence, each flask of BAEC or BPAEC was washed with 2 × 20 ml of sterile saline and incubated with 10 ml of a solution of trypsin (0.05%)/ethylene diamine tetraacetic acid (EDTA; 0.02%) (Flow Laboratories) until the cells had detached, usually 2–4 min. The cell suspension was then added to 2 ml of newborn calf serum to inactivate the trypsin, and twice spun (200 *g*; 4 min; 10°C) followed by resuspension in 5 ml of complete medium for BAEC and complete medium containing thymidine (10 μM) for BPAEC; 100 μl of the cell suspensions was then added to each of 48 Transwell membrane assemblies (Costar; 6.5 mm diameter; 3 μm pore size). These were then placed in 24 well plates, with each well containing 1 ml of complete medium for BAEC or complete medium containing thymidine (10 μM) for BPAEC, and incubated for a further 2–4 days.

For experimentation, membrane assemblies with cells attached were washed twice by immersion in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 2.4, HEPES (N-2-hydroxyethylpiperazine-N'-2 ethanesulphonic acid) 5 at 37°C at pH 7.4, and transferred to 24 well plates. Thereafter, 600 μl of the same Krebs solution was placed in each of the wells which formed the lower chamber and 100 μl of Krebs containing trypan blue-labelled albumin (4%) was placed above the endothelial monolayer. These volumes were chosen so as to avoid creation of a hydrostatic gradient across the monolayer. Drugs were then added to the top and bottom chambers and the plates placed on an orbital shaker and incubated under an atmosphere of air at 37°C. Any monolayers demonstrating visible leakage within the first 5 min were discarded and those remaining (97%) were incubated for 90 min. At the end of this time, a 100 μl aliquot was removed from each of the lower chambers and transfer of trypan blue-labelled albumin across the monolayers quantified by measuring optical density at 590 nm. In the Results, the transfer of albumin is expressed as a percentage of that which would have been achieved at equilibrium.

The trypan blue-labelled albumin complex was prepared by adding trypan blue (180 mg) and bovine serum albumin (4 g; fraction V; Sigma) to 100 ml of Krebs solution. Precipitation with trichloroacetic acid (6%) showed that the trypan blue was >99.8% albumin bound.

Measurement of intracellular free calcium ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured as previously described (Buchan & Martin, 1991b). Briefly, monolayers of first passage BAEC and BPAEC grown on glass coverslips were incubated for 45 min at 37°C with the penta-acetoxymethyl ester form of fura-2 (2 μM) in HEPES (20 mM)-buffered DMEM (Northumbria Biologicals) containing 1% bovine serum albumin (fraction V; Sigma). A coverslip containing fura-2 loaded cells was then transferred to HEPES (10 mM)-buffered Krebs solution, identical to that used for permeability studies except that KH_2PO_4 was omitted, for 20 min at room temperature to maximize conversion to the calcium-sensitive acid form of fura-2. The coverslip was then suspended across the diagonal of a quartz cuvette containing HEPES (10 mM)-buffered Krebs solution in a Perkin Elmer LS3B fluorimeter and maintained at 37°C with continuous stirring. The beam irradiated the cells without passing through the coverslip. The excitation monochromator was computer-driven between 340 and 380 nm every 3.8 s and fluorescence emission was collected at 509 nm. Background auto-fluorescence was determined at the end of each experiment by permeabilizing the cells to divalent cations with ionomycin (1 μM) and adding Mn^{2+} (2 mM) to quench intracellular fura-2 fluorescence. Following subtraction of auto-fluorescence, the corrected fluorescence values obtained following excitation at 340 nm were divided by those obtained at 380 nm, giving a corrected ratio (R). $[Ca^{2+}]_i$ was then calculated by the computer by the equation of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})\ S_{f2}}{(R_{max} - R)\ S_{b2}}$$

The maximal (R_{max}) and minimal (R_{min}) fluorescence ratios were determined to be 16.3 and 0.8, respectively. S_f and S_{b2} are the fluorescence values obtained following excitation at 380 nm in the absence of calcium and the presence of saturating levels of calcium, respectively, and the ratio of these two values was calculated to be 7.3. The K_d for the fura-2-calcium complex was assumed to be 225 nM at 37°C.

Drugs

Atriopeptin II, 8 bromo cyclic 3':5' guanosine monophosphate (8 bromo cyclic GMP), 4α-phorbol 12,13-didecanoate (4α-PDD), 4β-phorbol 12-myristate 13-acetate (PMA) and thrombin (bovine) were obtained from Sigma, Poole, Dorset. Forskolin, fura-2 penta-acetoxymethyl ester and ionomycin were obtained from Novabiochem, Cambridge, U.K. Solutions of drugs were made in distilled water except for forskolin, fura-2-acetoxymethyl ester and ionomycin which were dissolved in dimethylsulphoxide and PMA and 4α-PDD which were dissolved in 100% ethanol.

Statistical analysis

Results are expressed as the mean ± s.e.mean and comparisons were made by Student's t test or the Mann-Whitney test when there was unequal variance in samples. A probability of 0.05 or less was considered significant.

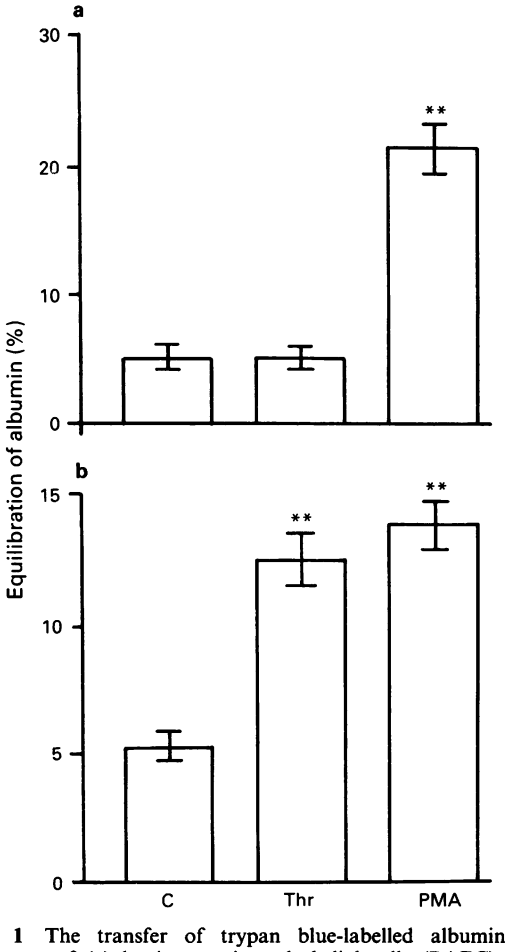

Figure 1 The transfer of trypan blue-labelled albumin across monolayers of (a) bovine aortic endothelial cells (BAEC) and (b) bovine pulmonary artery endothelial cells (BPAEC) during a 90 min incubation period in the absence of drugs (C) and following stimulation with thrombin (Thr; 1 u ml^{-1}) or 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM). Values given are means and vertical bars indicate the s.e.mean of 6 observations. $^{**}P<0.01$ indicates a significant difference from control (C).

Results

Effects of thrombin and a phorbol ester on endothelial barrier function

Resting transfer of trypan blue-labelled albumin across monolayers of BPAEC and BAEC in the 90 min incubation period was typically 2–10% (Figures 1–5). Treatment with thrombin (1 u ml^{-1}) during the 90 min period increased albumin transfer across monolayers of BPAEC, but not BAEC, whereas 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM) increased albumin transfer across monolayers of both endothelial cell types (Figure 1). The ability of PMA to

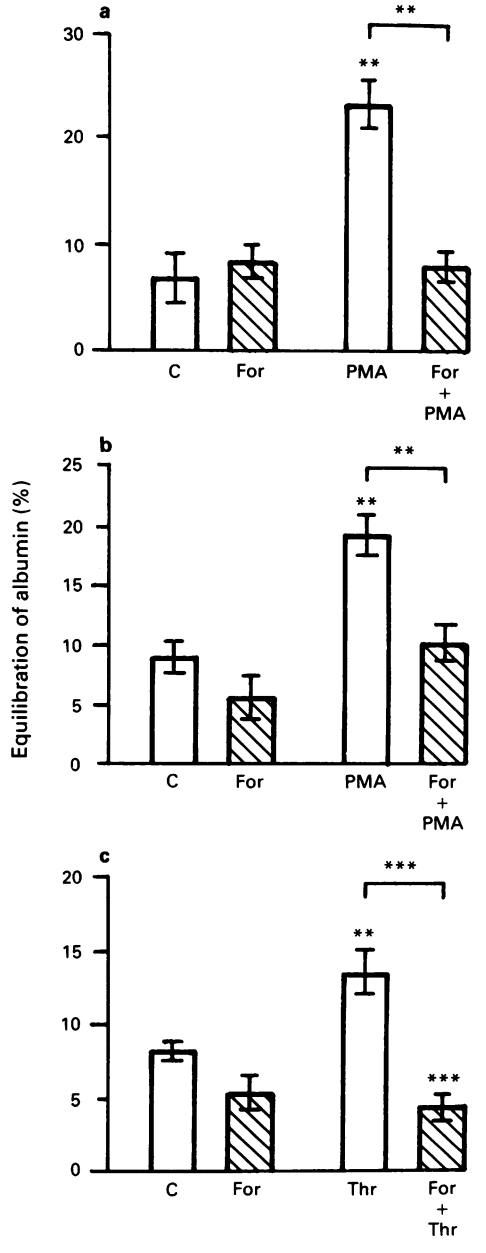

Figure 2 The effects of forskolin (For; 30 μM) on resting transfer of trypan blue-labelled albumin and on transfer stimulated by 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM) across monolayers of (a) bovine aortic endothelial cells (BAEC) and (b and c) bovine pulmonary artery endothelial cells (BPAEC) during a 90 min incubation. The effect of forskolin on albumin transfer stimulated by thrombin (Thr; 1 u ml^{-1}) across monolayers of bovine pulmonary artery endothelial cells is also shown. Values given are means and vertical bars indicate the s.e.mean of 6–12 observations. $^{**}P<0.01$; $^{***}P<0.001$ indicates a significant difference from control (C) or between groups joined by a bracket.

increase albumin transfer was not shared with the inactive phorbol ester, 4α-phorbol 12,13-didecanoate (600 nM; data not shown).

Effects of cyclic nucleotides on endothelial barrier function

Forskolin (30 μM), which activates the catalytic subunit of adenylate cyclase (Seaman & Daly, 1981), had no effect on resting transfer of albumin across monolayers of BPAEC or BAEC, inhibited the increase in albumin transfer stimulated by thrombin (1 u ml^{-1}) and PMA (600 nM) across BPAEC, and inhibited the increase stimulated by PMA (600 nM) across BAEC (Figure 2).

Neither atriopeptin II (100 nM), an activator of particulate guanylate cyclase, nor 8 bromo cyclic GMP (30 μM), a membrane permeant analogue of cyclic GMP, had any effect on resting or PMA (600 nM)-stimulated transfer of albumin across monolayers of BAEC (Figure 3) or BPAEC (Figure 4), but both inhibited that stimulated by thrombin (1 u ml^{-1}) across monolayers of BPAEC (Figures 3 and 4).

Calcium mobilization in endothelial cells

In monolayers of BPAEC and BAEC the basal level of $[Ca^{2+}]_i$ was 106 ± 4 nM ($n = 101$) and 98 ± 4 nM ($n = 127$), respectively. Thrombin (1 u ml^{-1}) induced a biphasic eleva-

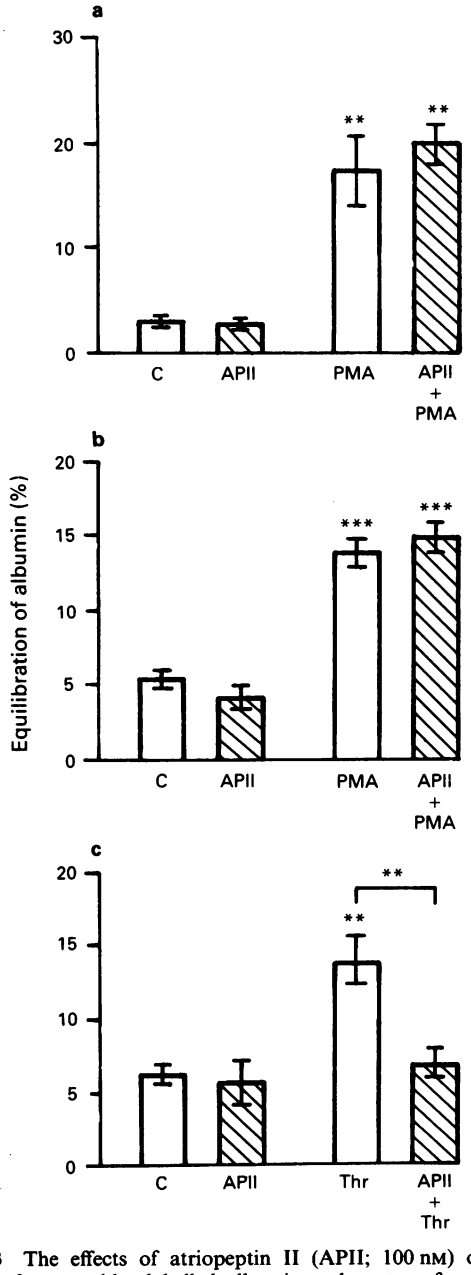

Figure 3 The effects of atriopeptin II (APII; 100 nM) on resting transfer of trypan blue-labelled albumin and on transfer stimulated by 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM) across monolayers of (a) bovine aortic endothelial cells (BAEC) and (b and c) bovine pulmonary artery endothelial cells (BPAEC) during a 90 min incubation. The effect of atriopeptin II on albumin transfer stimulated by thrombin (Thr; 1 u ml^{-1}) across monolayers of bovine pulmonary artery endothelial cells is also shown. Values given are means and vertical bars indicate the s.e.mean of 6 observations. $^{**}P < 0.01$; $^{***}P < 0.001$ indicates a significant difference from control (C) or between groups joined by a bracket.

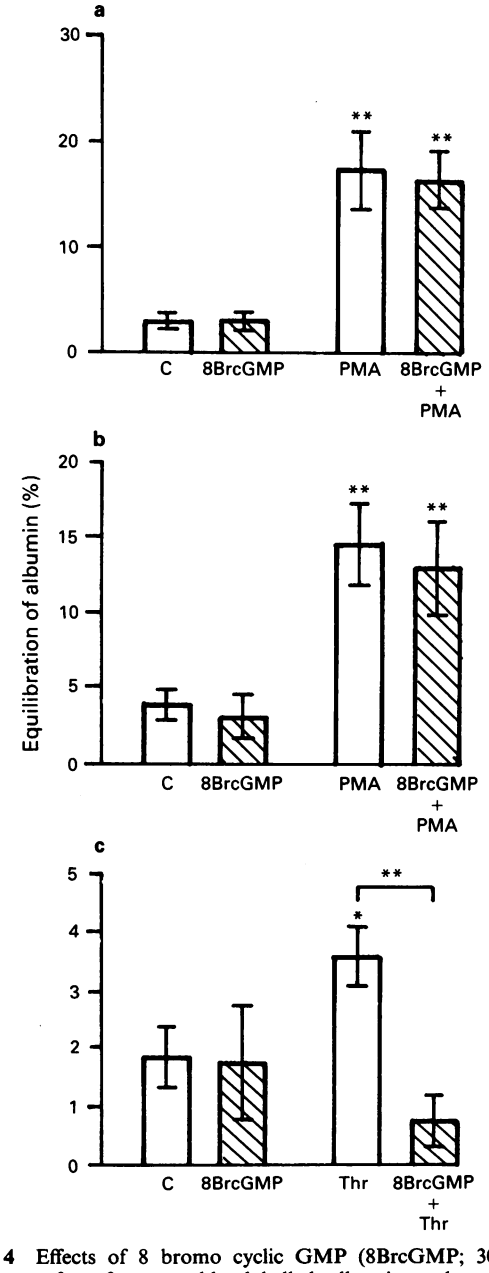

Figure 4 Effects of 8 bromo cyclic GMP (8BrcGMP; 30 μM) on resting transfer of trypan blue-labelled albumin and on transfer stimulated by 4β-phorbol 12-myristate 13-acetate (PMA; 600 nM) across monolayers of (a) bovine aortic endothelial cells (BAEC) and (b and c) bovine pulmonary artery endothelial cells (BPAEC) during a 90 min incubation period. The effect of 8 bromo cyclic GMP on albumin transfer stimulated by thrombin (Thr; 1 u ml^{-1}) across monolayers of bovine pulmonary artery endothelial cells is also shown. Values given are means and vertical bars indicate the s.e.mean of 6 observations. $^{*}P < 0.05$; $^{**}P < 0.01$ indicates a significant difference from control (C) or between groups joined by a bracket.

tion of $[Ca^{2+}]_i$ in both cell types consisting of a large initial peak at around 30 s which then fell to a more sustained plateau within 5 min (Figure 5); the peak and plateau levels were 350 ± 51 nM and 239 ± 21 nM ($n = 9$), respectively, for BPAEC, and 291 ± 30 nM and 180 ± 13 nM ($n = 20$), respectively, for BAEC. In contrast, PMA (1–1000 nM) had no effect on the basal level of $[Ca^{2+}]_i$ in BPAEC or BAEC.

Effects of cyclic nucleotides on thrombin-induced calcium mobilisation in BPAEC

Pretreatment of BPAEC with forskolin (30 μM; 5 min) had no effect on basal levels of $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) (data not shown). Addition of forskolin (30 μM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) did, however, lead to a further rapid increase in $[Ca^{2+}]_i$ of 67 ± 7 nM ($n = 6$), which remained stable for at least 5 min (Figure 5).

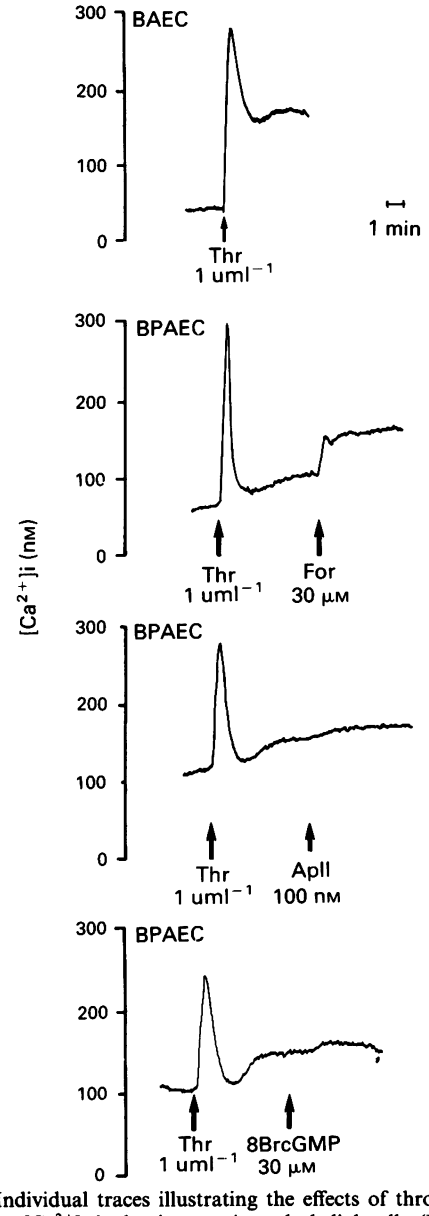

Figure 5 Individual traces illustrating the effects of thrombin (Thr; 1 u ml^{-1}) on $[Ca^{2+}]_i$ in bovine aortic endothelial cells (BAEC) and bovine pulmonary artery endothelial cells (BPAEC). The effects of adding forskolin (For; 30 μM), atriopeptin II (APII; 100 nM) or 8 bromo cyclic GMP (8BrcGMP; 30 μM) during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (Thr; 1 u ml^{-1}) in bovine pulmonary artery endothelial cells are also shown. Each trace is representative of at least 5 separate observations.

Pretreatment of BPAEC for 5 min with either atriopeptin II (100 nM) or 8 bromo cyclic GMP (30 μM) had no effect on basal levels of $[Ca^{2+}]_i$ or on the magnitude of the initial transient elevation of $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) (data not shown). Atriopeptin II (100 nM) and 8 bromo cyclic GMP (30 μM) were both also without effect when added during the plateau phase of the increase in $[Ca^{2+}]_i$ induced by thrombin (1 u ml^{-1}) (Figure 5).

Discussion

The major new finding in this study is that changes in endothelial barrier function can be dissociated from changes in levels of cytosolic calcium ($[Ca^{2+}]_i$). Evidence for this comes from the observation that thrombin stimulates calcium mobilization in both BPAEC and BAEC, yet increases albumin transfer across monolayers of only BPAEC. Furthermore, the phorbol ester, PMA, had no effect on basal levels of $[Ca^{2+}]_i$ in BPAEC or BAEC, yet stimulated albumin transfer across monolayers of both endothelial cell types. It is likely that the ability of phorbol esters to stimulate transendothelial transfer of macromolecular solutes results from activation of protein kinase C, since this action is not shared with phorbol esters which do not activate this enzyme, but is mimicked by synthetic diacylglycerols, and blocked by H7 (Gudgeon & Martin, 1989; Lynch *et al.*, 1990). It is possible, therefore, that activation of protein kinase C represents the major pathway by which inflammatory mediators induce plasma leakage.

At present, it is not clear how activation of protein kinase C inhibits endothelial barrier function, but it is likely to result from endothelial contraction (Antonov *et al.*, 1986; Grigorian & Ryan, 1987) and formation of inter-endothelial gaps. In vascular smooth muscle, activation of protein kinase C induces contraction by increasing the sensitivity of the contractile proteins to calcium (Itoh *et al.*, 1988), and it is possible that a similar mechanism operates in the endothelium. Alternatively, activation of protein kinase C may be responsible for the loss of peripheral bands of F-actin and the resultant disruption of cell-cell contacts (Garcia *et al.*, 1986; Minnear *et al.*, 1989) in a manner similar to that described for a kidney epithelial cell line (Schliva *et al.*, 1984).

Our finding that forskolin, which directly activates the catalytic subunit of adenylate cyclase (Seaman & Daly, 1981), inhibits increases in albumin transfer stimulated by PMA across monolayers of BPAEC and BAEC as well as that stimulated by thrombin across BPAEC is consistent with previous reports of elevated levels of cyclic AMP enhancing endothelial barrier function *in vivo* and *in vitro* (Marciniak *et al.*, 1978; Svensjö *et al.*, 1979; Killackey *et al.*, 1986; Minnear *et al.*, 1989; Gudgeon & Martin, 1989; Carson *et al.*, 1989; Langeler & Van Hinsbergh, 1991). Furthermore, our observation that atriopeptin II, a stimulant of endothelial particulate guanylate cyclase (Schini *et al.*, 1988; Martin *et al.*, 1988), and 8 bromo cyclic GMP inhibit thrombin-stimulated transfer of albumin across monolayers of BPAEC is also consistent with the ability of cyclic GMP to enhance barrier function (Yamada *et al.*, 1990; Lofton *et al.*, 1991).

The mechanisms by which elevations of cyclic AMP or cyclic GMP enhance endothelial barrier function are not clear, but are unlikely to result from inhibition of calcium mobilization, since atriopeptin II and 8 bromo cyclic GMP had no effect on thrombin-induced calcium mobilization and forskolin actually augmented this. This proposal is supported by the observation that elevation of cyclic AMP content inhibits histamine-induced transfer of albumin across monolayers of human umbilical vein endothelial cells but does not block the associated increase in $[Ca^{2+}]_i$ (Carson *et al.*, 1989). It is possible, however, that inhibition of barrier function is exerted through blockade of protein kinase C, since elevation of cyclic AMP content inhibits albumin transfer stimulated

by both thrombin and PMA. In contrast, elevation of cyclic GMP content inhibits albumin transfer stimulated by thrombin, but not PMA, suggesting a different mechanism of action from cyclic AMP. One possible explanation for this is that cyclic GMP may block the ability of thrombin to stimulate protein kinase C, and consistent with this is the ability of cyclic GMP to inhibit production of inositol (1,4,5) trisphosphate in porcine aortic endothelium (Lang & Lewis, 1991). We do not favour this explanation, however, since 8 bromo cyclic GMP and atriopeptin II had no effect on thrombin-induced mobilization of calcium in BPAEC, which presumably involves hydrolysis of phosphatidylinositol-4,5-bisphosphate (Jaffe *et al.*, 1987). It is possible, however, that albumin transfer is stimulated by activation of protein kinase C resulting from hydrolysis of phosphatidylcholine and not phosphatidylinositol-4,5-bisphosphate. This is suggested since diacylglycerol production from the former source is better sustained (Billah & Anthes, 1990) and would be more consistent with the relatively long time course (90 min) required to observe albumin transfer. If elevations of $[Ca^{2+}]_i$ and sustained production of diacylglycerol are subject to differential regulation in the endothelial cell, as in the neutrophil (Cronstein *et al.*, 1988; Cronstein & Haines, 1992), then it might be possible to explain our ability to block the increase in albumin transfer but not $[Ca^{2+}]_i$ stimulated by thrombin in BPAEC. Direct assessment of the differential effects of elevated levels of cyclic GMP on hydrolysis of phosphatidylinositol-4,5-bisphosphate and phosphatidylcholine will be required to test this hypothesis. A related problem that is also difficult to explain at present is the ability of thrombin to stimulate albumin transfer across monolayers of BPAEC but not BAEC. On the basis of the above scheme, it is possible that following stimulation with thrombin, only BPAEC generates diacylglycerol from phosphatidylcholine in the sustained manner necessary to stimulate albumin transfer. Alternatively, the two cell types could generate diacylglycerols, or contain different forms of protein kinase C (Thompson *et al.*, 1991).

In conclusion, the results of this study show that changes in endothelial barrier function can be dissociated from alterations in cytosolic calcium content and suggest protein kinase C as the primary regulator.

We are grateful for support from the British Heart Foundation and the Royal Society.

References

ANTONOV, A.S., LUKASHEV, M.E., ROMANOV, Y.A., TKACHUK, V.A., REPIN, U.S. & SMIRNOV, U.N. (1986). Morphological alterations in endothelial cells from human aorta and umbilical vein induced by forskolin and phorbol 12-myristate 13-acetate: A synergistic action of adenylate cyclase and protein kinase C activators. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 9704–9708.

BILLAH, M.M. & ANTHES, A.C. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.*, **269**, 281–291.

BUCHAN, K.W. & MARTIN, W. (1991a). Inhibition of thrombin-induced increases in endothelial permeability by forskolin and atriopeptin II. *Br. J. Pharmacol.*, **104**, 84P.

BUCHAN, K.W. & MARTIN, W. (1991b). Bradykinin induces elevations of cytosolic calcium through mobilisation of intracellular and extracellular pools in bovine aortic endothelial cells. *Br. J. Pharmacol.*, **102**, 35–40.

CARSON, M.R., SHASBY, S.S. & SHASBY, D.M. (1989). Histamine and inositol phosphate accumulation in endothelium: cAMP and a G protein. *Am. J. Physiol.*, **257**, L259–L264.

CASNOCHA, S.A., ESKIN, S.G., HALL, E.R. & MCINTYRE, L.V. (1989). Permeability of human endothelial monolayers: effect of vasoactive agonists and cAMP. *J. Appl. Physiol.*, **67**, 1997–2005.

CRONSTEIN, B.N. & HAINES, K.A. (1992). Stimulus-response uncoupling in the neutrophil. *Biochem. J.*, **281**, 631–635.

CRONSTEIN, B.N., KRAMER, S.B., ROSENSTEIN, E.D., KORCHAK, H.M., WEISSMANN, G. & HIRSCHHORN, R. (1988). Occupancy of adenosine receptors raises cyclic AMP alone and in synergy with occupancy of chemoattractant receptors and inhibits membrane depolarisation. *Biochem. J.*, **252**, 704–715.

GARCIA, J.G.N., SIFLINGER-BIRNBOIM, A., BIZIOS, R., DEL-VECCHIO, P.J., FENTON, J.W. & MALIK, A.B. (1986). Thrombin-induced increase in albumin permeability across the endothelium. *J. Cell. Physiol.*, **128**, 96–104.

GRIGORIAN, G.Y. & RYAN, U.S. (1987). Platelet-activating factor effects on bovine pulmonary artery endothelial cells. *Circ. Res.*, **61**, 389–395.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

GUDGEON, J.R. & MARTIN, W. (1989). Modulation of arterial endothelial permeability: studies on an *in vitro* model. *Br. J. Pharmacol.*, **98**, 1267–1274.

ITOH, T., KUBOTA, Y. & KURIYAMA, H. (1988). Effects of a phorbol ester on acetylcholine-induced Ca^{2+} mobilisation and contraction in the porcine coronary artery. *J. Physiol.*, **397**, 401–419.

JAFFE, E.A., GRULICH, J., WEKSLER, B.B., HAMPEL, G. & WATANABE, K. (1987). Correlation between thrombin-induced prostacyclin production and inositol triphosphate and cytosolic free calcium in cultured human endothelial cells. *J. Biol. Chem.*, **262**, 8557–8565.

KILLACKEY, J.J.F., JOHNSTONE, M.G. & MOVAT, H.Z. (1986). Increased permeability of microcarrier cultured endothelial monolayers in response to histamine and thrombin. A model for the *in vitro* study of increased vasopermeability. *Am. J. Pathol.*, **122**, 50–61.

LANG, D. & LEWIS, M.J. (1991). Inhibition of inositol 1,4,5,-triphosphate formation by cyclic GMP in cultured aortic endothelial cells of the pig. *Br. J. Pharmacol.*, **102**, 277–281.

LANGELER, E.G. & VAN HINSBERGH, V.W.M. (1991). Norepinephrine and iloprost improve barrier function of human endothelial cell monolayers: role of cAMP. *Am. J. Physiol.*, **260**, C1052–C1059.

LASKEY, R.E., ADAMS, D.J., JOHNS, A., RUBANYI, G.M. & VAN BREEMEN, C. (1990). Membrane potential and Na^+–K^+ pump activity modulate resting and bradykinin-stimulated changes in cytosolic free calcium in cultured endothelial cells from bovine atria. *J. Biol. Chem.*, **265**, 2613–2619.

LOFTON, C.E., BARON, D.A., HEFFNER, J.E., CURRIE, M.G. & NEWMAN, W.H. (1991). Atrial natriuretic peptide inhibits oxidant-induced increases in endothelial permeability. *J. Mol. Cell. Cardiol.*, **23**, 919–927.

LUM, H., DEL-VECCHIO, P.J., SCHNEIDER, A.S., GOLIGORSKY, M.S. & MALIK, A.B. (1989). Calcium-dependence of the thrombin-induced increase in endothelial albumin permeability. *J. Appl. Physiol.*, **66**, 1471–1476.

LYNCH, J.J., FERRO, T.J., BLUMENSTOCK, F.A., BROCKENAUER, A.M. & MALIK, A.B. (1990). Increased endothelial albumin permeability mediated by protein kinase C activation. *J. Clin. Invest.*, **85**, 1991–1998.

MAJNO, G. & PALADE, M.D. (1961). Studies on inflammation: I. The effect of histamine and serotonin on vascular permeability: An electron-microscopic study. *J. Biophys. Biochem. Cytol.*, **11**, 571–605.

MARCINIAK, D.L., DOBBINS, D.E., MACIEJKO, J.J., SCOTT, J.B., HADDY, F.J. & GREGA, G.J. (1978). Antagonism of histamine-induced edema formation by catecholamines. *Am. J. Physiol.*, **234**, H180–H185.

MARTIN, W. & LUCK, J.F. (1991). Enhancement of arterial endothelial barrier function by β-adrenoceptor agonists. *Br. J Pharmacol.*, **102**, 13.

MARTIN, W., WHITE, D.G. & HENDERSON, A.H. (1988). Endothelium-derived relaxing factor and atriopeptin II elevate cyclic GMP levels in pig aortic endothelial cells. *Br. J. Pharmacol.*, **93**, 229–239.

MINNEAR, F.L., DE MICHELE, M.A., MOON, D.G., RIEDER, C.L. & FENTON, J.W.II. (1989). Isoproterenol reduces thrombin-induced pulmonary endothelial permeability *in vitro*. *Am. J. Physiol.*, **257**, H1613–H1623.

PALADE, G.E. (1960). Transport in quanta across the endothelium of blood capillaries. *Anat. Record*, **136**, 254–264.

ROTROSEN, D. & GALLIN, J.I. (1986). Histamine type 1 occupancy increases endothelial cytosolic calcium, reduces F-actin, and promotes albumin diffusion across cultured endothelial monolayers. *J. Cell Biol.*, **103,** 2379–2387.

SCHINI, V., GRANT, N.J., MILLER, R.C. & TAKEDA, K. (1988). Morphological characterisation of cultured bovine aortic endothelial cells and the effects of atriopeptin II and sodium nitroprusside on cellular and extracellular accumulation of cyclic GMP. *Eur. J. Cell Biol.*, **47,** 53–61.

SCHLIVA, M., NAKAMURA, T., PORTER, K.R. & EUTENEUER, U. (1984). A tumor promoter induces rapid and co-ordinated reorganisation of actin and vinculin in cultured cells. *J. Cell Biol.*, **99,** 1045–1059.

SEAMAN, K. & DALY, J.W. (1981). Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. *J. Biol. Chem.*, **256,** 9799–9801.

SHASBY, D.M., LIND, S.E., SHASBY, S.S., GOLDSMITH, J.C. & HUNNINGHAKE, G.W. (1985). Reversible oxidant-induced increases in albumin transfer across cultured endothelium: Alterations in cell shape and calcium homeostasis. *Blood,* **65,** 605–614.

STELZNER, T.J., WEIL, J.V. & O'BRIEN, R.F. (1989). Role of cyclic adenosine monophosphate in the induction of endothelial barrier properties. *J. Cell. Physiol.*, **139,** 157–166.

SVENSJÖ, E., ARFORS, K.-E., RAYMOND, R.M. & GREGA, G.J. (1979). Morphological and physiological correlation of bradykinin-induced macromolecular efflux. *Am. J. Physiol.*, **236,** H600–H606.

THOMPSON, N.T., BONSER, R.W. & GARLAND, L.G. (1991). Receptor-coupled phospholipase D and its inhibition. *Trends Pharmacol. Sci.*, **12,** 404–408.

YAMADA, Y., FURUMICHI, T., FURUI, H., YOKOI, T., ITO, T., YAMAUCHI, K., YOKOTA, M., HAYASHI, H. & SAITO, H. (1990). Roles of calcium, cyclic nucleotides, and protein kinase C in regulation of endothelial permeability. *Arteriosclerosis,* **10,** 410–420.

(Received April 24, 1992
Revised June 26, 1992
Accepted July 27, 1992)

Br. J. Pharmacol. (1992), **107**, 939–944

Dissociation between biochemical and functional effects of the aldose reductase inhibitor, ponalrestat, on peripheral nerve in diabetic rats

[1]Norman E. Cameron & Mary A. Cotter

Department of Biomedical Sciences, University of Aberdeen, Marischal College, Aberdeen AB9 1AS

1 The aim of the study was to examine the effects in rats of two different doses of the aldose reductase inhibitor, ponalrestat, on functional measures of nerve conduction and sciatic nerve biochemistry.

2 After 1 month, streptozotocin-induced diabetes produced 22%, 23% and 15% deficits in conduction velocity of sciatic nerves supplying gastrocnemius and tibialis anterior muscles and saphenous sensory nerve respectively compared to controls. These deficits were maintained over 2 months diabetes.

3 Slower-conducting motor fibres supplying the interosseus muscles of the foot did not show a diabetic deficit compared to onset controls, however, there was a 13% reduction in conduction velocity after 2 months diabetes relative to age-matched controls, indicating a maturation deficit.

4 Resistance to hypoxic conduction failure was investigated for sciatic nerve trunks *in vitro*. There was an increase in the duration of hypoxia necessary for an 80% reduction in compound action potential amplitude with diabetes. This was progressive; after 1 month, hypoxia time was increased by 22% and after 2 months by 57%.

5 The effect of 1-month treatment with the aldose reductase inhibitor, ponalrestat, on the abnormalities caused by an initial month of untreated diabetes was examined. Two doses of ponalrestat were employed, 8 mg kg^{-1} day^{-1} (which is equivalent to, or greater than, the blockade employed in clinical trials), and 100 mg kg^{-1} day^{-1}.

6 Sciatic nerve sorbitol content was increased 7 fold by diabetes. Both doses were effective in reducing this; 70% for 8 mg kg^{-1} day^{-1}, and to within the control range for 100 mg kg^{-1} day^{-1}. However, 8 mg kg^{-1} day^{-1} produced only a modest lowering (44%) of the 8 fold increase in fructose content, indicating that flux through the polyol pathway remained substantially elevated. For 100 mg kg^{-1} day^{-1} ponalrestat, fructose content was within the normal range, indicating a profound inhibition of flux through the pathway.

7 Conduction velocity abnormalities in sciatic motor branches supplying gastrocnemius and tibialis anterior muscles, and sensory saphenous nerve were completely restored by treatment with ponalrestat at 100 mg kg^{-1} day^{-1}, whereas 8 mg kg^{-1} day^{-1} was completely ineffective. The maturation deficit for interosseus motor nerve was unaffected by treatment.

8 Neither 8 or 100 mg kg^{-1} day^{-1} ponalrestat reversed the increased resistance to hypoxic conduction failure resulting from the initial month of untreated diabetes. However, both doses prevented further increases in hypoxic resistance over the treatment period.

9 Three main conclusions were reached. First, substantial blockade of polyol pathway flux is necessary to reverse conduction velocity deficits and this degree of aldose reductase inhibition has not been achieved in clinical trials. Second, nerve content of fructose is a better biochemical indicator of likely functional benefit than that of sorbitol. Third, conduction velocity and hypoxic resistance were differentially affected by the two doses of ponalrestat, a finding that suggests differences in their aetiology.

Keywords: Neuropathy; nerve conduction; ischaemia; aldose reductase; polyol pathway; sorbitol; streptozotocin-induced diabetes; ponalrestat

Introduction

Defects in nerve function in diabetes have been linked to a hyperglycaemia-related increase in polyol pathway activity. Glucose is converted to the sugar alcohol, sorbitol, by the first pathway enzyme, aldose reductase, and sorbitol is subsequently metabolized to fructose by sorbitol dehydrogenase (Dvornik, 1987). It has been suggested that polyol pathway activation is responsible for a decrease in nerve *myo*-inositol concentration, leading to reduced Na-K ATPase pump activity (Green *et al.*, 1985) but substantial doubt has been cast over the obligatory involvement of a Na-K ATPase deficit in diabetic neuropathy (Bianchi *et al.*, 1987). Although several studies in animal models have demonstrated that aldose reductase inhibitors (ARIs) can prevent the slowing of nerve conduction characteristic of early diabetes (Mayer & Tomlinson, 1983; Cameron *et al.*, 1986a,b), their efficacy in either preventing deficits in the longer term (Willars *et al.*, 1988) or correcting established abnormalities has been questioned (Cameron *et al.*, 1989). Furthermore, clinical trials of ARIs have demonstrated only very modest benefits with respect to objective measures such as nerve conduction velocity (NCV), although the regeneration of damaged nerve fibres may be improved (Sima *et al.*, 1988). There are a number of potential explanations for the discrepancy between animal studies and clinical trials. Animal models may be unsuitable; the duration of diabetes is generally much shorter than in patients, there is less overt fibre damage in rat nerves than in biopsy samples from neuropathic patients, and, since they are much longer than those in rats, human nerves may, therefore, be more vulnerable to peripheral vascular disease. However, one factor that clearly differs between rat and human studies is the dose of ARIs employed, generally an order of magnitude

[1] Author for correspondence.

greater in the former. Thus, the main aims of this investigation were to ascertain whether a dose of an ARI similar to the upper limit used in the clinical trials was effective in diabetic rats, and to gauge what level of polyol pathway blockade might be needed to correct established conduction deficits.

In addition to reduced NCV, nerves in diabetic patients and animal models show an increased resistance to ischaemic conduction failure (RICF). This may be partially prevented by ARI treatment (Price *et al.*, 1988), although involvement of the polyol pathway in the aetiology of this abnormality has been disputed (Jaramillo *et al.*, 1984; Carrington *et al.*, 1991). An additional aim was to investigate this phenomenon.

Methods

Male Sprague-Dawley rats (Aberdeen University breeding colony), 19 weeks old at the start of the study were used. One group of non-diabetic animals acted as onset controls. Another was studied 2 months later, acting as age-matched controls. Others were given streptozotocin (45 mg kg^{-1} in 20 mmol l^{-1} sodium citrate buffer, pH 4.5, i.p.). Diabetes was verified 24 h later by estimating hyperglycaemia and glycosuria (Visidex II and Diastix; Ames, Slough). Animals were tested weekly, and weighed daily. They were rejected if blood glucose concentration was <20 mmol l^{-1} or if they showed a consistent increase in body weight over 3 days. Samples for plasma glucose measurement were also taken the day of final experiments.

Diabetic animals were divided into 4 groups. Two were untreated, acting as diabetic controls, and were studied after 1 or 2 months. Two further groups were left untreated for 1 month and were then given ponalrestat (Stribling *et al.*, 1985) either 8 or 100 mg kg^{-1} day^{-1} orally for a further month. The lower dose was chosen to be similar to the highest dose of ponalrestat (600 mg day^{-1}) given to patients in clinical trials (Florkowski *et al.*, 1991).

In final experiments (1–1.5 g kg^{-1} urethane anaesthesia i.p.), NCV was measured *in vivo* between the sciatic notch and knee for motor branches supplying tibialis anterior (peroneal division) and gastrocnemius (proximal tibial division) muscles and the interosseous muscle of the foot (distal tibial division). NCV in sensory nerves was measured in the saphenous nerve between groin and ankle. Methods have previously been described in detail (Cameron *et al.*, 1989).

RICF was measured *in vitro* (Cameron *et al.*, 1991b) after the NCV measurements. The contralateral sciatic trunk was removed and mounted on bipolar stimulating (proximal end) and recording (distal end) electrodes in a chamber filled with Krebs solution (composition, mM: Na^+ 144.0, K^+ 5.0, Ca^{2+} 2.5, Mg^{2+} 1.1, HCO_3^- 25.0, PO_4^{2-} 1.1, SO_4^{2-} 1.1) at 35°C containing 5.5 mmol l^{-1} glucose for nerves from non-diabetic rats and 40 mmol l^{-1} glucose for the diabetic groups. Previous experiments (Cameron, Cotter & D. Cox, unpublished observations) have shown that varying glucose concentration between 5.5 and 40 mmol l^{-1} does not have a significant effect on nerve hypoxic resistance under these conditions. Bathing fluid was gassed with 95% O_2:5% CO_2 (pH 7.35). Nerves were equilibrated for 30 min, then the chamber was refilled with mineral oil pregassed with 100% N_2 for 1 h, and N_2 gassing continued. Nerves were stimulated with just supramaximal pulses (1 Hz, 0.05 ms width, 10 mA) and compound action potential amplitude was monitored at 2 min intervals until it fell below 10% of its initial value. Sciatic nerves used for NCV measurements were rapidly dissected out before rats were killed. They were frozen in liquid nitrogen and then stored at −80°C. Nerve sugars and polyol concentrations were subsequently determined by gas chromatography of trimethyl-silyl derivatives prepared from aqueous deproteinized extracts (Stribling *et al.*, 1985).

Data are expressed as means ± s.e.mean. One-way analysis of variance was performed, followed by the Bonferroni corrected t test to assign differences to individual between-group comparisons when overall significance ($P<0.05$) was attained, using commercial software (Instat, Graphpad, San Diego, CA, U.S.A.).

Drugs

Streptozotocin and urethane were obtained from Sigma and ponalrestat was a gift from I.C.I. Pharmaceuticals.

Results

Body weights and final plasma glucose levels for control and diabetic rats are given in Table 1. Diabetes resulted in progressive weight loss, which after 2 months, was about 25%: controls showed a 16% weight gain over this period. Plasma glucose concentration was elevated approximately 5 fold by diabetes. Ponalrestat treatment at 8 and 100 mg kg^{-1} day^{-1} had no significant effect on these parameters.

Motor NCV results for tibialis anterior and gastrocnemius muscles are shown in Figure 1a and b respectively. There were no significant differences between onset and age-matched non-diabetic control groups. There was a decline in NCV for both nerve branches of around 22% over the first month of untreated diabetes, reaching 25% at 2 months ($P<0.001$ at both time points, for both nerves). When ponalrestat treatment was given to reverse the initial deficit, there was no significant effect with a dose of 8 mg kg^{-1} day^{-1}, but reversal was complete with 100 mg kg^{-1} day^{-1} ($P<0.001$ for both nerves).

Unlike the more proximal motor branches, the interosseous nerve was slower conducting, but increased during the experimental period such that age-matched NCV was greater by 13% than onset controls ($P<0.05$) (Figure 1c). This increase was halted by 2 months' diabetes ($P<0.01$) but there was no significant deficit compared to the onset control level. NCV was not restored to age-matched control values by ponalrestat at either dose level ($P<0.01$) and remained not significantly different from the onset control value. This contrasts with NCV changes in sensory saphenous nerves (Figure 1d) which showed a pattern similar to fast conducting motor nerves; with no significant difference between control groups, a 12% deficit with two months diabetes ($P<0.001$ compared to onset controls) and complete restoration by 100 ($P<0.001$) but not 8 mg kg^{-1} day^{-1} ponalrestat.

Figure 2 illustrates the data from RICF measurements. Initial compound action potential amplitudes are shown in the inset graph and did not differ significantly between groups. When hypoxic, the nerves of onset and age-matched control groups showed a rapid depression of compound action potential amplitude, after a short period of hyperexcitability (Seneviratne & Peiris, 1969). This decline was relatively prolonged in preparations from 1-month diabetic controls and more so after 2 months. Curves for the ponalre-

Table 1 Body weights and plasma glucose concentrations in control and diabetic rats

Group	n	*Weight* (g) *Start*	*Finish*	*Glucose* (mmol l^{-1})
Controls				
Onset	20	484 ± 12	–	6.4 ± 0.3
Age-matched	12	507 ± 19	589 ± 20	8.0 ± 0.5
Diabetic				
1 month	10	461 ± 6	413 ± 12	33.0 ± 3.1
2 month	20	514 ± 11	377 ± 11	40.6 ± 2.4
Ponalrestat-treated				
8 mg kg^{-1} day^{-1}	11	473 ± 9	357 ± 8	35.9 ± 2.2
100 mg kg^{-1} day^{-1}	11	481 ± 10	346 ± 14	38.3 ± 2.7

Data are means ± s.e.mean.

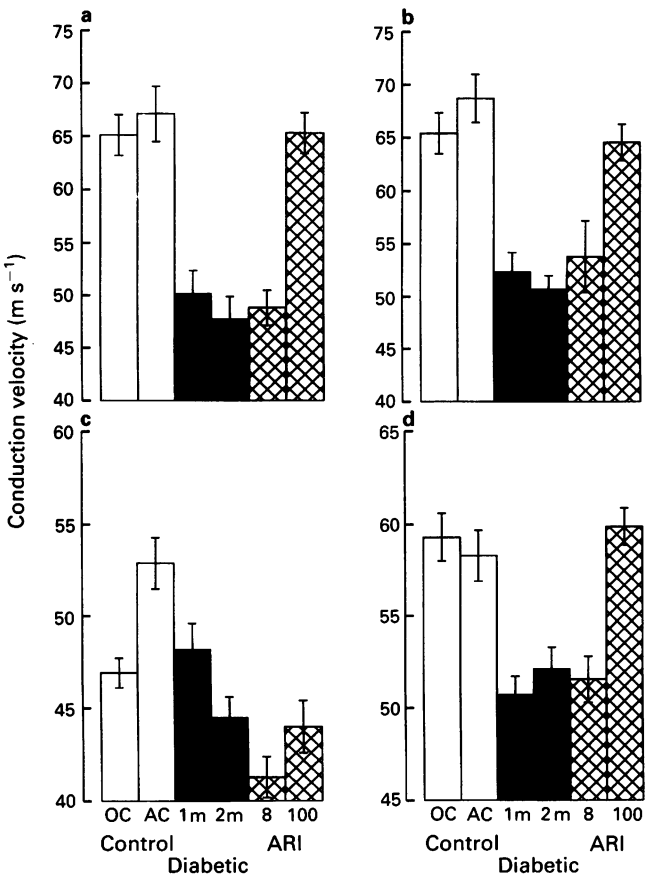

Figure 1 Conduction velocity in motor and sensory nerves for control diabetic and ponalrestat-treated diabetic rats: (a) tibialis anterior; (b) gastrocnemius; (c) interosseus muscles; (d) sensory saphenous nerve. Columns show means (± s.e.means, vertical bars). Controls (open columns): OC, onset controls ($n = 20$); AC, age-matched controls ($n = 12$); diabetic (closed columns), 1 month ($n = 10$), 2 month ($n = 20$); ARI, ponalrestat-treated diabetic (cross-hatched columns), 8 mg kg^{-1} day^{-1} ($n = 11$), 100 mg kg^{-1} day^{-1} ($n = 11$). For tibialis anterior, gastrocnemius and saphenous nerves, values were significantly reduced by 1 and 2 months' untreated diabetes compared to onset or age-matched controls ($P < 0.001$, all comparisons). In ponalrestat-treated diabetic groups, 8 mg kg^{-1} day^{-1} had no significant effect on conduction velocity whereas 100 mg kg^{-1} day^{-1} completely restored conduction ($P < 0.001$, compared to 2-month diabetic controls). For interosseous nerve, conduction velocity in 2 month diabetic controls was reduced compared to age-matched ($P < 0.01$) but not to onset controls. Ponalrestat treatment did not have a significant effect.

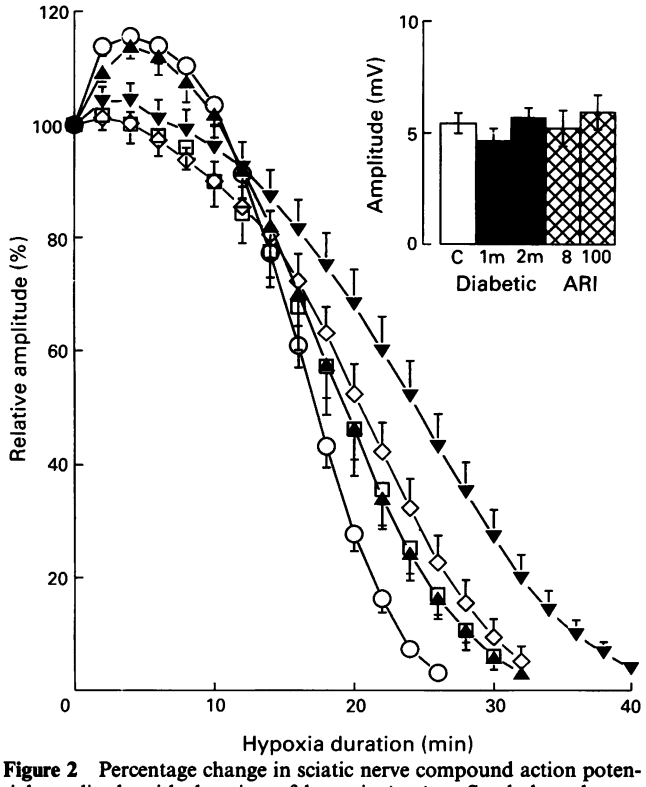

Figure 2 Percentage change in sciatic nerve compound action potential amplitude with duration of hypoxia *in vitro*. Symbols and error bars show group means ± s.e.means. Non-diabetic control, onset and age-matched controls pooled for clarity (○); diabetic control 1-month (▲), 2-month (▼); ponalrestat-treated diabetic groups, 8 mg kg^{-1} day^{-1} (◇), 100 mg kg^{-1} day^{-1} (□). The inset histogram shows initial sciatic nerve compound action potential amplitudes before the period of hypoxia for non-diabetic controls (C, open column bar), 1-month and 2-month diabetic controls (solid columns); and 8 mg kg^{-1} day^{-1} and 100 mg kg^{-1} day^{-1} ponalrestat-treated diabetic (cross-hatched columns) groups. There were no significant between-group differences in initial amplitude.

stat-treated rats lay close to that for the 1 month diabetic controls. This is reflected by the times taken for an 80% reduction in compound action potential amplitude, plotted as a histogram in Figure 3. It shows the progressive nature of the phenomenon with diabetes duration ($P < 0.01$ comparing 1 month diabetes with onset controls, $P < 0.001$ comparing 1- and 2-month diabetic groups). There was a good agreement between the 1-month diabetic controls and the treated groups, both of which were significantly different from the 2 months group ($P < 0.01$ for 8 mg kg^{-1} day^{-1}, $P < 0.001$ for 100 mg kg^{-1} day^{-1}). Thus, both levels of treatment prevented a further increase in hypoxic resistance, but neither reversed the initial deficit ($P < 0.001$ and $P < 0.01$ compared to onset controls for 8 mg kg^{-1} day^{-1} and 100 mg kg^{-1} day^{-1} respectively).

Sciatic nerve polyol concentrations are shown in Table 2. There were no significant differences between control groups so they have been pooled. Similarly, there were no differences between diabetes of 1 or 2 months' duration. With diabetes,

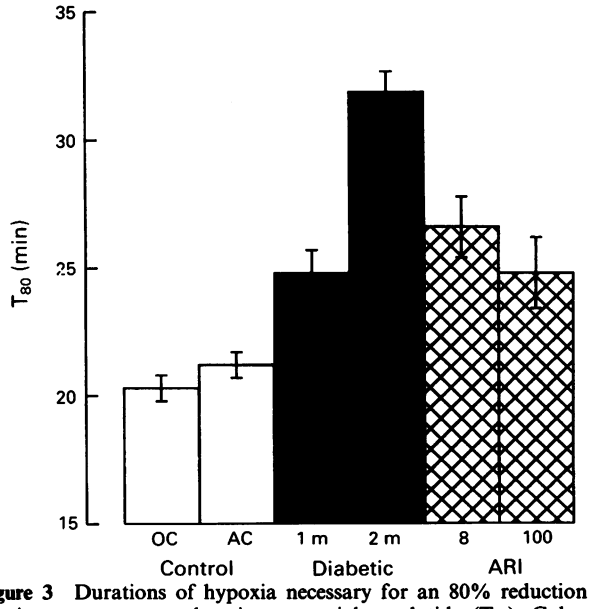

Figure 3 Durations of hypoxia necessary for an 80% reduction in sciatic nerve compound action potential amplutide (T_{80}). Columns show means (± s.e.means, vertical bars). Controls (open columns); OC, onset controls ($n = 20$); AC, age-matched controls ($n = 12$); diabetic (solid columns), 1 m, 1-month ($n = 10$); 2 m, 2-month ($n = 20$); ARI, ponalrestat-treated diabetic (cross-hatched columns), 8 mg kg^{-1} day^{-1} ($n = 11$), 100 mg kg^{-1} day^{-1} ($n = 11$). Untreated diabetes caused a progressive increase in T_{80} ($P < 0.01$ for 1-month group; $P < 0.001$ for 2-month group compared to onset controls). Treatment with 8 and 100 mg kg^{-1} day^{-1} ponalrestat prevented further increases in T_{80} between 1 and 2 months ($P < 0.001$ and $P < 0.001$ respectively compared to the 2-month diabetic group).

Table 2 Sciatic nerve polyol pathway metabolite and *myo*-inositol concentrations in control and diabetic rats

Group	n	*Sorbitol*	*Fructose*	myo-*Inositol*
Control	29	0.282 ± 0.022	0.898 ± 0.069	3.874 ± 0.342
Diabetic	27	1.979 ± 0.129[b]	7.595 ± 0.509[b]	2.067 ± 0.086[b]
Ponalrestat-treated				
8 mg kg^{-1} day^{-1}	11	0.725 ± 0.101[d]	4.634 ± 0.637[b,d]	2.710 ± 0.210[a]
100 mg kg^{-1} day^{-1}	11	0.104 ± 0.008[d,e]	0.428 ± 0.037[d,f]	3.160 ± 0.230[c]

Data are means ± s.e.means, expressed as nmol mg^{-1} nerve wet weight.
[a]$P<0.05$; [b]$P<0.001$: versus control group.
[c]$P<0.05$; [d]$P<0.001$: effect of ponalrestat treatment versus diabetic group.
[e]$P<0.05$; [f]$P<0.001$: effect of level of ponalrestat treatment, 100 mg kg^{-1} day^{-1} versus 8 mg kg^{-1} day^{-1}.

sorbitol concentration was increased 7 fold, and fructose showed a corresponding 8 fold elevation; 8 mg kg^{-1} day^{-1} ponalrestat reduced the excess sorbitol levels by 70%, but had a lesser effect (44%) on fructose concentration. By contrast, the 100 mg kg^{-1} day^{-1} dose reduced both sorbitol and fructose to within or below the control concentration range. Nerve *myo*-inositol concentration was decreased by 47% in diabetic animals; although the deficit was ameliorated to the extent of 60% with 100 mg kg^{-1} day^{-1} it was not significantly affected by treatment at 8 mg kg^{-1} day^{-1}.

Discussion

The data demonstrate two main points. First, a minor degree of polyol pathway inhibition (8 mg kg^{-1} day^{-1} ponalrestat), which largely blocked sorbitol accumulation but did not substantially reduce fructose concentration, also did not restore changes in motor or sensory NCV in diabetic rats. When the nerve fructose concentration was normalized by treatment with the high dose (100 mg kg^{-1} day^{-1}) of ponalrestat, indicating a substantial inhibition of polyol pathway flux, NCV was restored. In previous studies, we used an intermediate dose of ponalrestat (25 mg kg^{-1} day^{-1}) and found sorbitol concentrations were normal, fructose was somewhat elevated, and NCV changes only partially reversed (Cameron *et al.*, 1989). Taken together, this suggests that a high degree of pathway blockade is necessary for optimal effects on NCV, and that fructose concentration provides a better biochemical indicator than sorbitol concentration for potential functional improvements. Second, different measures of nerve function show differential sensitivity to polyol pathway inhibition since the low dose ARI had no effect on NCV whilst largely preventing a further increase in RICF.

In clinical trials, regardless of the ARI employed, polyol pathway inhibition was no better than found in rats with 8 mg kg^{-1} day^{-1} ponalrestat. For example, reductions of erythrocyte or nerve biopsy sorbitol concentration of about 50% have been reported (reviewed in Dvornik, 1987). This suggests a fairly low degree of blockade of the pathway, probably insufficient to test adequately the hypothesis that enhanced polyol pathway flux makes a major contribution to the aetiology of diabetic neuropathy. Thus, it is likely that the failure to find significant improvements in clinical trials of ponalrestat (Florkowski *et al.*, 1991) reflects the use of a drug dose that did not produce adequate inhibition of aldose reductase.

The lack of effect of ponalrestat (8 or 100 mg kg^{-1} day^{-1}) on interosseous NCV agrees with a previous finding with 25 mg kg^{-1} day^{-1} (Cameron *et al.*, 1989). The NCV deficit arises from comparison with age-matched rather than onset controls and can largely be explained by lack of nerve growth resulting in small diameter fibres that have a normal NCV for their size (Cameron *et al.*, 1986b). Thus, ponalrestat treatment cannot restore normal nerve growth in our experimental model. Other workers have found increases in interosseous NCV with ARI treatment, but usually in younger rats in a more rapid growth phase (Gillon *et al.*, 1983; Mayer & Tomlinson, 1983). In addition, methodological considerations may explain these conflicting results. Concentric bipolar recording electrodes were used in this investigation to ensure focal recording. In the other studies unipolar needle electrodes were used, thus, contamination by potentials from nearby muscles whose nerves respond more like gastrocnemius or tibialis anterior to treatment cannot be excluded.

The cause of increased RICF in diabetic nerves is disputed, there being two main schools of thought. According to the metabolic hypothesis, nerves are more resistant to ischaemia because a major requirement for ATP is to supply the Na-K ATPase pump. Na-K ATPase activity is reduced by around 50% in homogenates from diabetic nerves compared to controls (Das *et al.*, 1976; Lambourne *et al.*, 1988; Cameron *et al.*, 1991c). Diabetic nerves could, therefore, utilize energy stores more slowly and maintain function longer when oxidative metabolism is prevented. ARIs would be expected to prevent increased RICF as treatment improves Na-K ATPase activity (Greene *et al.*, 1985), although this has been disputed (Lambourne *et al.*, 1988), the contradictory results being explained by differences in dietary composition and measurement procedure (Sredy *et al.*, 1991). A suggested mechanism is that a diabetic deficit in nerve *myo*-inositol, as noted in this study, leads to reduced membrane phosphoinositide turnover, and less diacylglycerol-mediated activation of protein kinase C, which in turn increases Na-K ATPase activity (Greene *et al.*, 1985). ARI-treatment tends to restore *myo*-inositol levels, as noted for the 100 mg kg^{-1} day^{-1} dose in this study; therefore, it may be expected to restore Na-K ATPase activity.

The same polyol-pathway-dependent Na-K ATPase deficit has also been suggested to explain reduced NCV (Greene *et al.*, 1985). Thus, NCV and RICF changes should occur in parallel, whereas they were dissociated between the two ARI doses in this study. The time-course for NCV changes and RICF development also differ. NCV deficits develop over the first 2 weeks of diabetes, and have virtually reached asymptote by 1 month, with little further change to 4 months (Cameron *et al.*, 1989). In contrast, RICF shows a fairly linear increase with time, as noted in the present study for 1 and 2 months. In addition, other findings are at variance with this metabolic hypothesis. RICF was improved by 8 mg kg^{-1} day^{-1} ponalrestat, whereas *myo*-inositol levels were not significantly affected. In normal rats fed a galactose-enriched diet, the polyol pathway is stimulated (Dvornik, 1987); however, rather than reducing sciatic nerve Na-K ATPase activity, it is doubled (Lambourne *et al.*, 1988). This is accompanied by NCV and RICF abnormalities very similar to those noted for experimental diabetes (Low & Schmelzer, 1983; Cameron *et al.*, 1992), whereas the prediction is that these parameters would be normal or even supranormal.

An alternative vascular hypothesis better explains the data. Sciatic blood flow is reduced soon after the induction of diabetes in rats (Cameron *et al.*, 1991b), producing endoneurial hypoxia (Low *et al.*, 1987). In galactosaemic rats, nerve perfusion is also impaired (Myers & Powell, 1984; McManis *et al.*, 1986). Increased RICF under such conditions may be viewed as an adaptive response to improve ATP

supply by increased use of anaerobic metabolism (Low *et al.*, 1987). ARI treatment increases nerve blood flow (Yasuda *et al.*, 1989), which could be sufficient to restore ATP production and NCV. In addition, when polyol pathway flux is high, glucose is diverted through the pentose phosphate shunt to supply NADPH, a cofactor for aldose reductase (Dvornik, 1987); a process which requires ATP (Davidson & Murphy, 1985). Thus, polyol pathway activity may have both vascular and metabolic effects which contribute to RICF. When, however, these are corrected by ARIs there is no obvious adaptive stimulus to switch back to near total reliance on oxidative metabolism, given the glucose availability in diabetes. Thus, ARIs can restore NCV but, in a reversal experiment, simply halt the progressive increase in RICF.

The present results on RICF are in agreement with a previous study of partial prevention of the deficit by 25 mg kg^{-1} day^{-1} ponalrestat *in vivo* (Price *et al.*, 1988). In a recent report, however, Carrington *et al.* (1991) suggested that imirestat, a spiroimide-derived ARI, did not prevent the development of RICF, measured *in vitro*. We have replicated the present effect of ponalrestat (Cameron, Cotter & S. Hunter, unpublished observations), an acetic acid derivative, using a structurally unrelated sulphonylnitromethane compound (Mirrlees *et al.*, 1991). Spiroimide-derived ARIs stimulate Na-K ATPase independent of their effects on the polyol pathway (Garner & Spector, 1987), whereas ponalrestat does not. It is likely that such a chronic effect *in vivo* would cause increased ATP utilization, further encouraging the use of anaerobic metabolism, which would tend to increase RICF and cancel any beneficial effects of polyol pathway blockade. Between-study differences may also depend on diabetes duration, the experimental design, or the method of measurement. The investigation of Carrington *et al.* (1991) concerned preventative effects, over 1-month diabetes. Thus, that study examined the early stages of RICF development, whereas our investigation focused on later stages. In addition, Carrington *et al.* (1991) examined only the early stages of hypoxic conduction failure, evoked potentials being reduced by 20–40%, whereas this study examined reductions of $>80\%$ in all groups. Thus, the imirestat experiments monitored RICF in large myelinated fibres, which are most susceptible to diabetes (Cameron *et al.*, 1986b). In contrast, the present investigation examined all myelinated fibres; the differences between groups became more pronounced as evoked potential amplitude was reduced, indicating that smaller myelinated fibres benefit most from ARI treatment.

In conclusion, the data demonstrate polyol pathway involvement in the development of RICF in experimental diabetes. They also show that very high levels of polyol pathway blockade are necessary to normalize NCV. It is likely that ARI treatment of patients has been suboptimal and has not adequately tested the hypothesis that polyol pathway activity has an important role in the aetiology of diabetic neuropathy.

This work was supported in part by a grant from the British Diabetic Association. We would like to thank Dr Gordon Lees for constructive comments on the manuscript and Don Mirrlees and Jackie Stafford of ICI Pharmaceuticals for supplying the ponalrestat and for help with nerve polyol analyses.

References

BIANCHI, R., BOCCASAVIA, E., VITTADELLO, M., SCHIAVINATO, A. & GORIO, A. (1987). Sciatic nerve ATPase activity is unaffected in diabetic mutant C57BI/Ks (db/db) mice. *Diabetes*, **36**, 1082–1085.

CAMERON, N.E., COTTER, M.A. & HARRISON, J. (1986a). Effect of diabetes on motor conduction velocity in different branches of the rat sciatic nerve. *Exp. Neurol.*, **92**, 757–761.

CAMERON, N.E., LEONARD, M.B., ROSS, I. & WHITING, P. (1986b). The effects of Sorbinil on peripheral nerve conduction velocity, polyol concentrations and morphology in the streptozotocin-diabetic rat. *Diabetologia*, **29**, 168–174.

CAMERON, N.E., COTTER, M.A. & ROBERTSON, S. (1989). The effect of aldose reductase inhibition on the pattern of nerve conduction deficits in diabetic rats. *Q. J. Exp. Physiol.*, **74**, 917–926.

CAMERON, N.E., COTTER, M.A. & LOW, P.A. (1991a). Nerve blood flow in early experimental diabetes in rats: relation to conduction deficits. *Am. J. Physiol.*, **261**, E1–E8.

CAMERON, N.E., COTTER, M.A. & ROBERTSON, S. (1991b). Effects of essential fatty acid dietary supplementation on peripheral nerve and skeletal muscle function and capillarization in streptozocin diabetic rats. *Diabetes*, **40**, 523–539.

CAMERON, N.E., COTTER, M.A., FERGUSON, K., ROBERTSON, S. & RADCLIFFE, M.A. (1991c). Effect of chronic α-adrenergic receptor blockade on peripheral nerve conduction, hypoxic resistance, polyols, Na^+-K^+-ATPase activity, and vascular supply in STZ-D rats. *Diabetes*, **40**, 1652–1658.

CAMERON, N.E., COTTER, M.A, ROBERTSON, S. & COX, D. (1992). Muscle and nerve dysfunction in rats with experimental galactosaemia. *Exp. Physiol.*, **77**, 89–108.

CARRINGTON, A.L., ETTLINGER, C.B., CALCUTT, N.A. & TOMLINSON, D.R. (1991). Aldose reductase inhibition with imirestat – effects on impulse conduction and insulin-stimulation of Na^+/K^+-adenosine triphosphatase activity in sciatic nerves of streptozotocin-diabetic rats. *Diabetologia*, **34**, 397–401.

DAS, P.K., BRAY, G.M., AGUAYO, A.J. & RASMINSKY, M. (1976). Diminished ouabain-sensitive sodium-potassium ATPase activity in sciatic nerves of rats with streptozotocin-induced diabetes. *Exp. Neurol.*, **53**, 285–288.

DAVIDSON, W.S. & MURPHY, D.G. (1985). Aldehyde reductases and their involvement in muscular dystrophy. In *Enzymology of Carbonyl Metabolism 2: Aldehyde Dehydrogenase, Aldo-Keto Reductase, and Alcohol Dehydrogenase.* (Progress in Clinical and Biological Research. Series, Vol. 174). ed. Flynn, T.G. & Weiner, H. pp. 251–263. New York: A.R. Liss, Inc.

DVORNIK, D. (1987). Hyperglycemia in the pathogenesis of diabetic complications. In *Aldose Reductase Inhibition, an Approach to the Prevention of Diabetic Complications.* ed. Porte, D., pp. 69–151. New York: McGraw-Hill.

FLORKOWSKI, C.M., ROWE, B.R., NIGHTINGALE, S., HARVEY, T.C. & BARNETT, A.H. (1991). Clinical and neurophysiological studies of aldose reductase inhibitor ponalrestat in chronic symptomatic diabetic peripheral neuropathy. *Diabetes*, **40**, 129–133.

GARNER, M.H. & SPECTOR, A. (1987). Direct stimulation of Na^+-K^+-ATPase and its glucosylated derivative by aldose reductase inhibitor. *Diabetes*, **36**, 716–720.

GILLON, K.R.W., HAWTHORNE, J.N. & TOMLINSON, D.R. (1983). *Myo*-inositol and sorbitol metabolism in relation to peripheral nerve function in experimental diabetes in the rat: the effect of aldose reductase inhibition. *Diabetologia*, **25**, 365–371.

GREENE, D.A., LATTIMER, S., ULBRECHT, J. & CARROLL, P. (1985). Glucose-induced alterations in nerve metabolism: current perspectives on the pathogenesis of diabetic neuropathy and future directions for research and therapy. *Diabetes Care*, **8**, 290–299.

JARAMILLO, J., SIMARD-DUQUESNE, N. & DVORNIK, D. (1984). Resistance of the diabetic rat nerve to ischaemic inactivation. *Can. J. Physiol. Pharmacol.*, **63**, 733–737.

LAMBOURNE, J.E., BROWN, A.M., CALCUTT, N., TOMLINSON, D.R. & WILLARS, G.B. (1988). Adenosine triphosphatase in nerves and ganglia of rats with stretpozotocin-induced diabetes or galactosaemia; effects of aldose reductase inhibition. *Diabetologia*, **31**, 379–384.

LOW, P.A. & SCHMELZER, J.D. (1983). Peripheral nerve conduction studies in galactose-poisoned rats. *J. Neurol. Sci.*, **59**, 415–421.

LOW, P.A., TUCK, R.R. & TAKEUCHI, M. (1987). Nerve microenvironment in diabetic neuropathy. In *Diabetic Neuropathy*, ed. Dyck, P.J., Thomas, P.K., Asbury, A.K., Winegrad, A.I. & Porte, D. pp. 266–278. Philadelphia, PA, USA: W.D. Saunders Company.

MAYER, J.H. & TOMLINSON, D.R. (1983). Prevention of defects of axonal transport and nerve conduction by oral administration of *myo*-inositol or an aldose reductase inhibitor in streptozotocin-diabetic rats. *Diabetologia*, **25**, 433–438.

MCMANIS, P.G., LOW, P.A. & YAO, J.K. (1986). The relationship between nerve blood flow and intercapillary distance in peripheral nerve edema. *Am. J. Physiol.*, **251**, E92–E97.

MIRRLEES, D.J., WARD, W.H.J., SENNITT, C.M., COOK, P.N., CAREY, F., TUFFIN, D.P., BRITTAIN, D.R., PRESTON, J., HOWE, R., BROWN, S.P. & COOPER, A.L. (1991). Sulphonylnitromethanes – novel inhibitors of aldose reductase. *Diabetologia*, **34** (supplement 2), A21 (abstract).

MYERS, R.R. & POWELL, H.C. (1984). Galactose neuropathy – impact of chronic endoneurial edema on nerve blood flow. *Ann. Neurol.*, **16**, 587–594.

PRICE, D.E., AIREY, C.M., ALANI, S.M. & WALES, J.K. (1988). Effect of aldose reductase inhibition on nerve conduction velocity and resistance to ischemic conduction block in experimental diabetes. *Diabetes*, **37**, 969–973.

SENEVIRATNE, K.N. & PEIRIS, O.A. (1969). The effects of hypoxia on the excitability of isolated peripheral nerves of alloxan-diabetic rats. *J. Neurol. Neurosurg. Psychiatry*, **32**, 462–469.

SIMA, A.A.F., BRIL, V., NATHANIEL, V., McEWEN, T.A.J., BROWN, M.R., LATTIMER, S.A. & GREENE, D.A. (1988). Regeneration and repair of myelinated fibers in sural-nerve biopsy specimens from patients with diabetic neuropathy treated with sorbinil. *N. Engl. J. Med.*, **319**, 548–555.

SREDY, J., FLAM, B.R. & SAWICKI, D.R. (1991). Adenosine triphosphatase activity in sciatic nerve tissue of streptozocin-induced diabetic rats with and without high dietary sucrose: effects of aldose reductase inhibitors. *Proc. Soc. Exp. Biol. Med.*, **197**, 135–142.

STRIBLING, D., MIRRLEES, D.J., HARRISON, H.E. & EARL, D.C.N. (1985). Properties of ICI 128,436, a novel aldose reductase inhibitor and its effects on diabetic complications in the rat. *Metabolism*, **34**, 336–344.

WILLARS, G.B., TOWNSEND, J., TOMLINSON, D.R., COMPTON, A.M. & CHURCHILL, R.D. (1988). Studies on peripheral nerve and lens in long-term experimental diabetes: effects of the aldose reductase inhibitor Statil. *Metabolism*, **37**, 442–449.

YASUDA, H., SONOBE, M., YAMASHITA, M., TERADA, M., HATANAKA, I., HUITIAN, Z. & SHIGETA, Y. (1989). Effect of prostaglandin E_1 analogue TFC 612 on diabetic neuropathy in streptozocin-induced diabetic rats, comparison with aldose reductase inhibitor ONO 2235. *Diabetes*, **38**, 832–838.

(Received May 11, 1992
Revised July 23, 1992
Accepted July 27, 1992)

Br. J. Pharmacol. (1992), **107**, 945–955

Potassium channel modulation in rat portal vein by ATP depletion: a comparison with the effects of levcromakalim (BRL 38227)

[1]Thomas Noack, *Gillian Edwards, Petra Deitmer & *Arthur H. Weston

Department of Physiology, Philipps University, Deutschhausstrasse 2, W-3550 Marburg, Germany and *Smooth Muscle Research Group, Department of Physiological Sciences, University of Manchester, Manchester, M13 9PT

1 The effects of levcromakalim and of adenosine 5'-triphosphate (ATP) depletion on membrane potential and ionic currents were studied in freshly-dispersed smooth muscle cells of rat portal vein by use of combined voltage- and current-clamp techniques.

2 Levcromakalim (1 μM) induced a glibenclamide-sensitive, non-inactivating K-current (I_{KCO}) and simultaneously inhibited the slow, transient outward, delayed rectifier K-current (I_{TO}). Levcromakalim also hyperpolarized the portal vein cells by approximately 20 mV.

3 Reduction of intracellular ATP by removal of glucose and carboxylic acids from the recording pipette and of glucose from the bath fluid, induced a slowly-developing, non-inactivating and glibenclamide-sensitive K-current (I_{met}) within 60–300 s after breaking the membrane patch. I_{met} reached peak amplitude after 300–900 s, remained at a plateau for 200–800 s and then slowly ran down. At the peak of I_{met}, the cells were hyperpolarized by approximately 20 mV and their input conductance was increased by 42%.

4 At the time of maximum development of I_{met}, the delayed rectifier current, I_{TO}, was reduced by 48%.

5 In the absence of glucose and carboxylic acids, addition of 1 μM free ATP to the recording pipette almost doubled the magnitude of I_{met}. At a holding potential of −10 mV, I_{met} was increased from 124 ± 11 pA to 228 ± 54 pA whereas the time-course of development and run-down of I_{met} was unaffected.

6 During the development and after the run-down of I_{met}, levcromakalim (1–10 μM) failed to induce I_{KCO}.

7 Stationary fluctuation analysis of the current noise associated with I_{met} revealed a unitary conductance of between 10–20 pS in a physiological potassium gradient. A second contaminating current with an underlying unitary conductance of approximately 150 pS remained after I_{met} had run down.

8 It is concluded that I_{KCO} induced by levcromakalim and I_{met} are carried by the same population of relatively small conductance, glibenclamide-sensitive K-channels. The open state of these is increased by procedures designed to lower intracellular ATP concentrations.

9 The simultaneous inhibition of the delayed rectifier current (I_{TO}) by both levcromakalim and during the development of I_{met} is highly significant. It suggests that levcromakalim could modify the interaction of ATP with sites linked to more than one type of K-channel. This results in the opening of those channels which underlie I_{KCO} (and which are normally inhibited by ATP binding) together with the modulation of phosphorylation-dependent channels such as those which underlie I_{TO}.

Keywords: Levcromakalim; glucose; potassium channels; ATP; rat portal vein; delayed rectifier; metabolism; K-channel opener; unitary conductance; fluctuation analysis

Introduction

Levcromakalim (formerly BRL 38227) can be regarded as one of the prototypes of the group of agents known as the potassium (K) channel openers (Edwards & Weston, 1990). In a variety of smooth muscles, the effects of these substances can be inhibited by glibenclamide, a sulphonylurea derivative which acts as an inhibitor of ATP-sensitive K-channels (K_{ATP}) in the heart and pancreatic β-cell (Fosset *et al.*, 1988; Zünkler *et al.*, 1988). This inhibition has led to the view that a K_{ATP} is also the site of action of these agents in smooth muscle (see Quast & Cook, 1989).

Evidence in favour of this was apparently obtained by Standen *et al.* (1989) who reported that cromakalim opened a K_{ATP} with a unitary conductance of 135 pS in rat mesenteric artery. However, other workers have described the involvement of a K_{ATP} with a much lower conductance (10 pS: Kajioka *et al.*, 1991) while data from other groups has suggested that levcromakalim and related agents open the large conductance calcium-activated K-channel (Silberberg & van Breemen, 1990; Stockbridge *et al.*, 1991; Klöckner & Isenberg, 1992).

The conflicting results were largely obtained from electrophysiological studies which utilised isolated membrane patches from a variety of smooth muscles. In contrast, few investigators have characterized the currents generated by the K-channel openers, a factor which prompted us to study the effects of levcromakalim using the whole-cell patch clamp configuration (Noack *et al.*, 1992a,b). We found that in rat portal vein under voltage-clamp conditions, levcromakalim induced a voltage-independent, non-inactivating and glibenclamide-sensitive K-current with an underlying conductance in the range 10–20 pS. Furthermore, the hyperpolarization produced by levcromakalim in whole-cell current-clamp experiments (Noack *et al.*, 1992a) was similar to that measured with sharp microelectrodes following exposure of whole portal veins to cromakalim (Hamilton *et al.*, 1986).

The objective of the present experiments was to determine the conditions necessary for the generation of I_{KCO} in single cells of rat portal vein by modifying the composition of the recording pipette solution. Using this approach we hoped to

[1] Author for correspondence.

obtain some information about the possible ATP-sensitivity of this current. In addition, we also wished to characterize further the levcromakalim-induced inhibition of the delayed rectifier current (I_{TO}) which was one of the surprising findings of our previous study (Noack *et al.*, 1992a).

Methods

All experiments were performed on single smooth muscle cells isolated from portal veins which were removed from male Sprague-Dawley rats, previously killed by stunning and bleeding.

Production of isolated cells

Each portal vein (about 20 mm length) was carefully cleaned of fat and connective tissue with fine scissors in conjunction with a dissecting microscope. The vein was cut into small pieces and then incubated in a nominally Ca^{2+}-free physiological salt solution (PSS) for 30 min. The cell dispersion technique consisted of treatment of the tissue with purified collagenase and papain (for details see Noack *et al.*, 1992a). The cells were used for experiments within 12 h after separation, during which time they were stored at 6°C in Kraftbrühe (KB-medium; Klöckner & Isenberg, 1985). All experiments were performed at 26°C.

Single-cell electrophysiology

The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used in all experiments. All measurements were made with an amplifier which permitted fast switching between voltage- and current-clamp modes (see Noack *et al.*, 1992a). The settling time of the system was less than 500 μs. Patch pipettes were pulled from Pyrex glass (H 15/10, Jencons, U.K.) and had resistances of 3–4 MΩ when filled with the internal (intracellular) solution. Voltage commands and data acquisition were performed as described by Noack *et al.* (1992a). For cell stimulation and for recording and analyzing data the pCLAMP 5.5 programme was used (Axon Instruments, U.S.A.). Data acquisition and storage were as described by Noack *et al.* (1992a). The leak resistance ranged from 3 to 5 GΩ and these experimental values were used to estimate errors of potential measurement under whole-cell current-clamp.

The effects of levcromakalim and glibenclamide were investigated by adding the appropriate amount(s) of these agents to the main reservoir containing the external solution to ensure that responses were obtained under steady-state conditions. The bath (volume: 1 ml) was continuously perfused (1 ml min^{-1}) with fresh external solution using a pump (Microperpex, Pharmacia LKB, Freiburg, Germany); a second identical pump was used to remove excess solution from the recording chamber.

Drugs and solutions

The nominally Ca^{2+}-free PSS used for the cell separation comprised (mM): NaCl 137, KCl 2.7, $MgCl_2$ 1.0, glucose 5.6, HEPES 4.2, buffered with NaOH to pH 7.3. The PSS in the bath had the following composition (mM): NaCl 125, KCl 4.8, $MgCl_2$ 3.7, KH_2PO_4 1.2, glucose 11, HEPES 10, EGTA (ethylene glycol-*bis* β-aminoethyl ether tetra-acetic acid; Sigma) 1.0, buffered with NaOH to pH 7.3; aerated with O_2. The basic pipette (internal) solution (A) contained (mM): NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, glucose 11, HEPES 10, EGTA 1.2, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5. Pipette solution B had a composition similar to solution A but with the omission of glucose, oxalacetic acid, sodium pyruvate and sodium succinate. In addition NaCl was increased to 12 mM and KCl to 131 mM. Pipette solution C had the same composition as solution B but with the addition of 18.7 μM MgATP (Sigma) (calculated to give a free ATP concentration of 1 μM; Fabiato, 1988). After the addition of MgATP, solution C was buffered with KOH and used immediately. Assuming a contaminant concentration of 10 μM calcium in the external and in the pipette solutions, the addition of 1 mM and 1.2 mM EGTA to the bath and pipette solutions, respectively, should have produced an average free calcium concentration in these solutions of less than 1 nM (Fabiato, 1988).

Levcromakalim (SmithKline Beecham) and glibenclamide (Sigma) were first dissolved in dimethyl sulphoxide (DMSO; Sigma) to produce a concentrated stock solution (20 mM) from which dilutions were prepared with distilled water immediately before they were required.

Data presentation

Numerical values are given as mean ± s.e.mean with the number of observations in parentheses. The significance of differences between mean values was determined by an unpaired Student's *t* test: values of $P<0.05$ were taken to indicate a significant difference.

Results

Whole-cell currents in glucose-containing PSS and with pipettes containing carboxylic acids plus glucose

Whole-cell currents were elicited in single cells from the rat portal vein under conditions in which succinate, pyruvate, oxalacetate and glucose were included in the pipette solution (solution A; see Methods) and in which glucose was present in the extracellular calcium-free bathing fluid. On disruption of the membrane patch at the pipette tip, an outward current of magnitude between + 20 pA and + 50 pA was observed at a holding potential of − 10 mV. This current reached a steady state within about 300 s. A series of voltage protocols was then performed in which the cell was clamped at a holding potential of either − 10 mV or − 90 mV and then stepped to a range of test potentials from − 80 to + 50 mV in 10 mV increments. Under control conditions (i.e. with no drugs added to the extracellular bathing fluid) the currents observed at the holding potentials of − 10 mV or − 90 mV and those elicited at each test potential were reproducible for more than 1 h.

By use of the above voltage-step protocols at the two holding potentials, three major current components could be distinguished (Figure 1) : (i) a transient potassium current (I_A, similar to an A-current) with voltage-dependency (inactivated at − 10 mV) and with fast activation and inactivation kinetics, (ii) a transient, voltage-dependent potassium current (I_{TO}, inactivated at − 10 mV) with activation and inactivation time-courses slower than those of the transient A-like current. I_{TO} also exhibited a more positive voltage threshold for activation (at approximately − 30 mV) than that of I_A. These properties are characteristic of those of a delayed rectifier K-current and (iii) a complex of non-inactivating, voltage-independent background currents (activated in the potential range − 90 to + 50 mV) which determined the zero-current potential of the cell. These currents will be collectively termed I_{NI}.

Addition of levcromakalim to the extracellular bathing fluid induced an additional non-inactivating outward current (I_{KCO}) which was blocked by the addition of glibenclamide (EC_{50} 3 μM) to the extracellular bathing fluid. The characteristics of I_{KCO} were identical to those previously described by us (Noack *et al.*, 1992a). On switching from voltage-clamp to current-clamp at 0 pA, the membrane potential of the cells in the presence of levcromakalim (1 μM) was − 40 ± 3.8 mV, $n = 4$. This value was approximately 20 mV more hyperpolarized than immediately prior to exposure to levcromakalim (− 17.8 ± 2.6 mV, $n = 4$).

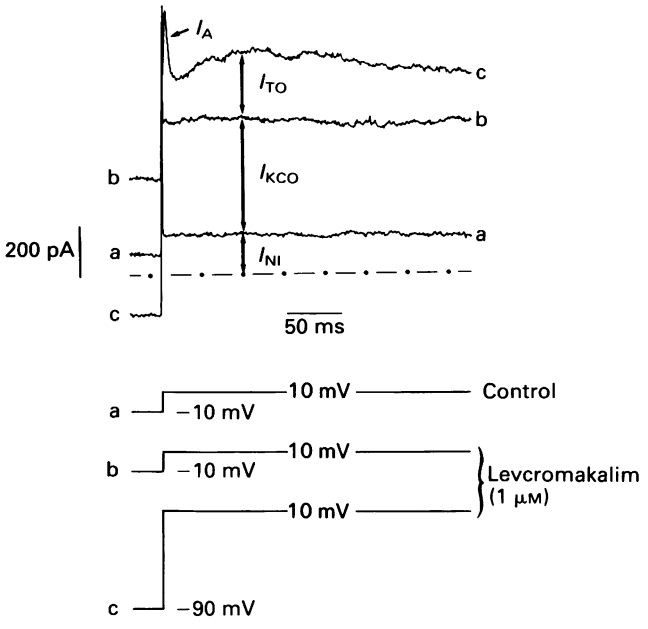

Figure 1 Typical whole-cell current traces obtained from a rat portal vein smooth muscle cell bathed in a calcium-free physiological salt solution (PSS; containing glucose and EGTA). The electrode PSS contained glucose and tricarboxylic acids. (a) On stepping the membrane potential from − 10 mV to + 10 mV, a non-inactivating current (I_{NI}) was observed (deviation from zero-current indicated by a dashed line). (b) When levcromakalim (1 μM) was present in the bathing fluid an additional non-inactivating outward current (I_{KCO}) was generated at the same test potential. Note also the additional current required to maintain the membrane potential at − 10 mV in the presence of levcromakalim. (c) When the holding potential was − 90 mV and levcromakalim (1 μM) was present in the bath PSS, a step depolarization to + 10 mV elicited a rapidly-inactivating K-current (I_A) and a slowly-inactivating K-current (I_{TO}) in addition to I_{KCO} and I_{NI}. Note that the amplitude of I_{TO} in the absence of levcromakalim is larger than that shown in this Figure (see Noack *et al.*, 1992a).

Whole cell currents in glucose-free PSS and with pipettes containing no carboxylic acids or glucose

In this series of experiments, whole-cell currents were monitored by use of a pipette solution devoid of succinate, pyruvate, oxalacetate and glucose (solution B; see Methods) and with the omission of glucose from the extracellular calcium-free bathing fluid. After breaking the membrane patch under these conditions and with a holding potential of − 10 mV, the cells developed an outward current initially similar to that observed with glucose-containing PSS and with carboxylic acids plus glucose in the recording pipette (Figure 2). However, after a time delay ranging from 60 s to 300 s, the outward current at the holding potential of − 10 mV markedly increased, reaching a maximum amplitude within 300 s to 900 s. This additional current component which was associated with the lack of metabolic substrates in both the pipette and bathing solutions was designated I_{met}. After reaching its peak, I_{met} remained at a nearly constant level for 200 s to 800 s. This current then declined slowly and within 600 s to 900 s (800 s ± 100 s, mean ± s.e.mean, $n = 3$), it reached a level which was not significantly different from that observed on initial breaking of the membrane patch. This slow decline of I_{met} will be termed 'run-down' and experiments designed to investigate the nature of this phenomenon are detailed later in this paper.

The magnitude of I_{met} varied with the holding potential. The large, slowly-developing outward current which characterized I_{met} at a holding potential of − 10 mV was reduced when the holding potential was − 50 mV. I_{met} was absent when the potential was − 80 mV, close to the calculated potassium equilibrium potential of − 83 mV in the present experiments (Figure 2). Such results indicate that the channels conducting I_{met} were selective for potassium ions. The four, large, peak-like deflections on stepping from − 80 mV to − 10 mV represent the activation and inactivation of I_{TO} (Figure 2).

The characteristics of I_{met} during its development phase were further studied by generating the voltage-step protocols already described from a holding potential of either − 10 mV (to evaluate changes in non-inactivating currents) or from − 90 mV (from which I_{NI}, together with I_{TO} and I_A could be elicited). The marked increase of holding current associated with the induction of I_{met} was clearly evident from those currents generated on stepping from a holding potential of − 10 mV (compare Figures 3a(ii) and 3b(ii)). Surprisingly, however, this increase in non-inactivating currents was not

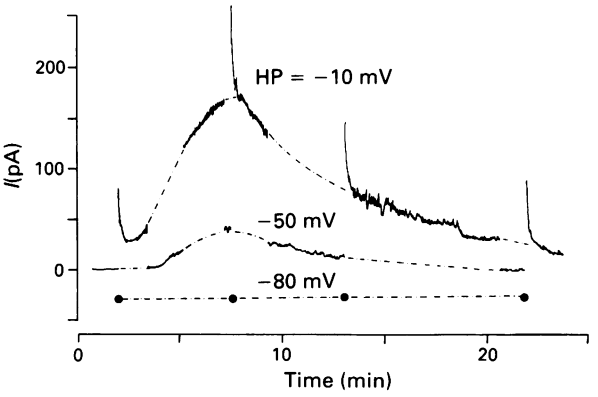

Figure 2 Typical whole-cell current recordings from rat portal vein showing the development of I_{met} and the effect of membrane potential on the magnitude of this current. The membrane potential was held at − 10 mV, − 50 mV or − 80 mV. With glucose and tricarboxylic acids absent from the pipette PSS (solution B), an outward current (I_{met}), evident at − 50 mV or − 10 mV, slowly developed and after a plateau phase it gradually declined. The zero on the time axis indicates the time when the membrane patch under the tip of the pipette was disrupted. The spike-like current deflections were obtained on stepping the membrane potential from − 80 mV to − 10 mV and indicate activation and inactivation of I_A and I_{TO}. The dotted lines on the various traces show the probable time-course of I_{met} at the indicated membrane potentials when the cell was held at a different potential.

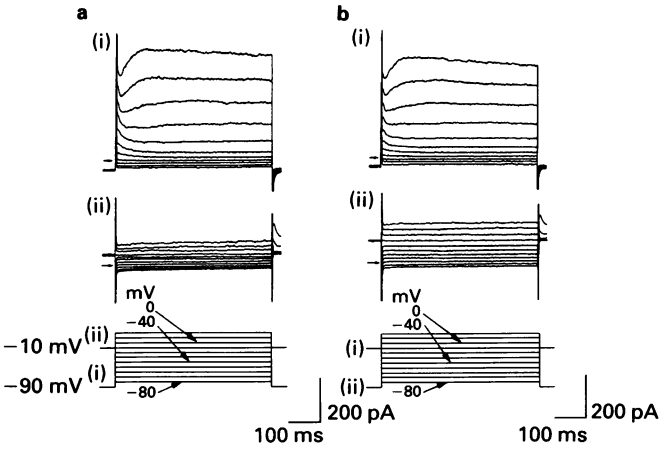

Figure 3 Effect of lack of glucose and tricarboxylic acids (pipette solution B) on whole-cell currents in smooth muscle cells from rat portal vein. (a) The cells were held at either − 90 mV (i) or − 10 mV (ii) and stepped for 500 ms to the different test potentials as indicated. The currents were recorded at a mean time of 175 ± 20 s after disrupting the patch under the pipette tip. (b) Conditions as in (a) but after a mean time of 540 ± 150 s after recording the traces in (a). Current traces in (a) and (b) are the computer-derived average from four individual cells.

evident in the evoked total currents generated by stepping to the same test potentials from a holding potential of − 90 mV (compare Figures 3a(i) and 3b(i)). This is a clear indication that an inactivating current component was reduced during the same time period.

To clarify this apparent anomaly, current-voltage relationships (*I-V* curves) were constructed using the voltage-step protocols already described. These were applied to the cells from holding potentials of either − 10 mV or − 90 mV and at times which corresponded to no apparent I_{met} (after breaking the membrane patch at the start of the experiment) or to the maximum I_{met} (Figure 4). Preliminary experiments showed that the major effects of the lack of metabolic substrates were exerted on the currents I_{TO} and I_{NI}. Therefore, for the construction of the *I-V* curves shown in Figure 4, the peak outward currents generated by each voltage step from the holding potentials of − 10 mV or − 90 mV were measured after inactivation of I_A, i.e. between 100 ms to 300 ms after the start of each depolarizing pulse.

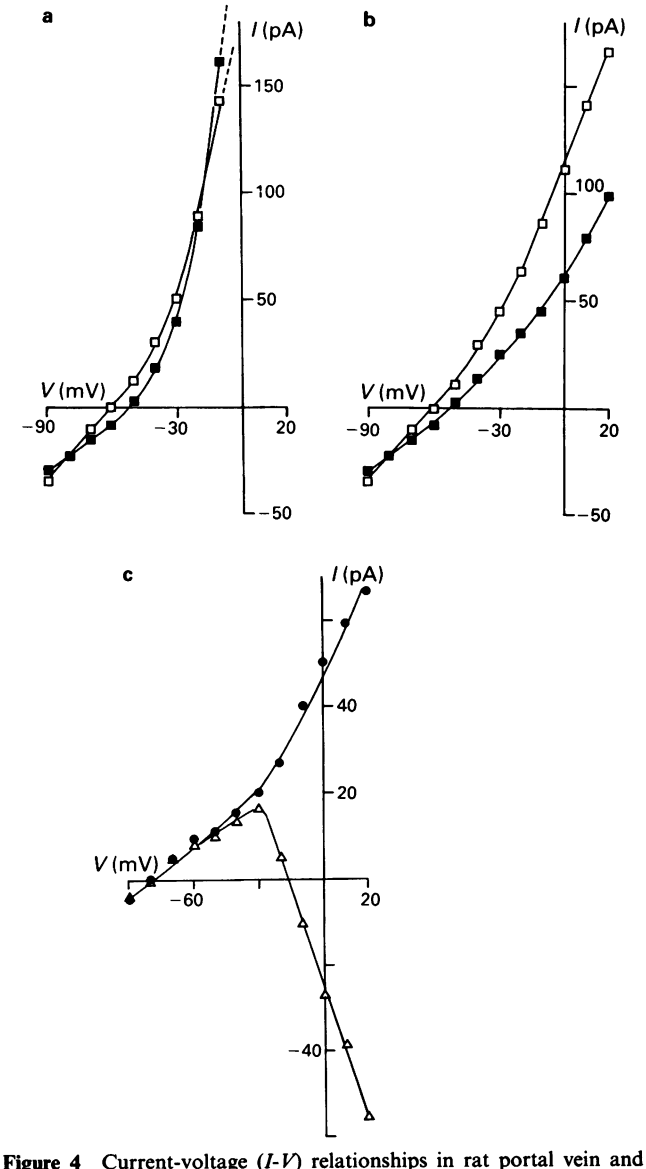

Figure 4 Current-voltage (*I-V*) relationships in rat portal vein and the effect of lack of glucose and tricarboxylic acids. (a) *I-V* relationship for the total current (measured at the peak of I_{TO}) initially (175 ± 20 s after membrane disruption, ■) and at the plateau phase of I_{met} (540 ± 150 s after membrane disruption, □). The holding potential was − 90 mV to reverse any inactivation of I_{TO}. The marked point of inflection of current at − 30 mV is due to activation of I_{TO}. (b) *I-V* relationship for the non-inactivating currents initially (■) and at the plateau phase of I_{met} (□). The holding potential was − 10 mV to ensure complete inactivation at the time-dependent current components. In all cells ($n = 4$), I_{met} had a reversal potential close to the potassium equilibrium potential (− 83 mV). (c) *I-V* relationships of the net current evoked by lack of metabolites (difference between curves shown in (a) and (b)) for the two holding potentials (Δ, − 90 mV; ●, − 10 mV). Note the marked inhibition of I_{TO} associated with the plateau phase of I_{met}. Each point is the mean derived from observations on 4 cells. Curves were fitted by eye.

From a holding potential of − 90 mV, these peak currents elicited at the start of an experiment represented the sum of the components I_{TO} and I_{NI}. They were taken as the control currents which could be elicited in the presumed absence of any modification of cell metabolism (Figure 4a). After induction of the maximum I_{met} resulting from the lack of glucose and carboxylic acids, the corresponding peak currents generated by identical voltage-step protocols from a holding potential of − 90 mV reflected the sum of I_{TO} and I_{NI}, which may have been modified, plus any new current component (I_{met}) which was not present at the start of the experiment (Figure 4a). It can be seen that the *I-V* curve elicited at the time of maximum I_{met} lies above the control curve in the voltage range between − 80 mV and − 30 mV but below it at potentials positive to − 30 mV. It crosses the control curve at approximately − 80 mV (the calculated E_K in these experiments was − 83 mV, thus indicating that I_{met} is a potassium current), and again at approximately − 30 mV, the threshold potential for I_{TO}.

The control *I-V* relationship for I_{NI} alone (Figure 4b) was constructed at the start of the experiment from a holding potential of − 10 mV and using the voltage-step protocols already described for $I_{TO} + I_{NI}$. After induction of the maximum I_{met}, the generated currents reflected the magnitude of I_{NI} (possibly modified by the lack of metabolites) together with that of any new current (I_{met}) not present under control conditions. The *I-V* curve elicited from a holding potential of − 10 mV at the time of maximum I_{met} ran above the control curve, crossed it near − 80 mV and showed outward rectification (Figure 4b).

The differences between control and test *I-V* curves which represent the net effects of metabolite removal on I_{TO} and I_{NI} are shown in Figure 4c. Comparison of the difference curves obtained at the two holding potentials clearly demonstrates that the lack of metabolites enhances a component of I_{NI} but markedly inhibits I_{TO}. Very similar effects (induction of a non-activating current, I_{KCO}, together with inhibition of I_{TO}) were also produced by levcromakalim in single rat portal vein cells (present study, data not shown; see also Noack *et al.*, 1992a).

Effect of lack of metabolic substrates on I_{TO}

Using the voltage protocols already described, it was impossible to elicit I_{TO} without contamination with I_{NI}. Thus I_{TO} was separated from I_{NI} by subtracting the current which was generated on stepping to test potentials from a holding potential of − 10 mV from that current obtained by stepping to the same test potentials but from a holding potential of − 90 mV. Using this procedure for 4 cells, the peak value of I_{TO} was derived at the time corresponding to maximum I_{met} and compared with the peak value of I_{TO} before I_{met} had become apparent. In these 4 cells, I_{met} reached a maximum after 14.8 ± 2 min, at which time I_{TO} was reduced by 48 ± 9%.

Effect of lack of metabolic substrates on membrane potential

To determine the extent to which I_{met} changed membrane properties, the effects of removal of metabolic substrates on both membrane current and membrane potential were investigated. This was performed with an amplifier which provided fast switching between voltage-clamp and current-clamp modes. The upper traces of Figure 5a show the mean ionic currents generated by 4 cells on stepping to 0 mV from

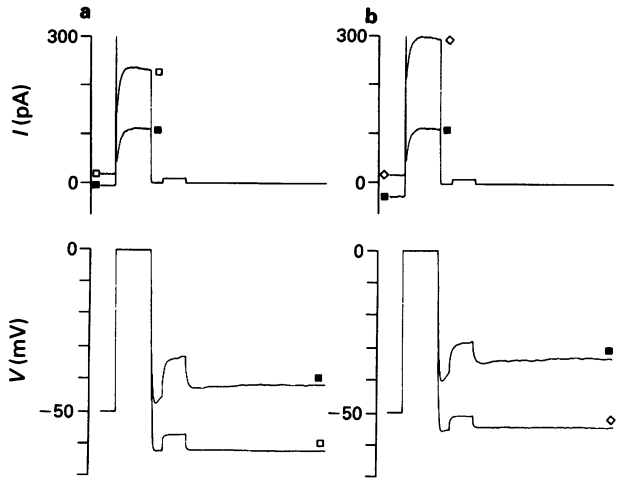

Figure 5 Effect of metabolic substrates on membrane currents and membrane potential in rat portal vein using the voltage-clamp/current-clamp protocol. (a) The superimposed current traces (upper part) show the effect of glucose and tricarboxylic acids (pipette solution B, see Methods) on membrane currents when the potential was stepped from a holding potential of − 50 mV to 0 mV (■, initially; □, plateau phase of I_{met}). Under current-clamp the membrane current was held at 0 pA apart from a current pulse of + 10 pA injected to give an indication of membrane input resistance. The voltage protocol and membrane potential (lower part) corresponds to the current traces above. (b) Same experimental protocol as in (a) with the exception that the pipette solution lacked glucose and tricarboxylic acids but contained 1 μM free ATP (pipette solution C, see Methods). Currents evoked at the start of the experiment (■). At the plateau phase of I_{met} (◇) the evoked current was increased in comparison to that shown in (a). The records in (a) and (b) are each computer-derived means from two sets of 4 different single cells.

a holding potential of − 50 mV under control conditions (at time 3.0 min ± 0.3 min after breaking the patch under the pipette tip) and at the time of maximum I_{met} development (12.7 min ± 2.6 min after breaking the patch under the pipette tip).

The data in Figure 5a were derived from 4 cells, each subjected to 10 identical voltage-current-clamp protocols. After 600 ms, the system was switched to current-clamp at 0 pA and the corresponding lower traces show the measured membrane potentials. At its time of maximum development, I_{met} increased the membrane potential to − 65.3 ± 3.5 mV from a mean zero current potential of − 43.5 ± 5.3 mV in 4 individual cells. The upward deflection in the voltage traces during current-clamp represents the electrotonic potential in response to a 10 pA current injection. Comparison of the electrotonic responses showed that the input conductance of the 4 cells depicted in Figure 5a was increased by a mean value of 42% in the presence of I_{met}, compared with controls.

Run-down of I_{met}: effect of addition of ATP to the recording pipette

In an attempt to prevent run-down of I_{met}, a series of voltage-clamp/current-clamp experiments was conducted in which glucose was removed from the PSS and in which 18.7 μM MgATP replaced the glucose and carboxylic acids in the pipette solution (pipette solution C). This concentration of ATP was calculated to give a free ATP concentration of 1 μM (Fabiato, 1988).

Under these conditions, the mean initial outward current at the holding potential of − 50 mV was − 13.6 pA ± 10.3 pA and the corresponding membrane potential under current clamp at 0 pA was − 34.7 mV ± 4.8 mV ($n = 4$). This mean holding current was thus more inward and the associated mean membrane potential was 9 mV more depolarized with 1 μM ATP in the pipette than when glucose and carboxylic acids were absent (+ 1.0 pA ± 3.9 pA and − 43.5 mV ± 5.3 mV, respectively, $n = 4$). With ATP in the pipette and at a holding potential of − 10 mV, the times to onset and the time-course of induction of I_{met} were as previously described for the glucose- and carboxylic acid-free experiments. In contrast, the maximum I_{met} with 1 μM ATP in the pipette (228 ± 54 pA, $n = 5$) was almost double that recorded in the absence of glucose and carboxylic acids (124 ± 11 pA, $n = 4$). Similarly, the outward currents evoked on stepping from − 50 mV to 0 mV at the time of maximum I_{met} were greater in the ATP-pipette group (Figure 5). The time-course of the 'run-down' of I_{met} was not modified by the presence of ATP in the pipette.

The effect of levcromakalim on membrane currents in the absence of glucose and carboxylic acids

The results so far described indicate a marked similarity between the effects on membrane currents induced by the lack of carboxylic acids and glucose (i.e. I_{met}) and those induced by levcromakalim (i.e. I_{KCO}, see Noack *et al.*, 1992a). To determine the extent to which these two currents could be induced independently, 4 cells were exposed to levcromakalim at different phases of the development of I_{met} with solution A in the patch pipette.

When levcromakalim (1 μM) was added to the bathing fluid during the development of I_{met}, there was neither a sudden change in the rate of development of I_{met} nor a difference in the maximum observed I_{met}. In addition, the run-down of I_{met} had the same time-course as if levcromakalim had not been present in the bathing fluid. After run-down, levcromakalim (1–10 μM) failed to induce I_{KCO}. In 2 out of 7 cells, I_{met} was not observed when ATP (1 μM free) was present in the pipette. In one of these cells, levcromakalim (1 μM) induced an I_{KCO} of 'normal' magnitude, an effect which was partially blocked by the addition of glibenclamide (1 μM). In the second of these cells, levcromakalim failed to induce I_{KCO}. Thus there was no evidence that levcromakalim could induce I_{KCO} in the presence of I_{met}. Furthermore, levcromakalim had no effect after I_{met} had run down.

Comparison between I_{met} and I_{KCO}

I_{KCO} is characterized by (i) its antagonism by glibenclamide ($EC_{50} = 3$ μM) and (ii) its relatively low single channel conductance (17pS): see Noack *et al.* (1992a). To determine any similarities between I_{met} and I_{KCO}, the ability of glibenclamide to modify I_{met} was evaluated and the single channel conductance underlying I_{met} was determined by stationary fluctuation analysis.

Effect of glibenclamide The ability of glibenclamide to modify I_{met} was tested in 4 cells at different stages of the development of this current. The resulting time-course of I_{met} was compared with that of I_{met} in time-matched experiments in which no glibenclamide was added. When glibenclamide (100 nM) was added to the bathing fluid during the development of I_{met}, no inhibition of the further increase of I_{met} to its maximum or modification of the subsequent run-down could be detected. In the presence of 300 nM glibenclamide, I_{met} seemed to run down slightly faster than under control conditions. However, when 1 μM glibenclamide was used, a marked step-like reduction in the time-course of I_{met} was detected (Figure 6).

Single channel conductance underlying I_{met} As can be seen qualitatively from Figure 2, the magnitude of current noise increased during the time-course of development of I_{met}. This increase was greater at a holding potential of − 10 mV than at − 50 mV because of the greater driving force on potassium ions at the more positive holding potential. In a previous paper, we used stationary noise fluctuation analysis

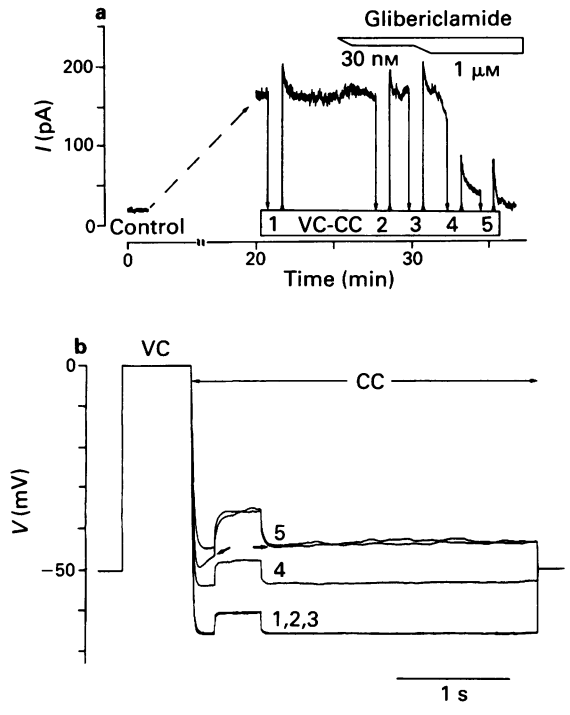

Figure 6 Effect of glibenclamide on I_{met} in rat portal vein. (a) Development of I_{met} (holding potential = − 10 mV) to its plateau phase using a pipette containing no glucose or carboxylic acids (pipette solution B). The addition of glibenclamide 30 nM had no effect. A higher concentration of glibenclamide (1 μM) suppressed I_{met} completely. During the different stages of the experiment, the clamp system was switched (↓) from voltage-clamp (VC) to current-clamp (CC), and (↑) *vice-versa*. The resulting membrane potential recordings under current clamp are shown in (b). (b) The hyperpolarization induced by I_{met} was 22.3 mV (VC-CC switch 1); this was unaffected by glibenclamide 30 nM (VC-CC switches 2 and 3), but completely reversed by glibenclamide 1 μM (VC-CC switches 4 and 5). The control membrane potential record is indicated as C near trace 5.

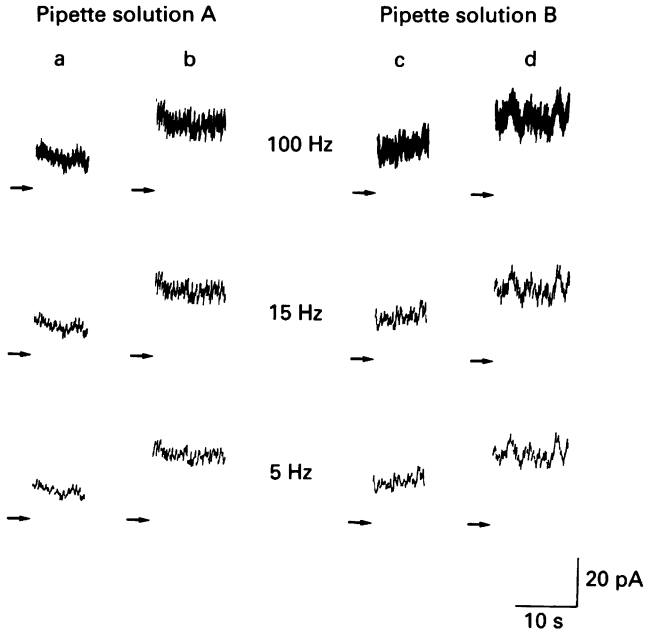

Figure 7 Comparison of typical current noise signals in the presence of levcromakalim (a,b: pipette solution A) with those obtained in the absence of glucose and tricarboxylic acids (c,d; pipette solution B). The holding potential was − 10 mV and the currents were filtered at the different low-pass frequencies as indicated. (a) Control noise prior to exposure to levcromakalim. (b) Levcromakalim, 1 μM induced a current with an increased high frequency noise component. (c) On breakthrough into the cell, the noise was similar to that seen in (a). However, when I_{met} became established (d), low frequency oscillations to different current levels were observed. Horizontal arrows show zero current position.

to estimate the single channel conductance of the current I_{KCO} induced by levcromakalim (see Noack *et al.*, 1992a for details) and this technique was again employed to determine the single channel conductance underlying I_{met}.

In the earlier analysis of I_{KCO} (Noack *et al.*, 1992a), we selected 2 s segments of the current trace. These were long enough to give a representative Gaussian distribution but short enough to prevent distortion during the development of I_{KCO}. In the present study, an identical procedure was successfully adopted for I_{KCO}. However, this was not possible for I_{met} because this current jumped during such 2 s time intervals from one level to another, a phenomenon which yielded Gaussian distributions with two or more peaks. These marked differences between I_{met} and I_{KCO} are clearly shown in the current traces in Figure 7. Although the high frequency fluctuations in both signals were essentially identical, a higher amplitude, but lower frequency component was also present with I_{met}. This lower frequency component shifted the Gaussian distributions in steps of about 5 pA or multiples of this value (Figure 8), whereas the distributions themselves were similar to those obtained for I_{KCO}.

The fact that both I_{met} and an additional current carried by a relatively large conductance potassium channel (using the Goldman-Hodgkin-Katz equation, a current of 6 pA at a holding potential of − 10 mV indicates a channel of unitary conductance of approximately 150 pS at 0 mV under quasi-physiological conditions) were induced by lack of glucose and carboxylic acids makes an accurate evaluation of the unitary conductance which underlies I_{met} rather difficult. However, assuming such a contaminating large conductance channel, fluctuation analysis yielded a value in the range of 10–20 pS ($n = 6$) which was in the same range as that of I_{KCO} obtained in the present and earlier investigations (see Noack *et al.*, 1992a; mean, 16.6 ± 1.6 pA, $n = 6$).

The current associated with the larger conductance component remained even when I_{met} had run down. Under these conditions, the magnitude of the former current at − 10 mV was approximately 20 pA; furthermore, such large current fluctuations were not observed at more negative holding potentials. These two factors suggest that the current associated with the larger conductance channel plays a secondary and minor role compared with that of I_{met} in the hyperpolarization which accompanies the lack of metabolic substrates.

Discussion

In both the present study and in two recent investigations (Noack *et al.*, 1992a,b), levcromakalim produced a marked increase in a non-inactivating K-current (I_{KCO}) in cells isolated from the rat portal vein. In these experiments the pipette solution contained substrates for glycolysis and the tricarboxylic acid (TCA) cycle to maintain the intracellular ATP concentration, $[ATP]_i$. Use of this procedure to prevent possible K-channel run-down was originally described in the Ca-channel studies of Klöckner & Isenberg (1985) in guinea-pig bladder. In previous smooth muscle patch clamp studies involving the K-channel openers, ATP (1–5 mM) (Beech & Bolton, 1989a; Standen *et al.*, 1989; Kajioka *et al.*, 1990; Okabe *et al.*, 1990), GTP (0.2–0.5 mM) (Beech & Bolton, 1989a; Clapp & Gurney, 1992) or glucose (Hu *et al.*, 1990; Kajioka *et al.*, 1991) was included in the solution bathing the intracellular side of the cell membrane. In addition, investigations using the RINm5F insulin-secreting cell line had also demonstrated that intracellular ATP was a prerequisite for

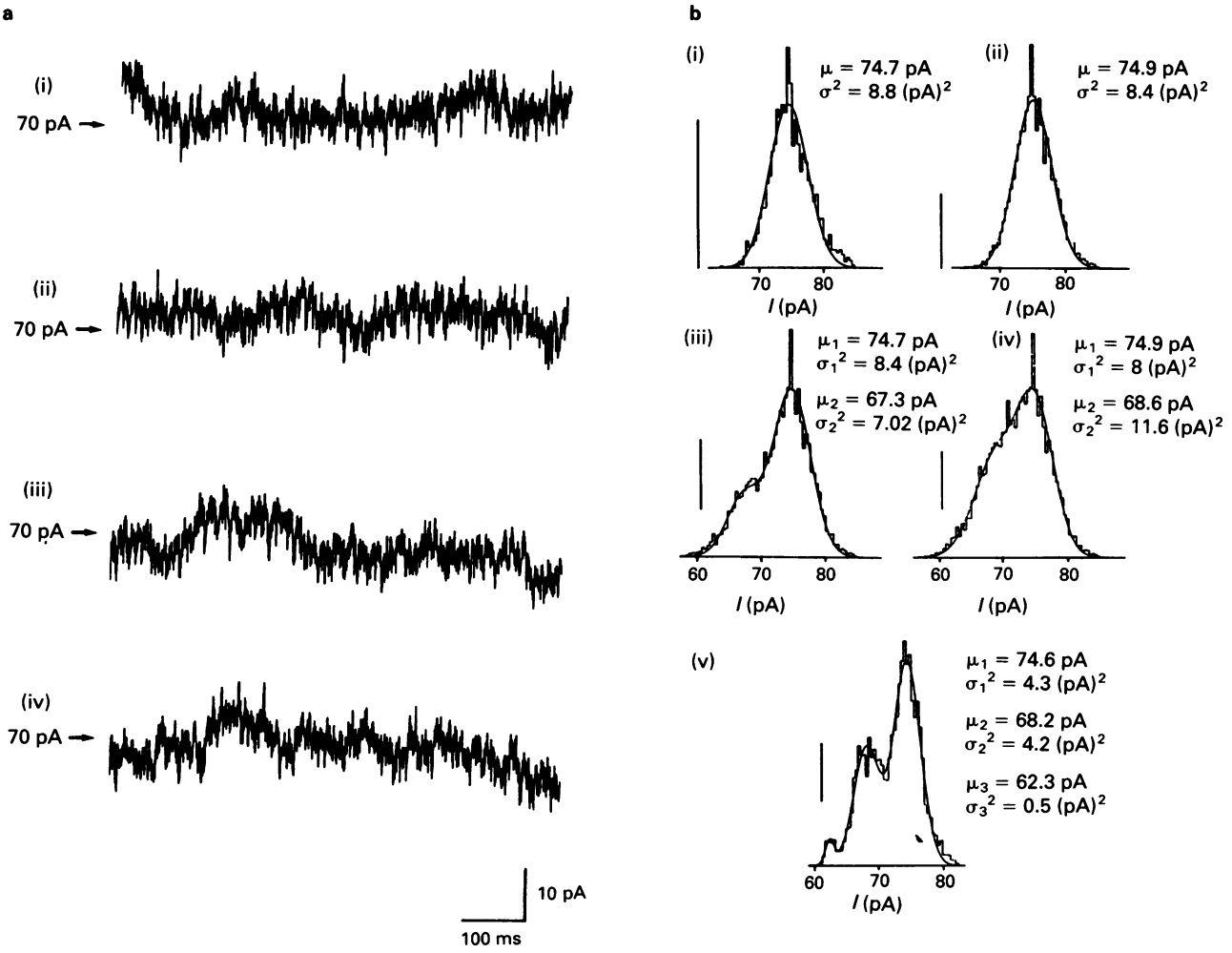

Figure 8 Analysis of the single channel current associated with I_{met} at a holding potential of − 10 mV. Signals were filtered at 10 kHz. (a) (i-iv) Segments, each 700 ms duration, of a continuous current trace obtained during the plateau phase of I_{met}. (b) Amplitude histograms of the current and the accompanying current noise derived from the traces shown in (a). (b i) Histogram of the current trace (a i); (b ii) Histogram of current traces (a i) and (a ii); (b iii) Histogram of current traces (a i), (a ii), and (a iii) showing a pronounced second Gaussian distribution; (b iv) Histogram of all current traces, (a i–iv), demonstrating an increase in the second Gaussian distribution; (bv) Histogram of the same current traces as in (b iv) but with filtering of the current signal at 40 Hz. Note the three peaks in the Gaussian distributions with differences between the means of 6.4 pA and 5.9 pA indicating the presence of a high unitary conductance current in addition to I_{met}. The numbers show the mean (μ) and variance (σ^2) of the current associated with each peak. The values for μ and σ^2 immediately on breaking the membrane patch were 45 pA and 1.5 (pA)^2, respectively. Vertical bars in (b) indicate 100 events.

K-channel opening by diazoxide or cromakalim (Dunne *et al.*, 1987; 1990). Thus, to obtain more information about the conditions necessary to generate I_{KCO} in rat portal vein, we examined the action of levcromakalim in the absence of substrates capable of generating intracellular ATP. To ensure that the results were comparable with those of our earlier studies (Noack *et al.*, 1992a,b), Ca-free conditions together with EGTA buffering were used. Such conditions also minimize the possibility of Ca-overloading due to loss of mitochondrial calcium and failure of Ca-pumping which can occur in the absence of intracellular ATP (Fuhrmann *et al.*, 1985; Klöckner & Isenberg, 1992).

I_{KCO}, I_{met} and $[ATP]_i$

With a pipette solution devoid of glucose and of substrates for the TCA cycle, whole-cell voltage- and current-clamp recordings showed that the cells initially exhibited a normal input conductance and complement of potassium currents shortly after breaking the membrane within the pipette. However, within a few minutes of breakthrough, a slowly-developing outward current (I_{met}) was observed, the characteristics of which could best be studied when the cell was clamped at membrane potentials positive to the potassium equilibrium potential. After maintaining a plateau level for several minutes, I_{met} slowly declined, a process which we considered to be due to 'run-down' similar to that displayed in other tissues by the ATP-sensitive K-channel (K_{ATP}) in the absence of MgATP (see Ashcroft, 1988). I_{met} was prominent at a holding potential of − 10 mV, smaller at − 50 mV and was essentially absent at − 80 mV, properties which suggested that the underlying ion channel was selective for potassium.

The development of I_{met} was associated with an increase in current noise density. Stationary fluctuation analysis of this noise indicated that the channel underlying I_{met} had a relatively small unitary conductance, estimated to lie in the range 10–20 pS when measured under conditions of a physiological K^+ gradient. This conductance was thus in the same range as that associated with the levcromakalim-induced I_{KCO} (17 pS) in both the present and a previous series of experiments (Noack *et al.*, 1992a), when using a pipette solution containing substrates for the TCA cycle and glucose. It was not possible to be more precise in the estimate of the unitary

conductance associated with I_{met}, since the current level exhibited amplitude jumps, indicating the presence of an additional, larger conductance component (see later).

Further similarities between I_{met} and I_{KCO} were observed in experiments using glibenclamide or current-clamp conditions. In the former, I_{met} was markedly inhibited in the presence of glibenclamide, a feature also characteristic of I_{KCO} (see Noack *et al.*, 1992a). In current-clamp mode, the membrane hyperpolarized and the membrane resistance diminished during the development of I_{met}, phenomena also seen in the presence of levcromakalim (present study; Noack *et al.*, 1992a). These data are the first to demonstrate clearly that the absence of metabolic substrates for ATP production induces a K-current and membrane hyperpolarization in smooth muscle. Furthermore, I_{KCO} could not be induced after run-down of I_{met} and exposure to levcromakalim did not modify the time-course of development of I_{met}. Collectively, these data strongly suggest that both I_{KCO} and I_{met} are carried by a single population of relatively small-conductance K-channels.

ATP- and metabolism-modulated K-currents: previous studies

Data which seemed consistent with an ATP-modulated K-current in smooth muscle have recently been presented by Clapp & Gurney (1992). In this study, whole-cell currents in pulmonary arterial cells were apparently inhibited by Na_2ATP in the pipette solution or when ATP was released into this solution from a caged compound. However, Na_2ATP or the liberated ATP could have chelated any free intracellular calcium, thus inhibiting calcium-dependent K-channels (see Klöckner & Isenberg, 1992). Indeed, Clapp & Gurney (1992) detected a time-dependent K-current (carried by the large conductance calcium-activated K-channel, BK_{Ca}) which was markedly reduced by Na_2ATP. From this work it is therefore difficult to differentiate between the possible inhibitory effects of ATP on a presumed ATP-sensitive K-channel and those produced by inhibition of calcium-sensitive K-channels via a simultaneous reduction in intracellular calcium due to chelation. A similar possible misinterpretation appears to be responsible for the claimed ATP-dependency of BK_{Ca} described by Silberberg & van Breemen (1990) (see Klöckner & Isenberg, 1992).

In a recent, preliminary report, Silberberg & van Breemen (1992) have also demonstrated the induction of a current (I_{mi}), apparently similar to I_{met}, following 'metabolic inhibition' (i.e. in the presence of 1 mM iodo-acetate and 50 μM dinitrophenol) in rabbit mesenteric artery cells. Based on the inhibition by glibenclamide of both I_{mi} and of the current induced by levcromakalim, Silberberg & van Breemen (1992) suggested that both currents could have been produced by the opening of K_{ATP} in this tissue. However, they were unable to exclude the possibility that a calcium-dependent K-channel carried the observed currents, and no data were presented to demonstrate the K^+ selectivity of the channel. Clapp & Gurney (1992) have also recently claimed that 'K_{ATP}' is the target for the actions of K-channel openers in rat pulmonary artery. This view appears to be based solely on the fact that the hyperpolarization stimulated by levcromakalim was reduced in the absence of $[ATP]_i$. However, under these conditions, the membrane potential of the cell lies closer to E_K (Clapp & Gurney, 1992), the limit to which levcromakalim could hyperpolarize the cell, and a reduced effect would thus be predicted.

Inhibition of I_{TO} and generation of I_{KCO} and I_{met}: a working hypothesis

In the present study, removal of the carboxylic acids and glucose from the pipette also produced a marked inhibition of the delayed rectifier current, I_{TO}. This is the first report of such inhibition and the parallel between this and the similar inhibition of I_{TO} induced by levcromakalim (Noack *et al.*, 1992a; present study) is very striking.

Current flow through delayed rectifier K-channels (in squid axon) is markedly reduced in the absence of ATP-dependent phosphorylation (Perozo & Bezanilla, 1991). Thus, in the absence of glucose and TCA substrates in the present study, the channel underlying I_{TO} could have become dephosphorylated, resulting in the observed reduction of I_{TO}. A simple working hypothesis for the mechanism underlying the inhibitory action of either levcromakalim or reduction of $[ATP]_i$ on I_{TO} would thus be that each reduces the proportion of delayed rectifier channels in a phosphorylated state. In the case of substrate removal, the dephosphorylation would result from a simple reduction in $[ATP]_i$. The effects of levcromakalim on I_{TO} could be explained in terms of an inhibition of ATP binding or of ATP production. Although there is some evidence that cromakalim can produce a reduction in $[ATP]_i$ (which is inhibited by glibenclamide), the slow time-course of this event, and the limited range of concentrations over which cromakalim was effective (Longman, 1989), suggests this may not be the mechanism of action of the K-channel openers. However, since the K-channels associated with both I_{KCO} and I_{met} seem identical, a net reduction of ATP binding could also explain the induction of I_{met} following substrate removal and the generation of I_{KCO} by levcromakalim, provided that these K-channels are normally inhibited by $[ATP]_i$.

The existence of such an ATP-sensitive K-channel, K_{ATP}, both in the pancreatic β-cell and in cardiac and skeletal muscle has already been widely described (Noma, 1983; Cook & Hales, 1984; Rorsman & Trube, 1985; Spruce *et al.*, 1985). Moreover, in these tissues this channel is also opened by the K-channel openers (Escande *et al.*, 1988; Arena & Kass, 1989; Findlay *et al.*, 1989; Dunne *et al.*, 1990; Sauviat *et al.*, 1991). Thus, the inescapable conclusion from the present study must be that a K_{ATP}-type channel is most likely to be the channel which conducts I_{met} and which is also responsible for the hyperpolarizing action of the K-channel openers in the rat portal vein.

Phosphorylation of K_{ATP}

The ATP-sensitive K-channel described in other tissues is dually-modulated by ATP which is thought to act at two different sites each associated with the channel (see Ashcroft, 1988). Thus, phosphorylation of 'Site 1' by ATP is an absolute requirement for channel opening, with run-down possibly associated with dephosphorylation (see Ashcroft & Ashcroft, 1991). The second ATP-binding site (Site 2) is inhibitory. Since both hydrolysable and non-hydrolysable forms of ATP inhibit channel opening it seems likely that simple binding of ATP at, rather than phosphorylation of, this second site causes the reduced opening of the channel (Spruce *et al.*, 1987; Ashcroft & Kakei, 1989; Lederer & Nichols, 1989). In both the present study and in a previous investigation (Noack *et al.*, 1992a), levcromakalim increased I_{KCO}. If the opening of a K_{ATP} does underly the generation of I_{KCO} by levcromakalim, this agent could act directly or indirectly at Site 2 to reduce the *binding* of ATP to K_{ATP}. Furthermore, since levcromakalim may modulate the delayed rectifier by *dephosphorylation* (see above), it could also inhibit the interaction of ATP with the phosphorylation site (Site 1) of K_{ATP}.

If levcromakalim indeed modulates the interaction of ATP with its target K-channel at two different sites, two distinct effects of K_{ATP} would be predicted. One of these, dephosphorylation of Site 1, should induce channel run-down and effectively inhibit opening of K_{ATP} (consistent with the findings of Kozlowski *et al.*, 1989). The other, prevention of the binding of ATP to Site 2, would stimulate channel opening. In an earlier study, a maximally-effective concentration of levcromakalim (10 μM) produced a channel open probability of less than 0.4 (Noack *et al.*, 1992a). Such an observation is consistent with a modulating effect of K-

channel openers at both putative sites of action of ATP on K_{ATP} but further studies are required to confirm this possibility.

Run-down of I_{met}

To determine whether run-down of I_{met} was associated with channel dephosphorylation, we attempted to reverse the process by the addition of MgATP to the recording pipette. Use of this salt avoids intracellular calcium chelation and the accompanying problems of data interpretation evident in the studies of Clapp & Gurney (1992) and of Silberberg & van Breemen (1990). A low concentration of free ATP (1 μM) was chosen to minimize channel closure by an action of ATP at the postulated 'Site 2' (discussed above). Such a procedure increased the magnitude of I_{met} and the associated membrane hyperpolarization but had no effect on the time over which I_{met} could be measured. These results suggest that run-down due to dephosphorylation was occurring even during the development phase of I_{met} and that this was modified by the presence of the low concentration of free ATP. However, the inability of ATP to modify the overall time-course of I_{met} suggests that loss of other factors such as GTP or enzyme subunits also contributed to the run-down process (see Ribalet *et al.*, 1989).

High conductance contaminating current

Recordings of I_{met} were contaminated with another current associated with a large unitary conductance channel. This current manifested itself as a series of discrete jumps in the relatively smooth I_{met} trace. It was barely evident during the early stages of development of I_{met}, but became more prominent during the run-down and after disappearance of I_{met}. The conductance of the channel associated with the current steps was estimated to be approximately 150 pS at 0 mV under the quasi-physiological conditions employed and in the present study we did not examine the characteristics of this current further. However, it is tempting to speculate that the underlying channel could be the large conductance calcium-sensitive K-channel, BK_{Ca}. The opening of this channel is stimulated by dephosphorylation (Reinhart *et al.*, 1991) and in smooth muscle BK_{Ca} has a single channel conductance in the range 100–150 pS when measured under a physiological K^+ gradient (Bolton & Beech, 1992). Several workers have reported that the K-channel openers can open BK_{Ca} under patch-clamp conditions (Hermsmeyer, 1988; Hu *et al.*, 1990; Gelband *et al.*, 1990; Silberberg & van Breemen, 1990; Collier *et al.*, 1992; Klöckner & Isenberg, 1992). It is unlikely that the opening of such a channel could contribute to the observed effects of the K-channel openers in whole tissues or organisms since unlike the opening of BK_{Ca} (Beech & Bolton, 1989b), whole tissue effects are not inhibited by charybdotoxin (Winquist *et al.*, 1989; Wickenden *et al.*, 1991). If reduction of channel phosphorylation can account for the mechanism of action of the K-channel openers, then these agents could potentially influence the gating of BK_{Ca} by effectively reducing its degree of phosphorylation. A similar mechanism (i.e. stimulation of BK_{Ca} through protein dephosphorylation) has indeed been demonstrated for the K_{ATP} channel opener, somatostatin (White *et al.*, 1991). Thus the anomaly that the K-channel openers can open BK_{Ca} under patch clamp conditons (Hermsmeyer, 1988; Hu *et al.*, 1990; Gelband *et al.*, 1990; Silberberg & van Breemen, 1990; Collier *et al.*, 1992; Klöckner & Isenberg, 1992), yet have no demonstrable effect on this channel in whole tissues (Winquist *et al.*, 1989; Wickenden *et al.*, 1991) may simply be associated with the relatively low concentration of $[ATP]_i$ in the vicinity of the membrane under certain patch clamp conditions in contrast to the higher concentrations of ATP which exist *in vivo*.

Effect of $[ATP]_i$ *on membrane potential: relevance to hypoxia*

With glucose and carboxylic acids in the pipette, the membrane potential of the portal vein cells was low (– 17 mV; see also Noack *et al.*, 1992a). This value compares with that of – 35 mV on breaking the membrane patch in the absence of substrates for ATP, a value which increased to – 58 mV after I_{met} had fully developed. If it can be assumed that these extremes represent the influence of high (glucose plus TCA substrates) and low (I_{met} plateau) $[ATP]_i$, respectively, then the influence of $[ATP]_i$ on membrane potential becomes clear. Such data provide the first convincing support at a single cellular level that hypoxic vasodilatation (Daut *et al.*, 1990; von Beckerath *et al.*, 1991) and increase in ^{42}K efflux (Post & Jones, 1991) are linked to membrane hyperpolarization via an ATP-sensitive K-channel. Furthermore, this channel, like the hypoxic vasodilatation and increased ^{42}K-efflux, is glibenclamide-sensitive and seems also to be the main site of action of the K-channel openers.

Recently, there has been much speculation about the *physiological* role of such a K-channel, especially in cardiac muscle during hypoxia (see Escande & Cavero, 1992). Under normal conditions of smooth muscle oxygenation *in vivo*, it seems unlikely that the opening of such a channel could be modulated by changes in $[ATP]_i$, given the buffering capacity of creatine phosphate (Bueding *et al.*, 1967). Thus, if such a channel has a physiological role, then changes in $[ATP]_i$ may not be the normal mechanism by which opening and closing are effected. However, in pathological conditions, especially those involving prolonged hypoxia in vascular smooth muscle, ATP synthesis could be compromised in spite of buffering by creatine phosphate with a resulting powerful vasodilator stimulus.

Conclusions

The present study has clearly demonstrated that removal of sources of ATP production from the pipette results in the appearance of a glibenclamide-sensitive K-current (I_{met}) with an underlying relatively low unitary conductance. A K-current (I_{KCO}) with characteristics very similar to those of I_{met} is produced on exposure to levcromakalim. It thus seems reasonable to conclude that the same K-channel, which can be described as ATP-sensitive, underlies both events. Significantly, the same lack of metabolic substrates inhibited the delayed rectifier current (I_{TO}), a change also produced by levcromakalim. The data obtained in the present study collectively suggest that levcromakalim interferes with the interaction between ATP and certain types of K-channel. This results in the opening of those channels in which ATP-binding is associated with K-channel closure (such as K_{ATP}) and modulation of phosphorylation-dependent channels (such as those underlying I_{TO}). Experiments are in progress to test this proposal further and to determine whether all chemical types of K-channel opener share an identical mechanism of action.

This study was supported by grants from the Deutsche Forschungsgemeinschaft (Th.N. and P.D.) and from Pfizer Central Research (G.E. and A.H.W.).

References

ARENA, J.P. & KASS, R.S. (1989). Enhancement of potassium-sensitive current in heart cells by pinacidil: Evidence for modulation of the ATP-sensitive potassium channel. *Circ. Res.*, **65**, 436–445.

ASHCROFT, F.M. (1988). Adenosine 5'-triphosphate-sensitive potassium channels. *Annu. Rev. Neurosci.*, **11**, 97–118.

ASHCROFT, S.J.H. & ASHCROFT, F.M. (1991). Properties and functions of ATP-sensitive K-channels. *Cell. Signalling*, **2**, 197–214.

ASHCROFT, F.M. & KAKEI, M. (1989). ATP-sensitive K^+ channels in rat pancreatic β-cells: modulation by ATP and Mg^{2+} ions. *J. Physiol.*, **416**, 349–367.

BEECH, D.J. & BOLTON, T.B. (1989a). Properties of the cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. *Br. J. Pharmacol.*, **98**, 851–864.

BEECH, D.J. & BOLTON, T.B. (1989b). Two components of potassium current activated by depolarization of single smooth muscle cells from the rabbit portal vein. *J. Physiol.*, **418**, 293–309.

BOLTON, T.B. & BEECH, D.Z. (1992). Smooth muscle potassium channels: their electrophysiology and function. In *Potassium Channel Modulators: Pharmacological, Molecular and Clinical Aspects*. ed., Weston, A.H. & Hamilton, T.C. pp. 144–180. Oxford: Blackwell Scientific.

BUEDING, E., BÜLBRING, E., GERCKEN, G., HAWKINS, J.T. & KURIYAMA, H. (1967). The effect of adrenaline on the adenosine and creatine phosphate content of intestinal smooth muscle. *J. Physiol.*, **193**, 187–212.

CLAPP, L.H. & GURNEY, A.M. (1992). ATP-sensitive K^+ channels regulate resting potential of pulmonary arterial smooth muscle cells. *Am. J. Physiol.*, **262**, H916–H920.

COLLIER, M.L., TAYLOR, S.G. & WARD, J.P.T. (1992). The novel benzopyranol potassium channel activator BRL55834 activates two potassium channels in bovine airways smooth muscle. *Br. J. Pharmacol.* (Proceedings of the Dublin meeting: in press).

COOK, D.L. & HALES, C.N. (1984). Intracellular ATP directly blocks K^+ channels in pancreatic β cells. *Nature*, **311**, 271–273.

DAUT, J., MAIER-RUDOLPH, W., VON BECKERATH, N., MEHRKE, G., GUNTHER, K. & GOEDEL-MEINEN, L. (1990). Hypoxic dilation of coronary arteries is mediated by ATP-sensitive potassium channels. *Science*, **247**, 1341–1344.

DUNNE, M.J., ILLOT, M.C. & PETERSON, O.H. (1987). Interaction of diazoxide, tolbutamide and ATP^{4-} on nucleotide-dependent K^+ channels in an insulin-secreting cell line. *J. Membr. Biol.*, **99**, 215–224.

DUNNE, M.J., YULE, D.I., GALLACHER, D.V. & PETERSEN, O.H. (1990). Comparative study of the effects of cromakalim (BRL-34915) and diazoxide on membrane potential, $[Ca^{2+}]_i$ and ATP-sensitive potassium currents in insulin-secreting cells. *J. Membr. Biol.*, **114**, 53–60.

EDWARDS, G. & WESTON, A.H. (1990). Potassium channel openers and vascular smooth muscle relaxation. *Pharmacol. Ther.*, **48**, 237–258.

ESCANDE, D. & CAVERO, I. (1992). K^+ channel openers and 'natural' cardioprotection. *Trends Pharmacol. Sci.*, **13**, 269–272.

ESCANDE, D., THURINGER, D., LEGUERN, S. & CAVERO, I. (1988). The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent K^+ channels in isolated cardiac myocytes. *Biochem. Biophys. Res. Commun.*, **154**, 620–625.

FABIATO, A. (1988). Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol.*, **157**, 378–417.

FINDLAY, I., DEROUBAIX, E., GUIRAUDOU, P. & CORABOEUF, E. (1989). Effects of activation of ATP-sensitive K^+ channels in mammalian ventricular myocytes. *Am. J. Physiol.*, **257**, H1551–H1559.

FOSSET, M., DE WEILLE, J.R., GREEN, R.D., SCHMID-ANTOMARCHI, H. & LAZDUNSKI, M. (1988). Antidiabetic sulphonylureas control action potential properties in heart cells via high affinity receptors that are linked to ATP-dependent K^+ channels. *J. Biol. Chem.*, **263**, 7933–7936.

FUHRMANN, G.F., SCHWARZ, W., KERSTEN, R. & SDUN, H. (1985). Effect of vanadate, menadione and menadione analogs on the Ca^{2+}-activated K^+ channels in human red cells. Possible relations to membrane-bound oxidoreductase activity. *Biochem. Biophys. Acta.*, **820**, 223–234.

GELBAND, C.H., SILBERBERG, S.D., GROSCHNER, K. & VAN BREEMEN, C. (1990). ATP inhibits smooth muscle Ca^{2+}-activated K^+ channels. *Proc. R. Soc. Ser. B.*, **242**, 23–28.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HAMILTON, T.C., WEIR, S.W. & WESTON, A.H. (1986). Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. *Br. J. Pharmacol.*, **88**, 103–111.

HERMSMEYER, R.K. (1988). Pinacidil actions on ion channels in vascular muscle. *J. Cardiovasc. Pharmacol.*, **12**, S17–S22.

HU, S., KIM, H.S., OKOLIE, P. & WEISS, G.B. (1990). Alterations by glyburide of effects of BRL 34915 and P 1060 on contraction, ^{86}Rb efflux and maxi-K^+ channel in rat portal vein. *J. Pharmacol. Exp. Ther.*, **253**, 771–777.

KAJIOKA, S., OIKE, M. & KITAMURA, K. (1990). Nicorandil opens a calcium-dependent potassium channel in smooth muscle cells of the rat portal vein. *J. Pharmacol. Exp. Ther.*, **254**, 905–913.

KAJIOKA, S., KITAMURA, K. & KURIYAMA, H. (1991). Guanosine diphosphate activates an adenosine 5'-triphosphate-sensitive K^+ channel in the rabbit portal vein. *J. Physiol.*, **444**, 397–418.

KLÖCKNER, U. & ISENBERG, G. (1985). Calcium current of cesium loaded isolated smooth muscle cells (urinary bladder of the guinea-pig). *Pflügers Arch.*, **405**, 340–348.

KLÖCKNER, U. & ISENBERG, G. (1992). ATP suppresses activity of Ca^{2+}-activated K^+ channels by Ca^{2+} chelation. *Pflügers Arch.*, **420**, 101–105.

KOZLOWSKI, R.Z., HALES, C.N. & ASHFORD, M.L.J. (1989). Dual effects of diazoxide on ATP-K^+ currents recorded from an insulin-secreting cell line. *Br. J. Pharmacol.*, **97**, 1039–1050.

LEDERER, W.J. & NICHOLS, C.G. (1989). Nucleotide modulation of the activity of rat heart ATP-sensitive K^+ channels in isolated membrane patches. *J. Physiol.*, **419**, 193–211.

LONGMAN, S.D. (1989). Potassium channel activation in smooth muscle cells may be associated with decreases in intracellular ATP. *Br. J. Pharmacol.*, **98**, 888P.

NOACK, Th., DEITMER, P., EDWARDS, G. & WESTON, A.H. (1992a). Characterization of potassium currents modulated by BRL 38227 in rat portal vein. *Br. J. Pharmacol.*, **106**, 717–726.

NOACK, Th., EDWARDS, G., DEITMER, P., GREENGRASS, P., MORITA, T., ANDERSSON, P.-O., CRIDDLE, D., WYLLIE, M.G. & WESTON, A.H. (1992b). The involvement of potassium channels in the action of ciclazindol in rat portal vein. *Br. J. Pharmacol.*, **106**, 17–24.

NOMA, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature*, **305**, 147–148.

OKABE, K., KAJIOKA, S., NAKAO, K., KITAMURA, K., KURIYAMA, H. & WESTON, A.H. (1990). Actions of cromakalim on ionic currents recorded from single smooth muscle cells of the rat portal vein. *J. Pharmacol. Exp. Ther.*, **252**, 832–839.

PEROZO, E. & BEZANILLA, F. (1991). Phosphorylation of K^+ channels in the squid giant axon. A mechanistic analysis. *J. Bioeng. Biomembr.*, **23**, 599–613.

POST, J.M. & JONES, A.W. (1991). Stimulation of arterial 42K efflux by ATP depletion and cromakalim is antagonized by glyburide. *Am. J. Physiol.*, **260**, C375–C382.

QUAST, U. & COOK, N.S. (1989). Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends Pharmacol. Sci.*, **10**, 431–435.

REINHART, P.H., CHUNG, S., MARTIN, B.L., BRAUTIGAN, D.L. & LEVITAN, I.B. (1991). Modulation of calcium-activated potassium channels from rat brain by protein kinase A and phosphatase 2A. *J. Neurosci.*, **11**, 1627–1635.

RIBALET, B., CIANI, S. & EDDLESTONE, G.T. (1989). ATP mediates both activation and inhibition of K(ATP) channel activity via cAMP-dependent protein kinase in insulin-secreting cell lines. *J. Gen. Physiol.*, **94**, 693–717.

RORSMAN, P. & TRUBE, G. (1985). Glucose dependent K^+-channels in pancreatic β-cells are regulated by intracellular ATP. *Pflügers Arch.*, **405**, 305–309.

SAUVIAT, M.P., ECAULT, E., FAIVRE, J.F. & FINDLAY, I. (1991). Activation of ATP-sensitive K-channels by a K-channel opener (SR-44866) and the effect upon electrical and mechanical activity of frog skeletal muscle. *Pflügers Arch.*, **418**, 261–265.

SILBERBERG, S.D. & VAN BREEMEN, C. (1990). An ATP, calcium and voltage sensitive potassium channel in porcine coronary artery smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **17**, 517–522.

SILBERBERG, S.D. & VAN BREEMEN, C. (1992). A potassium current activated by lemakalim and metabolic inhibition in rabbit mesenteric artery. *Pflügers Arch.*, **420**, 118–120.

SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1985). Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature*, **316**, 736–738.

SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1987). Studies of the unitary properties of adenosine-5'-triphosphate-regulated potassium channels of frog skeletal muscle. *J. Physiol.*, **382**, 213–236.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

STOCKBRIDGE, N., ZHANG, H. & WEIR, B. (1991). Effects of K^+ channel agonists cromakalim and pinacidil on rat basilar artery smooth muscle cells are mediated by Ca^{2+}-activated K^+ channels. *Biochem. Biophys. Res. Commun.*, **181**, 172–178.

VON BECKERATH, N., CYRIS, S., DISCHNER, A. & DAUT, J. (1991). Hypoxic vasodilation in isolated, perfused guinea-pig heart: an analysis of the underlying mechanisms. *J. Physiol.*, **442**, 297–319.

WHITE, R.E., SCHONBRUNN, A. & ARMSTRONG, D.L. (1991). Somatostatin stimulates Ca^{2+}-activated K^+ channels through protein dephosphorylation. *Nature*, **351**, 570–573.

WICKENDEN, A.D., GRIMWOOD, S., GRANT, T.L. & TODD, M.H. (1991). Comparison of the effects of the K^+ -channel openers cromakalim and minoxidil sulphate on vascular smooth muscle. *Br. J. Pharmacol.*, **103**, 1148–1152.

WINQUIST, R.J., HEANEY, L.A., WALLACE, A.A., BASKIN, E.P., STEIN, R.B., GARCIA, M.L. & KACZOROWSKI, G.J. (1989). Glyburide blocks the relaxation response to BRL 34915 (cromakalim), minoxidil sulphate and diazoxide in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **248**, 149–156.

ZÜNKLER, B.J., LENZEN, S., MANNER, K., PANTEN, U. & TRUBE, G. (1988). Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic B-cells. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, 225–230.

(Received July 3, 1992
Revised July 23, 1992
Accepted July 27, 1992)

Br. J. Pharmacol. (1992), **107**, 956–963

Effects of PAF on excitatory neuro-effector transmission in dog airways

Kazuhiro Tashiro, Zhuoqiu Xie & [1]Yushi Ito

Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

1 Effects of PAF on excitatory neuro-effector transmission in smooth muscle cells of mucosa-free trachea and epithelium-intact bronchiole of the dog were investigated, by isometric tension recording, microelectrode and double sucrose-gap methods.

2 PAF (10^{-11}–10^{-7} M) dose-dependently enhanced the amplitude of contraction evoked by repetitive field stimulations (10 stimuli at 20 Hz) in both tracheal and bronchiolar tissues. At higher concentrations PAF ($>10^{-8}$ M) increased the amplitude of contraction to a greater extent in the bronchiole than in the trachea.

3 In both muscle tissues, in parallel to the amplitude of contraction, PAF markedly enhanced the amplitude of excitatory junction potentials (e.j.ps) evoked by a single field stimulation in a dose-dependent manner, with no change in the resting membrane potential or input membrane resistance of the smooth muscle cells. PAF (5×10^{-7} M) enhanced the amplitude of e.j.p. to a greater extent in the bronchiole than in the trachealis. In contrast, lyso-PAF (10^{-10}–10^{-7} M) showed no effect on e.j.p. amplitude in bronchiolar tissues. At a high concentration (10^{-7} M) lyso-PAF slightly enhanced the e.j.p. amplitude in tracheal tissue, however the lyso-PAF induced stimulation of e.j.p. amplitude in the trachea was small compared to that of PAF.

4 PAF (10^{-7} M) had no effect on the membrane depolarization induced by acetylcholine (ACh, 10^{-9}–10^{-5} M) and carbachol (10^{-9}–10^{-5} M) in tracheal smooth muscle cells.

5 The PAF-antagonists CV3988 (5×10^{-7} M) or WEB2086 (5×10^{-7} M) significantly enhanced the e.j.p. amplitude themselves, PAF (5×10^{-8} M) further enhanced the e.j.p. amplitude in the presence of WEB2086 (5×10^{-7} M) but not CV3988 (5×10^{-7} M). In contrast, the new PAF-antagonist, E 6123 (5×10^{-8} M), did not affect the e.j.p. amplitude itself, and completely inhibited the increase in e.j.p. amplitude caused by 5×10^{-8} M PAF. On the other hand, in the presence of the H_1-antagonist, mepyramine, PAF (5×10^{-8} M) further enhanced the e.j.p. amplitude.

6 The leukotriene synthesis inhibitor AA-861 (10^{-6} M) or leukotriene antagonist ONO1078 (10^{-7} M) inhibited the increase in e.j.p. amplitude caused by 5×10^{-8} M PAF, respectively.

7 In the presence of AA-861 (10^{-6} M), leukotriene B_4 (LTB_4, 10^{-8} M) or LTD_4 (10^{-8} M) slightly, and LTC_4 (10^{-8} M) markedly enhanced the e.j.p. amplitude. In contrast, LTE_4 (10^{-8} M) significantly suppressed the e.j.p. amplitude.

8 PAF (5×10^{-8} M) attenuated the depression phenomena of e.j.ps observed during double stimulus experiments at different time intervals (5–10 s), but had no effect on the summation of e.j.ps during repetitive field stimulation at a high frequency (20 Hz) in the trachealis.

9 These results indicate that PAF potentiates excitatory neuro-effector transmission mainly through stimulating the release of lipoxygenase products, mainly LTC_4 in the dog airway smooth muscle tissues.

Keywords: PAF; trachea, canine; bronchiole; excitatory neuro-effector transmission

Introduction

Platelet activating factor (PAF) is a phospholipid that was first derived from rabbit basophils upon IgE-mediated immunological challenge (Benveniste *et al.*, 1972). Later, it was shown that PAF can be synthesized and released by several immune and non-immune stimuli from a variety of cell types, including macrophages, platelets, monocytes and endothelial cells (Vargaftig *et al.*, 1981a,b; O'Flaherty & Wykle, 1983; Braquet *et al.*, 1987).

In the lung, PAF has potent effects on various tissues and cells. For example it increases pulmonary vascular pressure, induces pulmonary oedema (Hamasaki *et al.*, 1984) and causes a long lasting, non-specific increase in bronchial responsiveness in experimental animals (Mazzoni *et al.*, 1985; Barnes *et al.*, 1987; Robertson & Page, 1987; Robertson *et al.*, 1988). Recently, it was shown that PAF increases the responsiveness of dog airway smooth muscle to parasympathetic stimuli *in vivo* (Leef *et al.*, 1987; Bethel *et al.*, 1989). The authors concluded that airway hyperresponsiveness elicited by PAF results from regional stimulation and/or release of mediators that augment contractility of airway smooth muscle. To investigate further the mechanisms involved in increased responsiveness of airway smooth muscle, we performed comparative studies on the effect of PAF and lyso-PAF on the contraction and excitatory junction potential (e.j.p.) recorded from epithelium-intact bronchial and mucosa-free tracheal tissues, in response to electrical field stimulation (EFS) with isometric tension recording, double sucrose gap and microelectrode methods.

Methods

Adult mongrel dogs of either sex, weighing 10–13 kg were anaesthetized by i.v. administration of pentobarbitone (30 mg kg^{-1}). Segments of the cervical trachea were excised and whole pulmonary lobes were quickly resected from the main bronchus. A dorsal strip of transversely running tracheal smooth muscles was separated from the cartilage, and the mucosa and adventitial areolar tissue were carefully removed, leaving just smooth muscle tissue. The tracheal smooth muscle was cut to a width of 2.0–2.5 mm and a length of about 15 mm for use with the double sucrose gap

[1] Author for correspondence.

method. Small airways (about 1 mm in diameter) were carefully excised from the lung tissue under microscopic observation, and lung parenchyma and pulmonary vessels running along a bronchiolar branch were removed. Histological investigations revealed that the tissues used for the present experiments had a diameter of 0.8–1.1 mm, and were composed of smooth muscle layers and mucous membrane but lacked cartilage, thereby indicating that the tissue comprised bronchioles (Cumming, 1972; Inoue & Ito, 1986; Ito & Inoue, 1989). A piece of bronchiole (1 mm in diameter, 4–5 mm in length), and ring preparations (0.8–1 mm in diameter, 1–2 mm wide) were used for the microelectrode and tension recording experiments, respectively. The preparation was bathed in a modified Krebs solution of the following ionic concentration (mM), Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134.0, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.5. The solution was aerated with 97% O_2 and 3% CO_2 and the pH was 7.3–7.4. The double sucrose gap method was used to record simultaneously the membrane potential and tension development from the tracheal smooth muscles. The chamber used has been described elsewhere (Ito & Tajima, 1981). To produce neurogenic responses, EFS was applied between ring electrodes placed in the centre pool of the apparatus, using an electronic stimulator (Nihon Kohden SEN-7103). Single and repetitive stimulation was applied with current pulse of 50 μs in duration and 10–20 V in strength. The voltage of the current pulse was adjusted so that an e.j.p. of a defined amplitude was evoked by a single pulse. Drugs were dissolved in Krebs solution and applied to the tissue through the centre pool of the double sucrose gap apparatus, using a multi-way tap (dead-time approximately 30 s).

For intracellular recording of the membrane potential from a single cell, thin strips of tracheal tissue 10–15 mm in length 4–5 mm in width and 0.3–0.4 mm thick or a piece of intact bronchiole (1 mm in diameter, 4–5 mm in length) were used. The dog tracheal preparations used in these experiments were dissected entirely free from the overlying mucosal layer and its cartilagenous attachments. In the case of bronchioles, the airway tissues were dissected out from the lung tissue under microscopic observation, and smooth muscle cells were impaled with the microelectrode from the outer surface of the tissue so that the epithelial layer inside the lumen was intact.

A conventional microelectrode filled with 3 M KCl (30–50 MΩ) was used throughout the experiments. Field stimulation was applied to the nerves through a pair of Ag-AgCl wires 3–5 mm apart and placed so that a current pulse would pass transversely across the tissue. Single and repetitive stimuli at 20 Hz were applied, with a pulse of 30–100 μs duration and 30–50 V from an electronic stimulator (Nihon Kohden SEN-7103). The chamber in which the strips were mounted had a volume of 2 ml, and was superfused at a rate of 3 ml min^{-1} at a temperature of 35–36°C. To avoid recording artifacts due to twitch-like contraction of the muscle tissue, the preparation was pinned on a rubber plate in the chamber with insect pins 0.1 mm in diameter.

To measure the mechanical responses of the bronchiole, the ring preparations (0.8–1.0 mm in diameter, and 1 mm in length) were hooked over a pair of right-angled fine needles which were reduced in diameter by electrolysis to about 0.05 mm under microscopic observation. One of the needles was fixed at the bottom of the chamber and the other was connected to a manipulator. The other end of the needle was connected to an isometric tension transducer. The fixed needle was also used for electrical stimulation of the ring preparations.

Materials

The following drugs were used; indomethacin, acetylcholine hydrochloride, carbachol, physostigmine, leukotrienes B_4, C_4, D_4, E_4, PAF (Sigma, St. Louis, Mo, U.S.A.), guanethidine (Tokyo Kasei, Tokyo), tetrodotoxin (Sankyo, Tokyo), lyso-PAF (Bachem, California, U.S.A.), leukotriene synthesis inhibitor AA-861 (2,3,5-trimethyl-6-(12-hydroxy-5,1 O-dodecadiynyl)-1,4-benzoquinone; Takeda, Tokyo), leukotriene antagonist ONO 1078 (4-oxo-8-[p-(4-phenylbutyloxy)benzoylamino]-2-(tetrazol-5-yl)-4H-1-benzopyran hemihydrate; ONO, Osaka), PAF antagonists WEB2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-(3,2,f)(1,2,4)-triazolo) 4, 3-a) (1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone) (Boehringer Ingelheim), CV3988 ((**RS**)-2-methoxy-3-(octadecylcarbamoyloxy)propyl2-(3-thiazolio)ethyl phosphate; Takeda, Tokyo), and E 6123 ((**S**)-(+)-6-(2-chlorophenyl)-3-cyclopropanecarbonyl-8, 11-dimethyl-2,3,4,5-tetrahydro-8H-pyrido-[4',3' : 4,5] thieno[3,2f] [1,2,4] triazolo[4,3,-a][1,4]diazepine; Eisai, Ibaraki), mepyramine and atropine sulphate (Daiichi, Tokyo), FPL 55712 (7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxyl] -4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid; Fisons, Ltd., Loughborough, U.K.).

Statistical evaluation

Results (membrane potential, amplitude of contractions, relaxations or e.j.ps) are expressed as mean ± s.d. and were analyzed by Student's *t* test for paired (amplitude of contractions, relaxations or e.j.ps) or unpaired data (membrane potential) to estimate the significance of differences between means ($P<0.05$ was judged to be significant). For the measurement of resting membrane potential, 10–30 cells were impaled before and after drug application from the same preparations, and the experiments were repeated using 4–5 preparations, and the mean values were calculated from the collected data (unpaired data). Microelectrode or double-sucrose gap recordings of e.j.ps were obtained from the same cell or the same tissue throughout the experiments before and after the drug application, and data obtained from 5–8 cells or 4–8 preparations were used for the statistical analysis (paired data).

Results

Effects of PAF on contraction of dog trachea and bronchioles evoked by electrical field stimulation

The effects of PAF on the contractions evoked by electrical field stimulation (EFS) of cholinergic nerve fibres were studied in dog epithelium-free tracheal and epithelium-intact bronchiolar tissues, in the presence of indomethacin (10^{-5} M) and guanethidine (10^{-6} M) (see Methods).

Figure 1 shows the effects of PAF (10^{-11}–10^{-7} M) on the amplitude of contractions evoked by repetitive field stimulation (10 stimuli at 20 Hz) in the tracheal and bronchiolar tissues. PAF (10^{-11}–10^{-7} M) had no effect on the resting tension but enhanced the amplitude of contractions in the tracheal tissue dose-dependently to 1.3–1.9 times the control value. In the bronchioles, PAF (10^{-11}–10^{-7} M) also enhanced the amplitude of contractions in a dose-dependent manner to 1.3–2.7 times the control and in some preparations it induced a phasic increase in muscle tone. As the PAF-induced phasic contraction did not occur consistently, no quantitative analysis was performed. However, the PAF-induced phasic contraction was partially suppressed by atropine (10^{-6} M) or FPL 55712 (10^{-6} M).

Effects of PAF and lyso-PAF on the amplitude of the excitatory junction potential of trachea and bronchioles

To assess the mechanisms involved in the enhancing effects of PAF on twitch contractions, we observed its effects on e.j.p. amplitude with microelectrode and double sucrose gap methods.

In the trachea, low concentrations of PAF (10^{-11} or 5×10^{-10} M) significantly enhanced the e.j.p. amplitude to

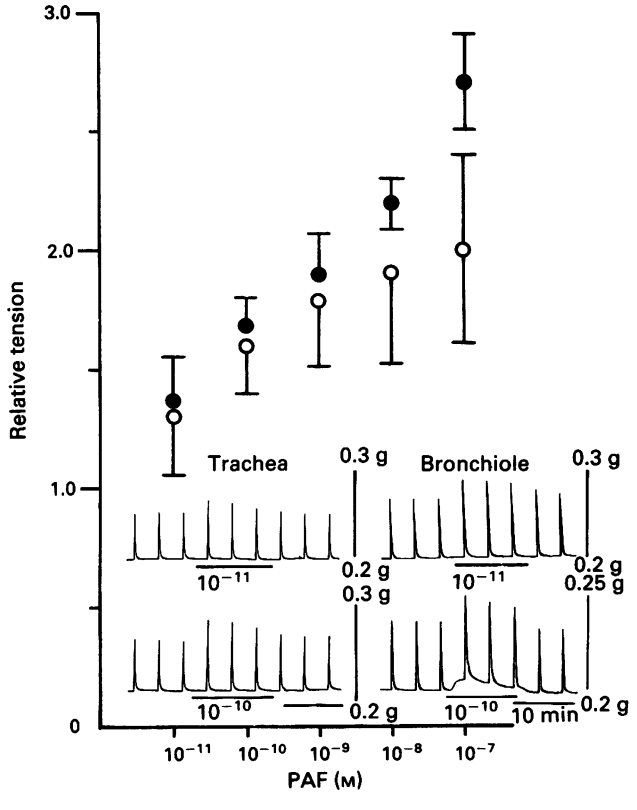

Figure 1 Effects of PAF (10^{-11}–10^{-7} M) on the contractions of dog trachea (○) and bronchiole (●) evoked by repetitive field stimulation (10 stimuli at 20 Hz). The amplitude of contractions evoked by 10 stimuli at 20 Hz in normal Krebs solution was taken as a relative amplitude of 1.0. Each point is the mean value of 8–12 experiments carried out with 7–10 preparations from 5 dogs and vertical bars indicate 2 × s.d.

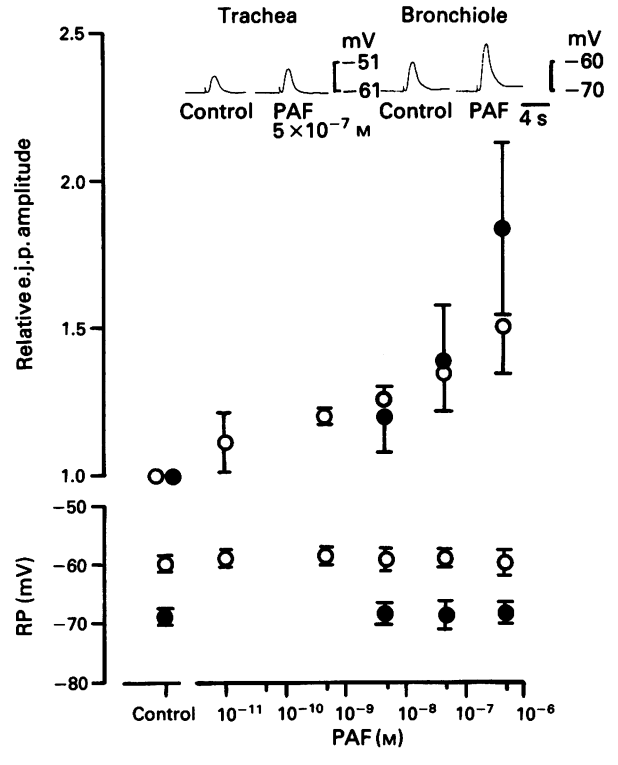

Figure 2 Effects of PAF (10^{-11}–10^{-7} M) on the amplitude of e.j.p. and resting membrane potential (RP) of smooth muscle cells of dog trachea (○) and bronchiole (●). The amplitude of e.j.p. evoked by a single field stimulation in normal Krebs solution was taken as a relative value 1.0. Each point is the mean value derived from 4–20 experiments using 4–8 preparations, and vertical bars indicate 2 × s.d. The e.j.p. and resting membrane potential were recorded by microelectrodes.

1.12 ± 0.08 ($n = 4$, $P < 0.05$), and 1.20 ± 0.02 ($n = 4$, $P < 0.01$) times the control value with no change in the resting membrane potential. In the bronchiole, on the other hand, relatively higher concentrations of PAF (5×10^{-9} M) were required to enhance the e.j.p. amplitude significantly to 1.21 ± 0.01 ($n = 4$, $P < 0.01$). The double sucrose gap method was also used to record e.j.ps and the following contractions, and confirmed that PAF (10^{-7} M) similarly enhanced the e.j.p. amplitude to 1.30 ± 0.20 ($n = 3$, $P < 0.001$) in the trachea with no change in the resting membrane potential and the input membrane resistance (data not shown).

Figure 2 summarizes the effects of PAF on the relative amplitude of the e.j.p. and the resting membrane potential of the smooth muscle cells in dog tracheal and bronchiolar tissues.

We also observed the effects of lyso-PAF on the amplitude of e.j.p. and resting membrane potential of the smooth muscle cells in both muscle tissues under the same condition. As shown in Figure 3, lyso-PAF (10^{-10}–10^{-7} M) showed no effect on e.j.p. amplitude and resting membrane potential in dog bronchiolar tissue. Similarly in the trachea, lyso-PAF (10^{-10}–10^{-8} M) did not affect the e.j.p. amplitude. However, at a higher concentration (10^{-7} M), it significantly enhanced the e.j.p. amplitude to 1.13 ± 0.06 ($n = 4$, $P < 0.05$) in the trachea, although the lyso-PAF-induced stimulation of e.j.p. amplitude was small compared to that of PAF.

Effects of PAF on acetylcholine or carbachol-induced depolarization

As relatively low concentrations of PAF ($< 5 \times 10^{-9}$ M) enhanced the e.j.p. amplitude and contractions evoked by EFS with no change in the membrane potential or input membrane resistance, it was of interest to observe the effects

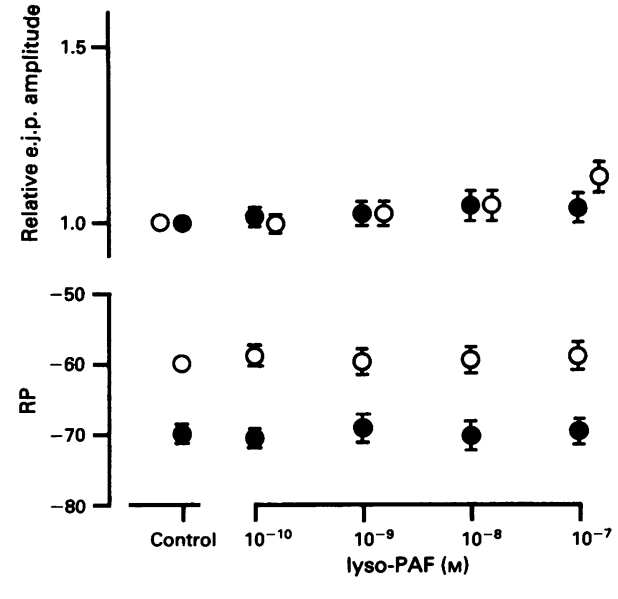

Figure 3 Effects of lyso-PAF (10^{-10}–10^{-7} M) on the amplitude of e.j.p. and resting membrane potential (RP) of smooth muscle cells of dog trachea (○) and bronchiole (●). The amplitude of e.j.p. evoked by a single field stimulation in normal Krebs solution was taken as a relative value 1.0. Each point is the mean value derived from 5–15 experiments using 5–8 preparations, and vertical bars indicate 2 × s.d. The e.j.p. and resting membrane potential were recorded by microelectrode.

of PAF on the sensitivity of smooth muscle cells to ACh. For this purpose, the effect of PAF (10^{-7} M) on the membrane depolarization induced by various concentrations of carbachol or ACh in the presence or absence of physostigmine (10^{-6} M) was examined in dog trachea. The membrane potential of the tracheal smooth smooth ranged between -57 mV and -61 mV, with a mean value of -59.2 ± 1.4 mV ($n = 20$). ACh ($> 10^{-9}$ M) depolarized the membrane dose-dependently, and the depolarization was enhanced in the presence of physostigmine (10^{-6} M), which produced a shift in the relationship between the dose of ACh and membrane potential to depolarized level (Figure 4). However, application of PAF did not affect the relationship between the membrane depolarization and dose of ACh in the presence or absence of physostigmine (data not shown). Similarly, carbachol ($> 10^{-9}$ M) dose-dependently depolarized the membrane of the tracheal smooth muscle cells, and PAF showed no effect on the carbachol-induced membrane depolarization (Figure 4).

Effects of PAF antagonists and mepyramine on the amplitude of the e.j.p.

To study further the mechanisms involved in the enhancing effects of PAF on e.j.p. amplitude, we used the PAF antagonists, WEB 2086, CV3988 and E6128. At a concentration of 5×10^{-8} M, WEB 2086 did not affect the e.j.p. amplitude and resting membrane potential of the dog trachea, but at an increased concentration (5×10^{-7} M) this agent significantly enhanced the e.j.p. amplitude to 1.20 ± 0.10 ($n = 4$, $P < 0.01$) times the control with no change in the membrane potential. In the presence of WEB 2086 (5×10^{-7} M), PAF (5×10^{-8} M) further enhanced the e.j.p. amplitude to 1.51 ± 0.06 ($n = 4$, $P < 0.01$), indicating that WEB 2086 did not antagonize the action of PAF. PAF-antagonist CV3988 ($> 5 \times 10^{-8}$ M) also enhanced the e.j.p. amplitude dose-dependently. After the pretreatment of the tissue with CV3988 (5×10^{-8} M), PAF (5×10^{-8} M) showed additional potentiating effects on e.j.p. amplitude. However, in the presence of an increased concentration of CV3988 (5×10^{-7} M), PAF (5×10^{-8} M) did not enhance the e.j.p. amplitude (Figure 5). However, it may be that the failure of PAF to enhance the e.j.p. amplitude in the presence of 5×10^{-7} M CV3988 is due to the drug having elicited the maximum possible stimulation of the e.j.p. itself. Therefore we used a new PAF antagonist, E6123, a member of the benzodiazepine class of PAF antagonists (Tsunoda *et al.*, 1990). E6123 (5×10^{-8} M) did not affect the e.j.p. amplitude and resting membrane potential of dog trachea, but completely suppressed the PAF (5×10^{-8} M)-induced stimulation of e.j.p. amplitude (Figure 5).

Similar experiments were repeated with mepyramine, since it is known that PAF stimulates the release of histamine from mast cells in the airway (see for example, Townley *et al.*, 1989) and it is known that released histamine stimulates the ACh release from the vagus nerve terminal through H_1-receptors (Inoue & Ito, 1986). However, in the presence of the H_1-receptor antagonist, mepyramine, PAF significantly enhanced the e.j.p. amplitude (Figure 5).

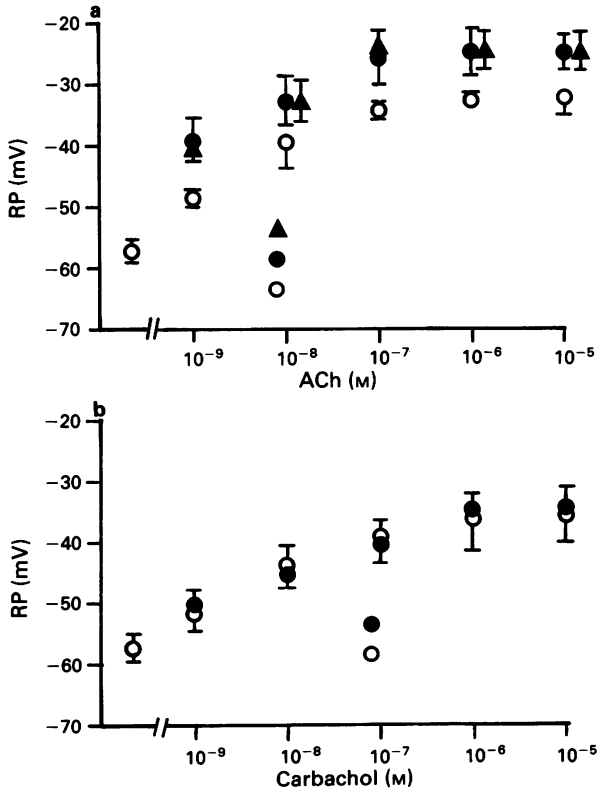

Figure 4 Effects of PAF (10^{-7} M) on the membrane depolarization induced by ACh (a) and carbachol (b) in the dog tracheal smooth muscle cells. In (a): (▲) ACh + physostigmine (10^{-6} M) + PAF (10^{-7} M); (●) ACh + physostigmine (10^{-6} M); (○) control. In (b): (●) carbachol + PAF (10^{-7} M); (○) control. Each point is the mean value of 15–30 experiments from 4–6 preparations. Bars indicate 2 × s.d.

Effects of lipoxygenase inhibitor AA-861, leukotriene antagonist ONO1078 and leukotrienes on the amplitude of e.j.p. of the dog trachea

It is also known that PAF stimulates the lipoxygenase pathway to produce leukotrienes in the perfused lung (Jancar *et al.*, 1989). Therefore we also observed the effects of lipoxygenase inhibitor AA-861 and leukotriene antagonist ONO-1078 (Yamai *et al.*, 1989) on the action of PAF on e.j.p. amplitude. AA-861 (10^{-6} M) or ONO1078 (10^{-7} M) did not affect the e.j.p. amplitude and resting membrane potential of dog trachea themselves, but completely inhibited the PAF-induced stimulation of e.j.p. amplitude (Figure 5).

It was of interest to observe the effects of exogenously applied leukotrienes on the amplitude of e.j.p. of the dog trachea, since AA-861 and ONO1078 suppressed the enhancing effects of PAF on e.j.p. amplitude.

In the presence of AA-861 (10^{-6} M), LTB_4 (10^{-8} M) or LTD_4 (10^{-8} M) slightly, and LTC_4 (10^{-8} M) markedly enhanced the e.j.p. amplitude. In contrast, LTE_4 (10^{-8} M) significantly suppressed the e.j.p. amplitude. Figure 6 summarizes the effects of leukotrienes on the e.j.p. amplitude of dog trachea in the presence of AA-861 (10^{-6} M).

Effects of PAF on the depression phenomenon of e.j.p.

When conditioning and test EFS were applied in tandem at intervals of 5–60 s, the amplitude of the test e.j.p. was always smaller than the conditioning e.j.p., showing a marked depression phenomenon of e.j.ps (Ito & Tajima, 1981). Figure 7 shows the mean amplitude of the second e.j.p. relative to the first e.j.p. PAF (5×10^{-8} M) reduced the depression phenomenon within the time intervals of 5–10 s.

Effects of PAF on the e.j.p. evoked by repetitive field stimulation

When repetitive field stimulation at high frequency (20 Hz) was applied, e.j.ps showed summation in dog trachea (Ito & Tajima, 1981; Ito & Yoshitomi, 1988; Hakoda & Ito, 1990).

Figure 8 shows the effects of PAF on the relationship between the relative amplitude of e.j.p. and number of stimuli. PAF had no effect on the relationship.

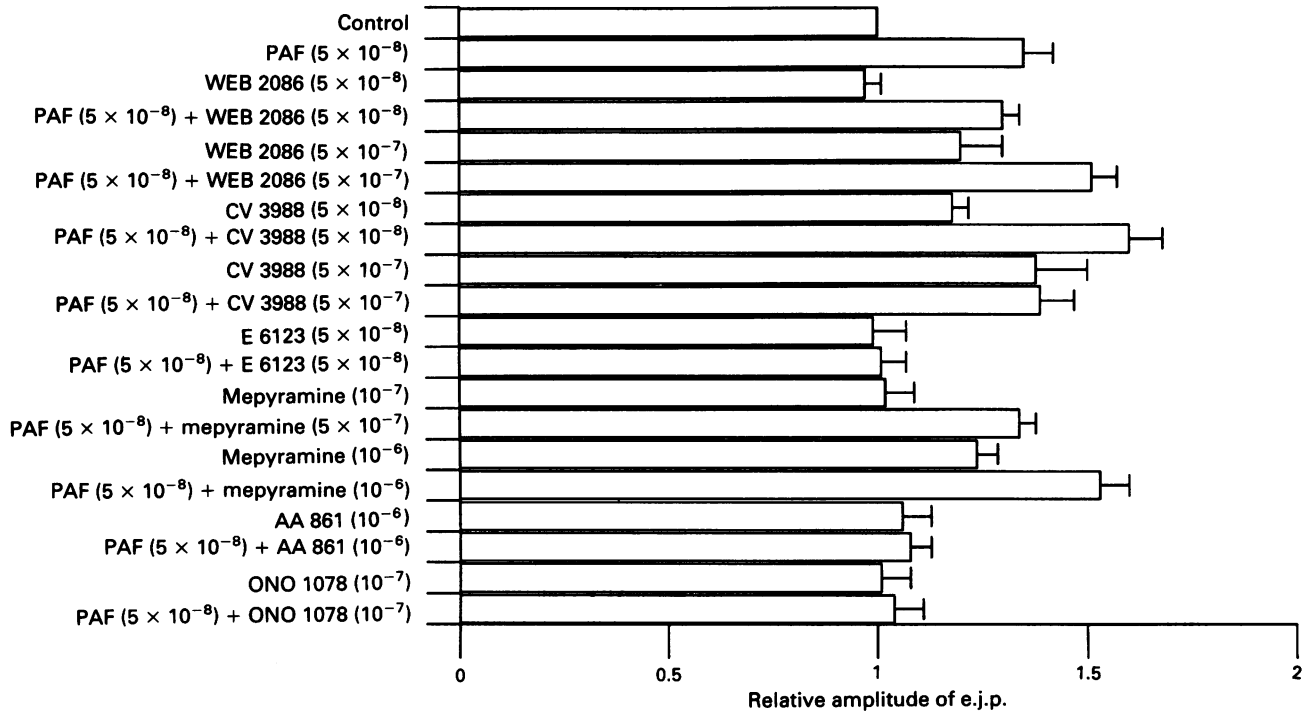

Figure 5 Effects of PAF (5×10^{-8} M), PAF-antagonists (WEB 2086, CV 3988 and E 6123), mepyramine, leukotriene synthesis inhibitor AA-861 (10^{-6} M) and leukotriene antagonist ONO 1078 (10^{-7} M) on the e.j.p. amplitude of dog trachea. The amplitude of e.j.p. evoked by a single field stimulation before application of drugs is defined as a relative amplitude of 1.0. Each value indicates mean of 4–8 experiments with 4–6 preparations and bars are s.d. All concentrations are molar.

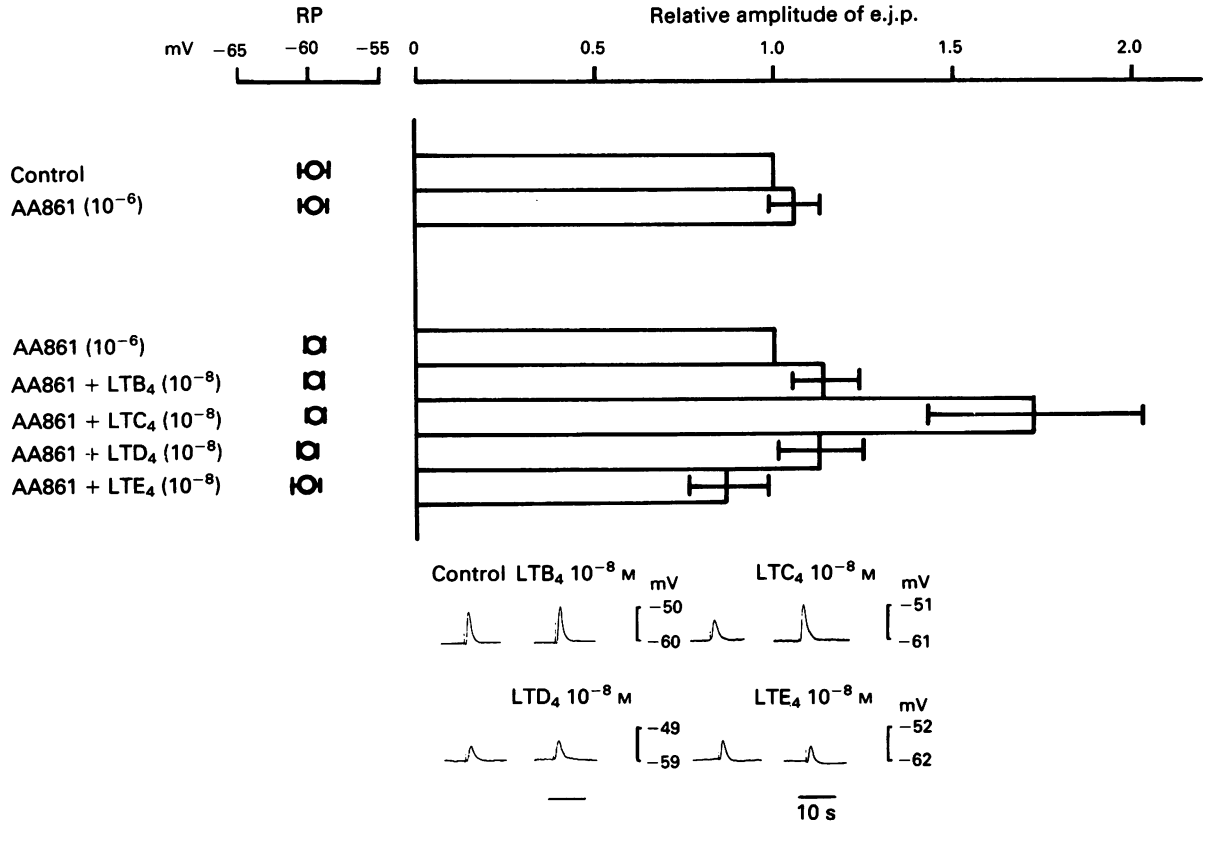

Figure 6 Effects of AA-861 (10^{-6} M) and various leukotrienes (LTB_4, C_4, D_4 and E_4, 10^{-8} M each) on the amplitude of e.j.p. recorded with microelectrodes in the dog trachea. Each column is the mean value of 4–7 experiments with 4–5 preparations and horizontal bars indicate 2 × s.d. All concentrations are molar.

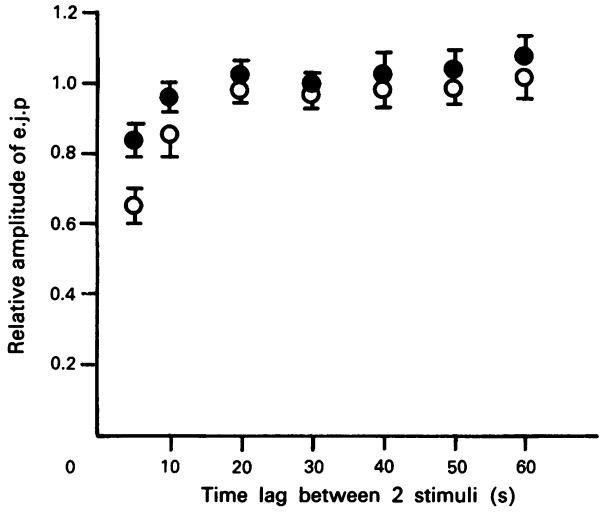

Figure 7 The effects of PAF (5×10^{-8} M) on relative changes in the amplitude of the test e.j.p. (the second e.j.p.) following the conditioning e.j.p. (the first e.j.p.) measured at various time intervals (5–60 s): (●) PAF 5×10^{-8} M; (○) control. Each point is the mean value obtained from 5–7 experiments with 5–7 preparations and vertical bars indicate s.d. or 2 × s.d.

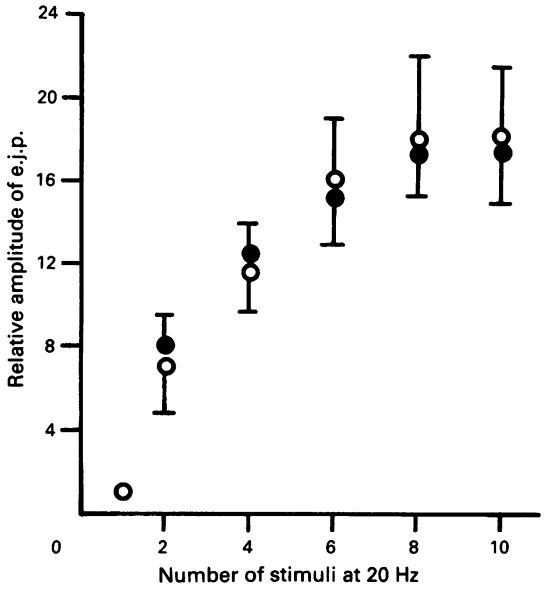

Figure 8 Effects of PAF (5×10^{-8} M) on the relationship between e.j.p. amplitude and the number of stimuli at 20 Hz: (○) control. (●) PAF (5×10^{-8} M). Each point is the mean value of 4–6 experiments with 4–6 preparations, vertical bars show s.d.

Discussion

The present study clearly shows an enhancing effect of PAF on the excitatory neuro-effector transmission in the dog trachea and bronchioles *in vitro*, presumably by increasing ACh release from the vagus nerve, since low concentrations of PAF (10^{-11}–10^{-9} M) significantly increased the amplitude of e.j.p. and contractions evoked by nerve stimulation with no change in resting membrane potential, input membrane resistance or ACh-sensitivity of the smooth muscle cells. The enhancing action of PAF on neuro-effector transmission in the airway seems due to specific action of PAF, since lyso-PAF showed practically no effect on e.j.p. amplitude in both tissues and PAF antagonist E6123 completely suppressed the PAF-induced stimulation of e.j.p. amplitude. Furthermore, it seems that effects of PAF on release of ACh from the vagus nerve terminal are not due to a direct action of PAF itself but through stimulation of lipoxygenase to produce leukotrienes in the tracheal and bronchiolar tissues. This view is mainly dependent on the observation that in the presence of the lipoxygenase inhibitor AA-861 or the leukotriene antagonist, ONO1078, PAF showed no effect on the e.j.p. amplitude.

Intravenous injections of PAF into the systemic circulation of living animals induces effects similar to an 'anaphylactic reaction' such as strong bronchoconstriction, acute inflammation and oedema, platelet aggregation and degranulation, and chemotaxis of neutrophils and eosinophils (Vargaftig *et al.*, 1981a,b; Page *et al.*, 1984). PAF-induced bronchoconstriction observed *in vivo* does not seem to be induced by a direct action of PAF on the bronchus, since the effects of PAF were less potent when the isolated lungs were perfused with cell-free solutions, or when isolated airway smooth muscle tissues were used in the experiments (Townley *et al.*, 1989). Thus the effects of PAF in lungs appear to depend on the method of application, which may reflect activation of different cell types.

Initially it was thought that platelets are essential for PAF to induce bronchoconstriction in various animal species, and in the dog it is largely dependent on secretion of 5-hydroxytryptamine released from platelets (Popovich *et al.*, 1988). However, it was also shown that in guinea-pig isolated lungs perfused with cell-free solutions, the injection of PAF induced a bronchoconstriction which was platelet-independent and blocked by cyclo-oxygenase inhibitors (Lefort *et al.*, 1984). Confirming this observation, there is a report indicating that the airway hyperresponsiveness induced by PAF may be dependent on thromboxane generation, since OKY-046, a thromboxane synthase inhibitor, suppressed PAF-induced bronchoconstriction and hyperresponsiveness in the dog (Chung *et al.*, 1986). PAF actually induces release of thromboxane A_2 (TXA_2) which preceded the increase in airway and pulmonary pressures (Lefort *et al.*, 1984), and inhalation of PAF increased the amount of a major urinary TXA_2 metabolite, 2,3-dinor-TXB_2 (Taylor *et al.*, 1991).

On the other hand, recent investigations indicate that when PAF was injected into isolated, perfused lungs, it stimulated the release of substance(s) causing contraction of bronchus, trachea and parenchymal strips which is unrelated to cyclo-oxygenase products (Jancar *et al.*, 1987; 1989). The authors concluded that a lipoxygenase product, possibly LTB_4 may be responsible for spasmogenic activity released by lungs following PAF stimulation, and that cyclo-oxygenase products subsequently released appear to result from the initial generation of LTB_4.

PAF causes vasoconstriction and oedema in non-sensitized rat lungs, possibly through the production of LTC_4 and D_4 (Voelkel *et al.*, 1982). As the present experiments were performed in the presence of indomethacin, it is possible to exclude the role of cyclo-oxygenase products on the enhancing actions of PAF on the excitatory neuro-effector transmission. Thus it is reasonable to assume that PAF stimulates 5-lipoxygenase to produce leukotrienes in the airway smooth muscle tissues, and in turn these leukotrienes enhance the excitatory neuro-effector transmission.

PAF and arachidonic acid may be released from a common precursor, 1-alkyl-2-acyl-glycero-3-phosphocholine, in various cells (Snyder, 1985). Thus it seems probable that PAF might act as a potential regulator of arachidonic acid metabolism and *vice versa*. Although the PAF-antagonist CV 3988 possesses weak agonistic activity at high concentrations, it is likely to stimulate the endogenous generation of leukotrienes (Chung *et al.*, 1986; Braquet *et al.*, 1987; Taylor *et al.*, 1991). These findings could be explained by effects of CV 3988 on the e.j.p. amplitude in the absence and presence of PAF. In addition, in the case of PAF, inhibition of leukotriene synthesis by AA-861 produced similar results on the e.j.p. amplitude as with the PAF-antagonist, CV 3988, in our experiments, i.e. suppressing the PAF actions on the e.j.p.

amplitude. Furthermore, in the presence of leukotriene antagonist, ONO1078, which is a specific LTC_4 and LTD_4 antagonist (Yamai *et al.*, 1989) PAF did not show any effect on e.j.p. amplitude. These data further reinforce the explanation for the role of leukotrienes in the actions of PAF on excitatory neuro-effector transmission.

WEB 2086 did not antagonize the stimulating action of PAF on excitatory neuro-effector transmission, whereas CV 3988 in relatively high concentrations only partially suppressed the action of PAF. However, PAF-induced stimulation of e.j.p. amplitude was completely inhibited by E 6123, a new member of the benzodiazepine class of PAF antagonists (Tsunoda *et al.*, 1990). Recently, it was reported that among various PAF antagonists E 6123 potently suppresses PAF inhalation-induced bronchoconstriction in guinea-pigs with an ED_{50} value of 1.3 $\mu g\,kg^{-1}$ which was lower than that of other PAF-antagonists such as WEB 2347 ($ED_{50} = 26$ $\mu g\,kg^{-1}$) and Y-24180 ($ED_{50} = 12\,\mu g\,kg^{-1}$) (Sakuma *et al.*, 1991). Thus, the stimulating action of PAF on excitatory neuro-effector transmission is due to a specific action of PAF through PAF-receptors.

In the present experiments, there was a tendency for the enhancing effects of PAF on the amplitude of e.j.p. and contractions evoked by EFS to be more pronounced in the bronchioles. As far as tracheal muscle was concerned, the mucosa was removed entirely to leave just smooth muscle tissue. In contrast, bronchioles were carefully excised from the lung tissue under microscopic observation and smooth muscle cells were impaled with a microelectrode from the adventitial surface of the tissue leaving the inner lumen intact. Thus, the experiments were performed with epithelium-free tracheal tissue and epithelium-intact bronchiolar tissues. It has been reported that the airway epithelium may contain surface receptors for PAF (Adler & Henke, 1991), and there is evidence that PAF stimulates the airway epithelium to produce cyclo-oxygenase products. Recent investigations also indicate that factor(s) released from epithelial cells in the airway control excitatory neuro-effector transmission possibly through lipoxygenase products of arachidonic acid (Xie *et al.*, 1992). Furthermore, LTC_4 applied exogenously in relatively low concentration enhanced the amplitude of e.j.p. and contractions evoked by EFS in the dog bronchiole. The present experiments indicate that PAF stimulates lipoxygenase in the airway smooth muscle cells and epithelial cells to produce leukotrienes and these modify excitatory neuro-effector transmission in the tracheal and bronchiolar tissues. PAF causes both acute bronchoconstriction (Leef *et al.*, 1987; Smith *et al.*, 1988) and persistent airway hyperresponsiveness of the airway (Chung *et al.*, 1986; Christman *et al.*, 1987). Thus, it is reasonable to assume that leukotrienes released from airway smooth muscle or epithelial cells in response to application of PAF contribute to both acute bronchoconstriction and hyperresponsiveness of the airway.

The authors wish to thank Ono, Boehringer Ingelheim, Fisons and Eisai Pharmaceuticals for the gift of ONO1078, WEB2086, FPL55712 and E6123.

References

ADLER, K.B. & HENKE, D.C. (1991). Effects of inflammatory mediators on epithelial function and integrity. In *Mediators of Pulmonary Inflammation*, Vol. 54. ed. Bray, M.A. & Anderson, W.H. pp. 377–402. New York: Marcel Dekker Inc.

BARNES, P.J., GRANDORDY, B.M., PAGE, C.P., RHODEN, K.J. & ROBERTSON, D.N. (1987). The effect of platelet-activating factor on pulmonary beta-adrenoceptors. *Br. J. Pharmacol.*, **90**, 709–715.

BENVENISTE, J., HENSON, P.M. & COCHRANE, C.G. (1972). Leukocyte-dependent histamine release from rabbit platelets: the role of IgE, basophils, and a platelet-activating factor. *J. Exp. Med.*, **136**, 1356–1377.

BETHEL, R.A., CURTIS, S.P., LIEN, D.C., IRVIN, C.G., WORTHEN, G.S., LEFF, L.R. & HENSON, P.M. (1989). Effect of PAF on parasympathetic contraction of canine airways. *J. Appl. Physiol.*, **66**, 2629–2634.

BRAQUET, P., TOUQUI, L., SHEN, T.Y. & VARGAFTIG, B.B. (1987). Perspectives in platelet-activating factor research. *Pharmacol. Rev.*, **39**, 97–145.

CHRISTMAN, B.W., LEFFERTS, P.L. & SNAPPER, J.B. (1987). Effect of platelet-activating factor on aerosol histamine responsiveness in awake sheep. *Am. Rev. Respir. Dis.*, **135**, 1267–1270.

CHUNG, K.F., AIZAWA, H., LEIKAUF, G.D., UEKI, I.F., EVANS, T.W. & NADEL, J.A. (1986). Airway hyperresponsiveness induced by platelet-activating factor: Role of thromboxane generation. *J. Pharmacol. Exp. Ther.*, **236**, 580–584.

CUMMING, G. (1972). Airway morphology and its consequences. *Clin. Resp. Physiol.*, **8**, 527–532.

HAKODA, H. & ITO, Y. (1990). Modulation of cholinergic neurotransmission by the peptide VIP, VIP antiserum and VIP antagonists in dog and cat trachea. *J. Physiol.*, **428**, 133–154.

HAMASAKI, Y., MOJARAD, M., SAGA, T., TAI, H.-H. & SAID, S.I. (1984). Platelet-activating factor raises airway and vascular pressures and induces edema in lungs perfused with platelet-free solution. *Am. Rev. Respir. Dis.*, **129**, 742–746.

INOUE, T. & ITO, Y. (1986). Characteristics of neuro-effector transmission in the smooth muscle layer of dog bronchiole and modifications by autacoids. *J. Physiol.*, **370**, 551–565.

ITO, Y. & INOUE, T. (1989). Contracture and change in membrane potential produced by sodium removal in the dog trachea and bronchiole. *J. Appl. Physiol.*, **67**, 2078–2086.

ITO, Y. & TAJIMA, K. (1981). Actions of indomethacin and prostaglandins on neuro-effector transmission in the dog trachea. *J. Physiol.*, **319**, 379–392.

ITO, Y. & YOSHITOMI, T. (1988). Autoregulation of acetylcholine release from vagus nerve terminals through activation of muscarinic receptors in the dog trachea. *Br. J. Pharmacol.*, **93**, 636–646.

JANCAR, S., THÉRIAULT, P., BRAQUET, P. & SIROIS, P. (1987). Comparative effects of platelet activating factor, leukotriene D_4 and histamine in guinea pig trachea, bronchus and lung parenchyma. *Prostaglandins*, **33**, 199–208.

JANCAR, S., THÉRIAULT, P., LAUZIÉRE, M., BRAQUET, P. & SIROIS, P. (1989). Paf-induced release of spasmogens from guinea-pig lungs. *Br. J. Pharmacol.*, **96**, 153–162.

LEEF, A.R., WHITE, S.R., MUNOZ, N.M., POPOVICH, K.J., SHIOYA, T. & STIMLER-GERARD, N.P. (1987). Parasympathetic involvement in PAF-induced contraction in canine trachealis in vivo. *J. Appl. Physiol.*, **62**, 599–605.

LEFORT, J., ROTILIO, D. & VARGAFTIG, B.B. (1984). The platelet-independent release of thromboxane A_2 by PAF-acether from guinea-pig lungs involves mechanisms distinct from those for leukotriene. *Br. J. Pharmacol.*, **82**, 565–575.

MAZZONI, L., MORLEY, J., PAGE, C.P. & SANJAR, S. (1985). Induction of airway hyper-reactivity by platelet-activating factor in the guinea-pig. *J. Physiol.*, **365**, 107P.

O'FLAHERTY, J.L. & WYKLE, R.L. (1983). Biology and biochemistry of platelet-activating factor. *Clin. Rev. Allergy*, **1**, 353–367.

PAGE, C.P., ARCHER, C.B., PAUL, W. & MORLEY, J. (1984). PAF-acether: a mediator of inflammation and asthma. *Trends Pharmacol. Sci.*, **5**, 239–241.

POPOVICH, K.J., SHELDON, G., MACK, M., MUNOZ, N.M., DENBERG, P., BLAKE, J., WHITE, S. & LEFF, A.R. (1988). Role of platelets in contraction of canine trachealis muscle elicited by PAF in vitro. *J. Appl. Physiol.*, **65**, 914–920.

ROBERTSON, D.N. & PAGE, C.P. (1987). Effect of platelet agonists on airway reactivity and intrathoracic platelet accumulation. *Br. J. Pharmacol.*, **92**, 105–111.

ROBERTSON, D.N., COYLE, A.J., RHODEN, K.J., GRANDORDY, B., PAGE, C.P. & BARNES, P.J. (1988). The effect of platelet activating factor on histamine and muscarinic receptor function in guinea-pig airways. *Am. Rev. Resp. Dis.*, **137**, 1317–1322.

SAKUMA, Y., TSUNODA, H., SHIRATO, M., KATAYAMA, S., YAMATSU, I. & KATAYAMA, K. (1991). Pharmacological effects of oral E6123, a novel PAF antagonist, on biological changes induced by PAF inhalation in guinea pigs. *Prostaglandins*, **42**, 463–472.

SMITH, L.J., RUBIN, A.E. & PATTERSON, R. (1988). Mechanisms of platelet activating factor-induced bronchoconstriction in humans. *Am. Rev. Respir. Dis.*, **137**, 1015–1019.

SNYDER, F. (1985). Chemical and biochemical aspects of platelet activating factor: a novel class of acetylated ether-linked cholinephospholipids. *Med. Res. Rev.*, **5**, 107–140.

TAYLOR, I.K., WARD, P.S., TAYLOR, G.W., DOLLERY, C.T. & FULLER, R.W. (1991). Inhaled PAF stimulates leukotriene and thromboxane A_2 production in humans. *J. Appl. Physiol.*, **71**, 1396–1402.

TOWNLEY, R.G., HOPP, R.J., AGRAWAL, D.K. & BEWTRA, A.K. (1989). Platelet-activating factor and airway reactivity. *J. Allergy Clin. Immunol.*, **83**, 997–1010.

TSUNODA, H., SAKUMA, Y., HARADA, K., MURAMOTO, K., KATAYAMA, S., HORIE, T., SHIMOMURA, N., CLARK, R., MIYAZ AWA, S., OKANO, K., MACHIDA, Y., KATAYAMA, K. & YAMATU, I. (1990). Pharmacological activities of a novel thinodiazepine derivative as a platelet-activating factor antagonist. *Arzneim-Forsch. Drug Res.*, **40**, 1201–1205.

VARGAFTIG, B.B., LEFORT, J., WAL, F. & CHIGNARD, M. (1981a). Role of the metabolites of arachionate in platelet-dependent and independent experimental bronchoconstriction. *Bull. Eur. Physiopathol. Respir.*, **17**, 723–736.

VARGAFTIG, B.B., CHIGNARD, M., BENVENISTE, J., LEFORT, J. & WAL, F. (1981b). Background and present status of research in platelet-activating factor (Paf-acether). *Ann. N.Y. Acad. Sci.*, **350**, 119–137.

VOELKEL, N.F., WORTHEN, S., REEVES, T.J., HENSON, P.M. & MURPHY, R.C. (1982). Non-immunological production of leukotrienes induced by platelet-activating factor. *Science*, **218**, 286–288.

XIE, Z., HAKODA, H. & ITO, Y. (1992). Airway epithelial cells regulate membrane potential, neurotransmission and muscle tone of the dog airway smooth muscle. *J. Physiol.*, **449**, 619–639.

YAMAI, T., WATANABE, S., MOTOJIMA, S., FUKUDA, T. & MAKINO, S. (1989). The significance of leukotriene in antigen-induced late asthmatic response. *Am. Rev. Respir. Dis.*, **139**, a462.

(Received March 5, 1992
Revised July 7, 1992
Accepted July 28, 1992)

Br. J. Pharmacol. (1992), **107**, 964–969

Enhanced responsiveness of ovalbumin-sensitized guinea-pig alveolar macrophages to tachykinins

[1]Sandra Brunelleschi, Astrid Parenti, Elisabetta Ceni, Alberto Giotti & *Roberto Fantozzi

Department of Pharmacology, Univ. Florence, Viale G.B. Morgagni, 65-50134 Firenze, Italy and *Institute of Pharmacology, Univ. Turin, Via Pietro Giuria, 9-10125 Torino, Italy

1 We have evaluated the ability of substance P (SP), neurokinin A (NKA) and the selective NK_2 receptor agonist [β-Ala^8]-NKA(4–10) to induce superoxide anion (O_2^-) production and prostanoid (prostaglandin E_2, thromboxane B_2) release from alveolar macrophages (AMs) isolated from control or actively sensitized guinea-pigs.

2 The dose-response curves for NKA and SP were shifted to the left (three orders and one order of magnitude, respectively) in AMs isolated from sensitized animals, with no variation in maximal effects.

3 By evaluating the effects of [β-Ala^8]-NKA(4–10), we observed that not only was the concentration-response curve shifted to the left in both the functional parameters examined, but also maximal effects were significantly enhanced in AMs isolated from sensitized guinea-pigs.

4 This varied responsiveness seems to be specific for tachykinins, as it was not reproduced by another AM stimulant, the bacterial peptide N-formylmethionyl-leucyl-phenylalanine (fMLP).

5 Only small amounts of β-glucuronidase were released following tachykinin or ovalbumin stimulation both in control and sensitized AMs.

6 These results indicate that AMs isolated from sensitized guinea-pigs show an increased responsiveness to NK_2 receptor stimulation and further stress the role played by AMs in allergic lung diseases.

Keywords: Tachykinin receptors; alveolar macrophages, ovalbumin sensitization

Introduction

Alveolar macrophages (AMs), which play a key role in the local inflammatory process associated with bronchial asthma (Rankin, 1989), can be activated by different agonists *in vitro*. Upon stimulation, AMs undergo a respiratory burst, secrete regulatory cytokines (especially, interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α)) and lysosomal enzymes, release biologically active lipids, e.g., PAF (platelet activating factor), cyclo-oxygenase and lipoxygenase metabolites (Sibille & Reynolds, 1990).

We have previously shown that mammalian tachykinins dose-dependently evoke superoxide anion (O_2^-) generation from guinea-pig AMs: a comparative evaluation of the effects of natural tachykinins and selective synthetic peptide analogues indicated that macrophage activation is mainly mediated by NK_2 receptors (Brunelleschi *et al.*, 1990).

Neurogenic inflammation, which involves the release of neuropeptides, including the tachykinins, substance P (SP) and neurokinin A (NKA), from capsaicin-sensitive primary neurones is known to contribute to airway disease, namely asthma (Barnes *et al.*, 1991; Frossard & Advenier, 1991). Saria *et al.* (1983) and Matsuse *et al.* (1991) reported that capsaicin pretreatment provides significant protection against antigen-induced bronchoconstriction and airway hyperresponsiveness in sensitized guinea-pigs. Dusser *et al.* (1989) demonstrated that the bronchoconstrictor responses to SP and capsaicin, but not to acetylcholine, are potentiated during viral respiratory infections in the guinea-pig, thus suggesting that airway smooth muscle responsiveness to sensory neuropeptides could be impaired in pathophysiological conditions.

Binding sites for IgG and IgE have been demonstrated on both rabbit and human AMs (Arendt & Mannick, 1973; Spiegelberg *et al.*, 1983; Rossman *et al.*, 1986; Kindt *et al.*, 1991) as well as an impaired responsiveness to β-adrenoceptor stimulation in AMs obtained from sensitized guinea-pigs (Bachelet *et al.*, 1989; Beusenberg *et al.*, 1989; 1991). Furthermore, IgE-stimulated canine AMs enhance the neurally-mediated contraction of bronchial smooth muscle *in vitro*: this effect appears to be associated with release of mediators (namely thromboxane B_2 (TXB_2)) from immunologically stimulated AMs (Tamaoki *et al.*, 1991).

The present study was undertaken to assess the effects of natural (SP and NKA) and synthetic ([β-Ala^8]-NKA(4–10); a selective NK_2 receptor agonist) tachykinins in AMs obtained from control or ovalbumin-sensitized guinea-pigs and to compare these effects with those elicited, in the same preparations, by the bacterial peptide N-formylmethionyl-leucyl-phenylalanine (fMLP).

Methods

Sensitization procedure

Male guinea-pigs (380–500 g) were actively sensitized by two s.c. injections at a 2-week interval of 0.5 ml of saline containing 10 μg ovalbumin dispersed in 1 mg $Al(OH)_3$ according to Pretolani *et al.* (1989). Animals were used for the experiments 7–10 days after the second injection. Control animals were injected with the same volume of saline and $Al(OH)_3$.

Sensitization of the animals was verified by measuring ovalbumin-induced contractile responses in guinea-pig tracheal spirals obtained from the same guinea-pigs, by use of techniques described by Brunelleschi *et al.* (1987).

Harvesting and purification of guinea-pig alveolar macrophages

AMs were harvested from control or ovalbumin-sensitized guinea-pigs as previously described (Brunelleschi *et al.*, 1990). Briefly, guinea-pigs were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.) and the trachea was cannulated. Twelve successive bronchoalveolar lavages were performed with 5 ml aliquots of phosphate buffered saline (pH 7.4, 37°C) injected with a plastic syringe through a polyethylene

[1] Author for correspondence.

cannula inserted into the trachea. Fluid of the two first lavages was discarded, as it was found to contain a large quantity of cells other than macrophages (especially, epithelial cells and eosinophils). After centrifugation and lysis of erythrocytes by hypotonic shock, the bronchoalveolar lavage pellet was resuspended in MEM (Minimum Essential Medium with Earle's salts) supplemented with 5% foetal calf serum, 20 mM HEPES, 2 mM glutamine, 50 $\mu g\,ml^{-1}$ streptomycin and 5 $u\,ml^{-1}$ penicillin and microscopically evaluated (May-Grunwald/Giemsa). Cell viability, as assessed by trypan blue exclusion, was >95%.

Aliquots of cell suspension ($0.8-1 \times 10^6$ cells) were plated in 6-well tissue culture plates (35 mm diameter, Costar, Cambridge) and allowed to adhere for 2 h at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed by washing monolayers three times with MEM.

Activation of guinea-pig alveolar macrophages

Adherent AMs, obtained from both control or sensitized animals, were incubated with fMLP, ovalbumin, SP, NKA or the synthetic NK_2 receptor agonist, [β-Ala^8]-NKA(4–10) at 37°C in a CO_2 incubator for different times, according to the functional parameter evaluated (see below). Challenge with the different stimuli used was terminated by placing the culture plates on ice.

Determination of superoxide anion (O_2^-) production from guinea-pig alveolar macrophages

In these experiments, adherent AMs were incubated (final volume 2 ml) with the stimuli for 30 min at 37°C in a humidified atmosphere containing 5% CO_2 in the presence of cytochrome C (1 $mg\,ml^{-1}$), as previously described (Brunelleschi *et al.*, 1990).

Production of O_2^- was measured by the superoxide dismutase-inhibitable cytochrome C reduction, the absorbance changes being recorded at 550 nm in a Perkin Elmer 552S spectrophotometer. Release of O_2^- was expressed as nmol cytochrome C reduced per 10^6 AMs per 30 min, using an extinction coefficient of 2.1×10^{-4} M min^{-1}.

To avoid interference with spectrophotometric recordings of O_2^- production, monolayers were incubated in MEM (supplemented by HEPES, glutamine and antibiotics; see above) without phenol red.

Determinations of prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) production in guinea-pig alveolar macrophages

The concentrations of TXB_2 and PGE_2 in the cell supernatant were determined by specific enzyme-immunoassay (EIA) after suitable dilutions (1:2 to 1:100) in EIA buffer without prior extraction or purification, according to the manufacturer's instructions. The threshold sensitivities of these assays were: 9 $pg\,ml^{-1}$ for PGE_2 and 20 $pg\,ml^{-1}$ for TXB_2.

In these experiments adherent AMs were challenged with the stimuli in a CO_2 incubator at 37°C, in the presence of MEM without phenol red, supplemented by HEPES, glutamine and antibiotics. The incubation time was 18 h, according to Hartung & Tokya (1983), and was confirmed by preliminary time-course studies (see Results). Supernatants were collected, immediately frozen and lyophilized before being evaluated for their prostanoid content. Results are expressed as ng TXB_2 per 10^6 AMs or pg PGE_2 per 10^6 AMs.

Determination of β-glucuronidase release from guinea-pig alveolar macrophages

Adherent AMs ($1-1.2 \times 10^6$) were incubated for 10 min with cytochalasin B (5 $\mu g\,ml^{-1}$) at 37°C in a CO_2 incubator and then challenged for 30 min with the stimuli, according to Hartung *et al.* (1986). The reaction was stopped by placing monolayers on ice and the supernatants were collected. Pellets were treated with 0.1% Triton X-100 to lyse the cells.

β-Glucuronidase (EC 3.2.1.31) activity was chosen as a marker of lysosomal enzymes according to Arnoux *et al.* (1987) and was measured spectrophotometrically after 16 h incubation at 37°C, with phenolphthalein glucuronide used as a substrate (Fishman, 1974). Total macrophage enzyme activity was calculated by summation of the activity in the supernatant with that in the pellet; enzyme release was expressed as the percentage of the total enzyme activity.

Data analysis

Experiments were performed in duplicate or triplicate and basal values (e.g., values obtained in the absence of any treatment) were subtracted from all determinations. All results are expressed as mean ± s.e.mean; statistical significance was evaluated by Student's *t* test.

Chemicals

The compounds used and their sources were: SP and NKA (Peninsula: St. Helens, Merseyside); fMLP and Triton X-100 (Serva: Heidelberg, Germany); HEPES (2-4-(2-hydroxyethyl)-1-piperazino-ethan-sulfonic acid) and cytochalasin B (Aldrich Chemical Co: Milwaukee, WI, U.S.A.); superoxide dismutase, cytochrome C type III, phenolphthalein glucuronide and ovalbumin (Sigma: St. Louis, MO, U.S.A.); MEM with or without phenol red, glutamine, heat-inactivated foetal calf serum, penicillin and streptomycin (Gibco: Paisley). [β-Ala^8]-NKA(4–10) was synthesized at Menarini Laboratories, Firenze (Italy); [Pro^9]SP sulphone was kindly supplied by Prof. D. Regoli, University of Sherbrooke (Canada). EIA kits for PGE_2 and TXB_2 measurements were purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.).

Results

Effect of ovalbumin sensitization in the bronchoalveolar population

No significant differences in body weight or the number of cells in bronchoalveolar lavages were observed between control or sensitized animals. Control and sensitized animals provided a mean of $27.6 \pm 2.2 \times 10^6$ and $29.8 \pm 4 \times 10^6$ cells per guinea-pig, respectively ($n = 6$). The different cell populations present in lavage fluid from control and sensitized animals are listed in Table 1: a significant increase in the number of eosinophils and lymphocytes was induced by the sensitization procedure. In contrast, the percentage of AMs present in bronchoalveolar lavage from sensitized guinea-pigs was lower ($P<0.05$) than in control animals, but the absolute number of macrophages did not differ significantly ($24.8 \pm 3 \times 10^6$ in controls, $23 \pm 3.5 \times 10^6$ in sensitized animals).

Table 1 Cell populations in the bronchoalveolar lavage from control or ovalbumin-sensitized guinea-pigs

	% Composition	
	Control	*Sensitized*
Macrophages	88.7 ± 0.1	78 ± 2*
Lymphocytes	10.2 ± 0.5	17.2 ± 2*
Eosinophils	0.7 ± 0.5	5.3 ± 1**
Neutrophils	0.6 ± 0.4	0.7 ± 0.5

Data are mean ± s.e.mean for 6 animals.
*$P<0.05$ vs control animals; **$P<0.01$ vs control animals (Student's unpaired *t* test).

Superoxide anion generation in alveolar macrophages obtained from control or sensitized guinea-pigs

Basal O_2^- production (e.g., O_2^- production in the absence of any treatment) was not different in AMs obtained from either experimental group (1.2 ± 0.2 vs 1.35 ± 0.3 nmol cytochrome C reduced per 10^6 AMs; $n = 8$) and was subtracted from all values.

The challenge of AMs isolated from sensitized guinea-pigs with ovalbumin (100 μg ml^{-1}) resulted in O_2^- generation (3 ± 0.5 nmol cytochrome C reduced per 10^6 AMs; $n = 8$); the antigen was devoid of activity in AMs obtained from control animals (data not shown).

We previously found that NKA and SP dose-dependently evoked O_2^- production from guinea-pig AMs (Brunelleschi *et al.*, 1990): these effects were not significantly varied in the presence of 10 μM thiorphan or a cocktail of inhibitors (thiorphan, captopril, bestatin, each 1 μM, data not shown). When AMs isolated from sensitized animals were challenged with SP, a leftward displacement of the dose-response curve by approximately one order was observed, ED_{50} values being 0.56 nM in control and 0.04 nM in sensitized cells (Figure 1a). The amount of maximal activation was similar in both experimental groups (2.33 ± 0.3 vs 2.37 ± 0.7 nmol cytochrome C reduced per 10^6 AMs; $n = 6$) and was achieved at 10 nM in sensitized and 100 nM in control cells (Figure 1a). When AMs were challenged with NKA, the leftward shift in the concentration-response curve observed with cells from sensitized animals was more than two orders of magnitude and ED_{50} values were 0.14 pM and 0.09 nM in sensitized and control cells respectively (Figure 1b). Maximal O_2^- production, which did not differ significantly between the two groups, was observed at 0.1 nM NKA in sensitized and 0.1 μM NKA in control animals (Figure 1b). The synthetic peptide analogue, [β-Ala^8]-NKA(4–10), which is a selective NK_2 receptor agonist (Rovero *et al.*, 1989), dose-dependently (0.01 nM–0.1 μM) evoked O_2^- production in AMs from control guinea-pigs. The dose-response curve for this NK_2 receptor agonist was shifted to the left in experiments performed on AMs from sensitized animals: ED_{50} values were 3 pM and 0.9 nM in sensitized and control cells respectively (Figure 1c). Unlike results with natural tachykinins, the extent of O_2^- production induced by [β-Ala^8]-NKA(4–10) was higher in AMs isolated from sensitized animals and maximal activation (4.08 ± 0.8 nmol cytochrome C reduced per 10^6 AMs; $n = 5$) was achieved at 1 nM (Figure 1c). In contrast, no significant variation in the dose-response curves for [Pro^9]-SP sulphone, a selective NK_1 agonist, was observed between control and sensitized AMs (data not shown). As previously reported (Brunelleschi *et al.*, 1990), [Pro^9]-SP sulphone acted at high concentrations (0.1 μM–1 μM) only.

The bacterial peptide fMLP, which interacts with specific receptors in AMs (Daniele *et al.*, 1982), evoked O_2^- production in both control and ovalbumin-sensitized guinea-pig AMs. Neither maximal effects (18.4 ± 2.1 vs 18 ± 1.7 nmol cytochrome C reduced per 10^6 AMs; $n = 5$) nor ED_{50} values (10.9 vs 11.6 nM) were significantly different in control and sensitized cells (Figure 2).

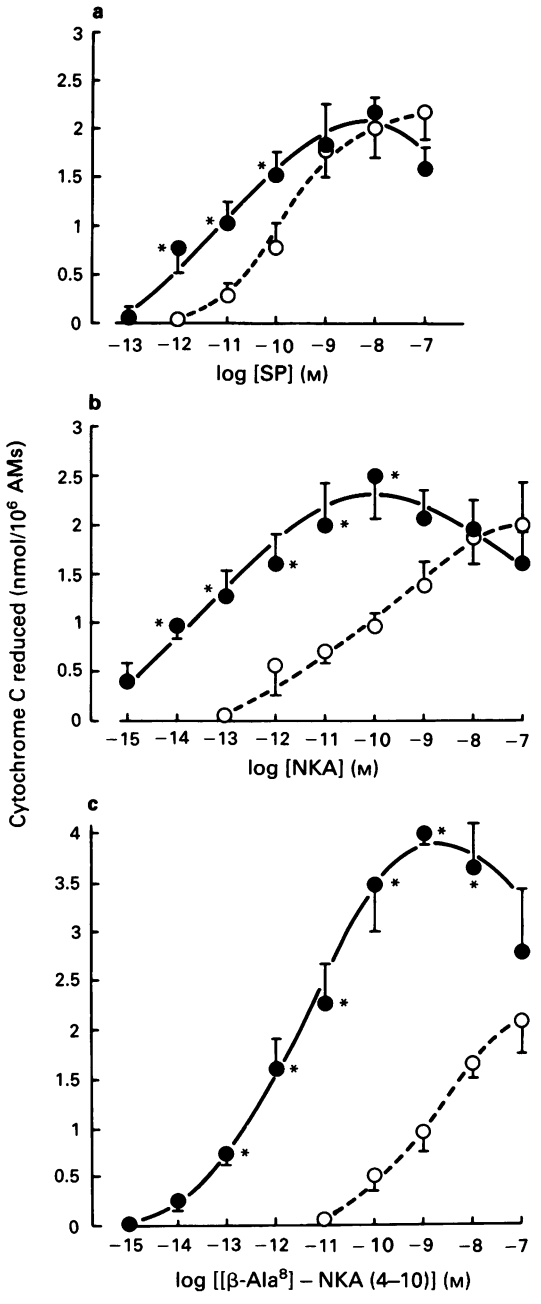

Figure 1 Tachykinin-evoked superoxide anion generation in alveolar macrophages isolated from control (○) or ovalbumin-sensitized (●) guinea-pigs. (a) Dose-response curve to substance P (SP) ($n = 6$). (b) Dose-response curve to neurokinin A (NKA) ($n = 8$). (c) Dose-response curve to [β-Ala^8]-NKA(4–10) ($n = 5$). Cells were challenged for 30 min with tachykinins. Data show mean values (± s.e.mean, vertical bars). *Denotes $P < 0.01$ (Student's t test for paired samples).

Prostanoid release in alveolar macrophages obtained from control or sensitized guinea-pigs

Prostanoid release from AMs of both experimental groups was evaluated after 18 h incubation with the stimuli. Preliminary time-course studies revealed that basal prostanoid release (which was subtracted from all determinations) increased with time (data not shown). PGE_2 release evoked by a fixed concentration of 0.1 μM NKA amounted to 60, 90, 125, 220 and 230 pg per 10^6 AMs, after 1, 6, 12, 18 and 24 h, respectively (means of two determinations in triplicate). The release of PGE_2 by 0.1 μM fMLP was 380–400 pg per 10^6 AMs, reached a plateau after 6 h of incubation and remained stable until 18 h. Therefore, the 18 h incubation period was selected in these experiments.

Basal TXB_2 release differed significantly ($P < 0.01$) between the two groups: 13.9 ± 1.9 and 27.2 ± 5 ng per 10^6 AMs ($n = 3$), in control and sensitized AMs, respectively. These values were subtracted from all determinations with stimuli. Ovalbumin 100 μg ml^{-1} released 14.43 ± 8.7 ng per 10^6 AMs ($n = 3$) from sensitized AMs, with no effect on control cells. Moreover, TXB_2 release evoked by 0.1 μM fMLP was not modified in either group: 28.5 ± 4 and 21 ± 5 ng per 10^6 AMs ($n = 3$), in sensitized and control cells, respectively.

By measuring tachykinin-evoked TXB_2 release, we confirmed the pattern of results seen when O_2^- generation

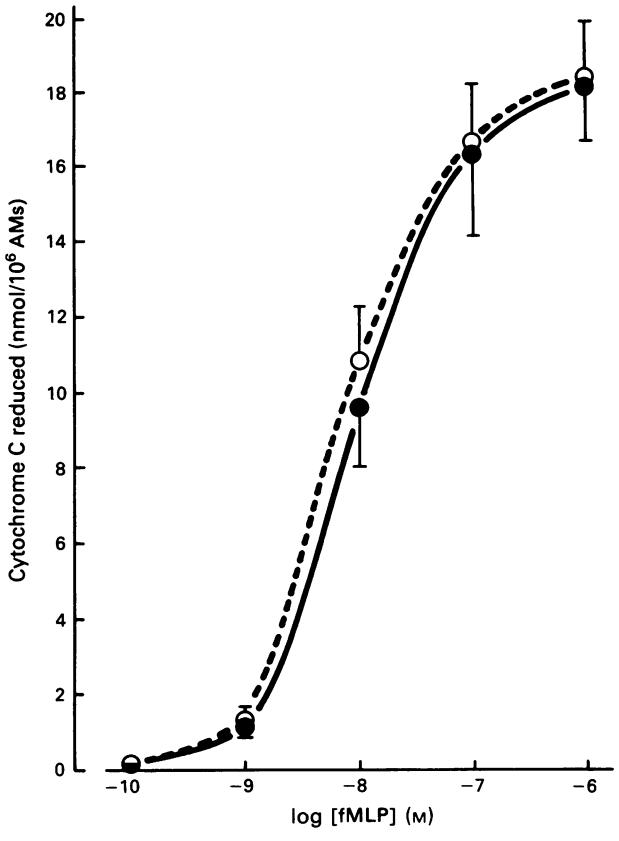

Figure 2 Superoxide anion generation evoked by N-formylmethionyl-leucyl-phenylalanine (fMLP) in alveolar macrophages (AMs) isolated from control (○) or ovalbumin-sensitized (●) guinea-pigs. Data are mean (± s.e.mean, vertical bars) of 5 experiments.

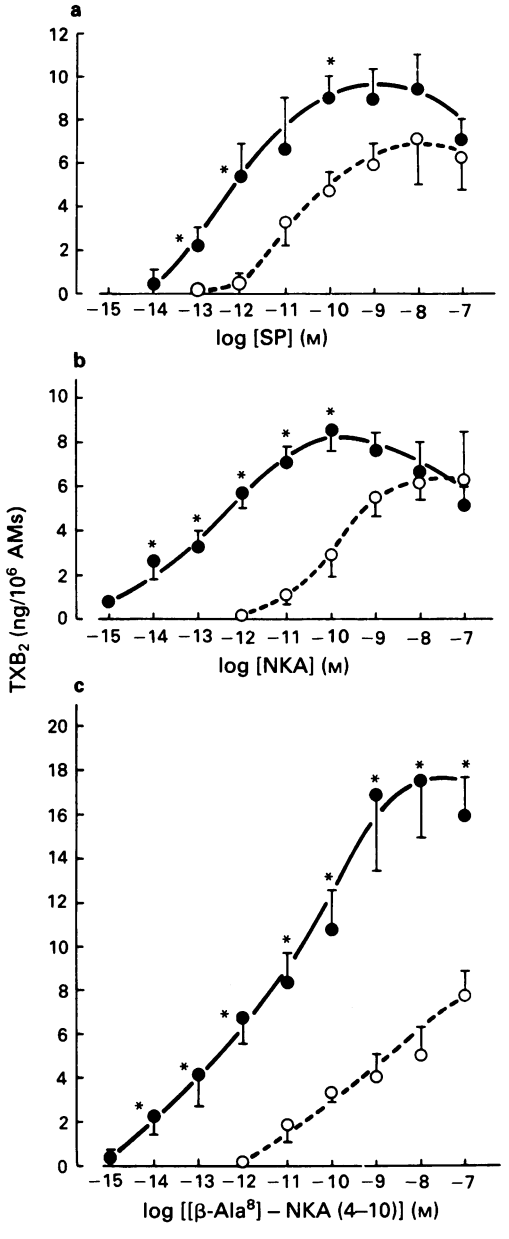

Figure 3 Tachykinin-evoked thromboxane B_2 release in alveolar macrophages (AMs) isolated from control (○) or ovalbumin-sensitized guinea-pigs (●). (a) Dose-response curve to substance P (SP). (b) Dose-response curve to neurokinin A (NKA). (c) Dose-response curve to [β-Ala⁸]-NKA(4–10). Data show mean (± s.e.mean, vertical bars) of three experiments in duplicate. *Denotes $P < 0.01$.

was measured. As depicted in Figure 3a, SP dose-dependently evoked TXB_2 release from control or sensitized AMs, a greater than one order of magnitude leftward shift in its dose-response curve being observed in sensitized animals. ED_{50} values were 70 pM in control and 1 pM in sensitized AMs. Maximal release, which was similar in both experimental groups, was obtained at 10 nM SP (Figure 3a). When AMs were stimulated by NKA, the leftward shift in the dose-response curve observed with cells harvested from sensitized animals was about three orders of magnitude and ED_{50} values were 0.2 pM and 0.4 nM in sensitized and control AMs, respectively (Figure 3b). Maximal TXB_2 release, which was similar in both experimental groups (8.2 ± 0.4 and 6.2 ± 2.5 ng per 10^6 AMs; $n = 3$), was measured at 0.1 nM NKA in sensitized and 0.1 μM NKA in control AMs (Figure 3b). Moreover, the results obtained by evaluating, in both groups, the effect of the NK_2 receptor agonist [β-Ala⁸]-NKA(4–10) matched those observed by measuring O_2^- production (see above): ED_{50} values were 1 nM in control and 10 pM in sensitized AMs and the evoked TXB_2 release was significantly higher in AMs from sensitized animals (Figure 3c).

Also, by evaluating tachykinin-evoked PGE_2 release from AMs of both groups, we observed that dose-response curves in sensitized cells were shifted leftwards (data not shown). Maximal PGE_2 release induced by both SP and NKA was similar and did not differ significantly between the two experimental groups (SP = 207 ± 41 and 264 ± 30 pg per 10^6 AMs in control or sensitized cells; NKA = 216 ± 37 and 293 ± 63 pg per 10^6 AMs in control or sensitized cells; $n = 4$). ED_{50} values were as follows: SP = 0.48 nM in control and 8 pM in sensitized AMs; NKA = 0.2 nM in control and 1 pM in sensitized AMs. Basal PGE_2 release, which was subtracted from all determinations, was 630 ± 76 pg per 10^6 AMs in control and 472 ± 72 pg per 10^6 AMs in sensitized cells ($n = 4$). A total of 369 ± 68 pg PGE_2 per 10^6 AMs in sensitized animals and 311 ± 93 pg PGE_2 per 10^6 AMs in control animals was released by 0.1 μM fMLP ($n = 4$). Ovalbumin-evoked PGE_2 release was detected only in sensitized AMs and amounted to 138 ± 48 pg per 10^6 AMs ($n = 4$).

β-Glucuronidase release in alveolar macrophages obtained from control or sensitized guinea-pigs

Basal enzyme release did not differ significantly between the two experimental groups (6.4 ± 0.8% and 7.2 ± 1% in control and sensitized AMs, respectively; $n = 6$). Basal values were subtracted from all determinations.

SP, NKA and the NK_2 receptor agonist [β-Ala⁸]-NKA (4–10) caused a net enzyme release at micromolar concentrations only: the extent of β-glucuronidase release was very small in both experimental groups (SP 100 μM = 5.3 ± 1.3% vs 6 ± 1.5%; SP 10 μM = 2 ± 0.2% vs 2.5 ± 0.5%; NKA

10 μM = 3.5 ± 0.4% vs 3.1 ± 0.5%; NKA 1 μM = 2.5 ± 0.5% vs 2.8 ± 0.6%; [β-Ala^8]-NKA(4–10) 10 μM = 2.8 ± 0.3 vs 3.5 ± 0.6% in control and sensitized AMs, respectively; $n = 6$).

Ovalbumin (100 μg ml^{-1}) released 3.5 ± 0.7% of total β-glucuronidase content in sensitized cells, but had no effect on control cells.

The peptide fMLP (0.1 μM), which is more potent than tachykinins in evoking O_2^- production, released 6.4 ± 0.7% and 7.5 ± 0.4% of the total β-glucuronidase activity in control and sensitized cells, respectively ($n = 6$) and the calcium ionophore A23187 1 μM released 20 ± 1.7% and 23 ± 1.8% in control and sensitized AMs, respectively ($n = 6$).

Discussion

Our study indicates that AMs isolated from ovalbumin-sensitized guinea-pigs exhibit an increased responsiveness to tachykinins. By assessing O_2^- production and prostanoid release, concentration-response curves for NKA and SP are shifted leftwards, with no variation in maximal response: the left shift is far more pronounced for NKA (about three orders of magnitude) than for SP (only one order of magnitude). The involvement of NK_2 receptors in this event is further confirmed by the results obtained with a selective NK_2 receptor agonist: the concentration-effect curve for [β-Ala^8]-NKA(4–10) is not only shifted leftwards, but also maximal activation is strongly enhanced in AMs isolated from sensitized animals. In contrast, no variation is observed between AMs obtained from control or sensitized guinea-pigs by evaluating the effects of the selective NK_1 agonist [Pro^9]-SP sulphone or those of the bacterial peptide fMLP. However, when we evaluated the ability of mammalian tachykinins to induce lysosomal enzyme release from AMs, only minimal amounts of β-glucuronidase could be detected and tachykinins acted at concentrations higher than those needed to induce O_2^- generation or prostanoid release. This fact warrants some explanations. Firstly, lysosomal enzyme release is not a very sensitive parameter of AM activation compared to O_2^- generation. According to Brieland *et al.* (1987), rat AMs secrete lysozyme and N-acetyl-β-D-glucosaminidase either spontaneously or following zymosan stimulation. However, when data are expressed as net enzyme release, only 1.5% of the total lysozyme activity is observed after challenge with opsonized zymosan (5 μg ml^{-1}; Brieland *et al.*, 1987). Moreover, Joseph *et al.* (1983) observed that the release of the lysosomal acid hydrolase, β-glucuronidase, from passively sensitized human AMs or AMs obtained from asthmatic patients is not significantly different and amounts to 4–8% after a 30 min contact time with the specific antigen, *in vitro*. Similar results were also reported by Arnoux *et al.* (1987).

The fact that micromolar concentrations of tachykinins are needed to induce lysosomal enzyme secretion from guinea-pig AMs whereas nanomolar concentrations evoke O_2^- generation and prostanoid release suggests that different pattern(s) of cell activation exhibit a varied coupling to second messengers for the release of the selected mediator. In many cell types, the interaction of agonists with specific membrane receptors stimulates phospholipase C, yielding inositol 1,4,5-trisphosphate (IP_3, which in turn mobilizes intracellular calcium) and 1,2-diacylglycerol (which directly activates protein kinase C, PKC) (Nishizuka, 1984; Berridge & Irvine, 1984). Enzyme exocytosis is primarily a calcium-dependent response, while the respiratory burst may also occur in the absence of added calcium in the medium.

Sakata *et al.* (1987) suggested that in guinea-pig peritoneal macrophages, arachidonic acid release plays an essential role in the activation of the O_2^- generating system, with PKC having a significant but not an obligatory role, in the respiratory burst. In guinea-pig AMs, the stimulation of arachidonic acid release by fMLP involves several mechanisms and is regulated by both protein kinase C and A, the latter exerting a negative modulation (Kadiri *et al.*, 1990).

Most of the physiological effects of tachykinins depend on their terminal sequence, involving NK_1, NK_2 and NK_3 receptors. Activation of NK_2 receptors is coupled to a guanine nucleotide-binding protein (G-protein) and stimulates phosphatidylinositol hydrolysis in different tissues (Frossard & Advenier, 1991). However, little information is available concerning the signal transduction mechanisms afforded by tachykinins in inflammatory cells, and only SP-induced effects have been investigated. In human neutrophils, activation of the respiratory burst by SP is insensitive to 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7, a PKC inhibitor), indicating that PKC is not obligatory for the activation of NADPH-oxidase, and is only partially inhibited by pertussis toxin (Serra *et al.*, 1988). SP also induces a cytochalasin B-dependent secretion of lysosomal enzymes from human neutrophils (Serra *et al.*, 1988).

In this study, the varied responsiveness of AMs to tachykinins *in vitro* follows a sensitization procedure which, according to Garcez Do Carmo *et al.* (1986), Pretolani *et al.* (1989) and Desquand *et al.* (1991), causes serum levels of both IgG and IgE to be significantly enhanced. AMs obtained from actively sensitized guinea-pigs have been shown to possess an impaired adenylate cyclase activity, conflicting results being reported (Bachelet *et al.*, 1989; Beusenberg *et al.*, 1989; 1991). While Bachelet *et al.* (1989) noted that PGE_2 and β-adrenoceptor agonists are less effective in increasing the intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) content of AMs from ovalbumin-sensitized compared to control guinea-pigs, Beusenberg *et al.* (1989, 1991) demonstrated that both agonists stimulate adenylate cyclase activity more effectively in AMs obtained from sensitized animals. These authors also reported that sensitization and antigen challenge do not affect β-adrenoceptor binding and suggested that the enhanced adenylate cyclase responsiveness results from alterations in the α_s-subunit of a G-protein (Beusenberg *et al.*, 1991).

In our experiments, AMs isolated from sensitized guinea-pigs demonstrate an enhanced responsiveness to tachykinins (and, especially to NKA and NK_2 receptor stimulation), but not to the bacterial peptide fMLP, thus showing selectivity. We can, therefore, suggest a role for NK_2 receptor stimulation in immunomodulation, since O_2^- generation and prostanoid release evoked by the peptide analogue [β-Ala^8]-NKA (4–10) are significantly enhanced in AMs obtained from sensitized guinea-pigs. The involvement of NK_2 receptors in the modulation of primary antibody response has been recently discussed by Eglezos *et al.* (1991). In their experiments, rats treated neonatally with capsaicin to destroy primary afferent nerves presented a diminished antibody response: NKA was twelve times more potent than SP in restoring the immune response (Eglezos *et al.*, 1991). Furthermore, in keeping with the observations by Nohr & Weihe (1991), who demonstrated the presence of tachykinin- and calcitonin gene-related peptide-fibres in alveolar walls of different species, including the guinea-pig, and suggested that tachykinins released from alveolar fibres might influence epithelial and inflammatory cells, we think it possible to hypothesize that the binding of immunoglobulins to guinea-pig AM membrane may be the trigger for increased responsiveness to tachykinins.

In conclusion, these results indicate that AMs isolated from actively sensitized guinea-pigs show enhanced responsiveness to tachykinins and point to AMs as important cells for the development of allergic lung disease.

We wish to thank Dr C.A. Maggi (Menarini Pharmaceuticals, Florence, Italy) for the kind gift of [β-Ala^8]-NKA(4–10), Prof. D. Regoli for supplying [Pro^9]-SP sulphone and Mr Paolo Ceccatelli for his excellent technical assistance. This work was supported in part by a grant from Centro di Ricerche Interuniversitario 'Ipossie' c/o Dept. Pharmacology, Univ. Florence, Florence (Italy).

References

ARENDT, W.P. & MANNICK, M. (1973). The macrophage receptor for IgG: number and affinity of binding sites. *J. Immunol.*, **110**, 1455–1463.

ARNOUX, B., JOSEPH, M., SIMOES, M.H., TONNEL, A.B., DUROUX, P., CAPRON, A. & BENVENISTE, J. (1987). Antigenic release of paf-acether and β-glucuronidase from alveolar macrophages of asthmatics. *Bull. Eur. Physiopathol. Respir.*, **23**, 119–124.

BACHELET, M., MASLIAH, J., MALANCHERE, E., LEFORT, J., BEREZIAT, G. & VARGAFTIG, B.B. (1989). Reduced responsiveness of bronchoalveolar cells from sensitized guinea-pigs to cyclic AMP-stimulating effects of prostaglandin E_2 and β-adrenoceptor agonists. *Pulmonary Pharmacol.*, **2**, 41–44.

BARNES, P.J., BARANIUK, J.N. & BELVISI, M.G. (1991). Neuropeptides in the respiratory tract. *Am. Rev. Respir. Dis.*, **144**, 1187–1198.

BERRIDGE, M.J. & IRVINE, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*, **312**, 315–321.

BEUSENBERG, F.D., ADOLFS, M.J.P., VAN SCHAIK, A., VAN AMSTERDAM, J.G.C. & BONTA, I.L. (1989). Antigen challenge modifies the cyclic AMP response of inflammatory mediators and β-adrenergic drugs in alveolar macrophages. *Eur. J. Pharmacol.*, **174**, 33–41.

BEUSENBERG, F.D., LEURS, B., VAN SCHAIK, A., VAN AMSTERDAM, J.G.C. & BONTA, I.L. (1991). Sensitization enhances the adenylyl cyclase responsiveness in alveolar macrophages. Changes induced at post-receptor level. *Biochem. Pharmacol.*, **42**, 485–490.

BRIELAND, J.K., KUNKEL, R.G. & FANTONE, J.C. (1987). Pulmonary alveolar macrophage function during acute inflammatory lung injury. *Am. Rev. Respir. Dis.*, **135**, 1300–1306.

BRUNELLESCHI, S., HAYE-LEGRAND, I., LABAT, C., NOREL, X., BENVENISTE, J. & BRINK, C. (1987) Platelet-Activating-Factor-acether-induced relaxation of guinea-pig airway muscle: role of PGE_2 and the epithelium. *J. Pharmacol. Exp. Ther.*, **243**, 356–363.

BRUNELLESCHI, S., VANNI, L., LEDDA, F., GIOTTI, A., MAGGI, C.A. & FANTOZZI, R. (1990). Tachykinins activate guinea-pig alveolar macrophages: involvement of NK_1 and NK_2 receptors. *Br. J. Pharmacol.*, **100**, 417–420.

DANIELE, R.P., DIAMOND, M.S. & HOLIAN, A. (1982). Demonstration of a formylpeptide receptor on lung macrophages. Correlation of binding properties with chemotaxis and release of superoxide anion. *Am. Rev. Respir. Dis.*, **126**, 274–280.

DESQUAND, S., LEFORT, J., DUMAREY, C. & VARGAFTIG, B.B. (1991). Interference of BN 52021, an antagonist of PAF, with different forms of active anaphylaxis in the guinea-pig: importance of the booster injection. *Br. J. Pharmacol.*, **102**, 687–695.

DUSSER, D.J., JACOBY, D.B., DJOKIC, T.D., RUBINSTEIN, I., BORSON, D.B. & NADEL, J.C. (1989). Virus induces airway hyperresponsiveness to tachykinins: role of neutral endopeptidase. *J. Appl. Physiol.*, **67**, 1504–1511.

EGLEZOS, A., ANDREWS, P.V., BOYD, R.L. & HELME, R.D. (1991). Tachykinin-mediated modulation of the primary antibody response in rats: evidence for mediation by an NK-2 receptor. *J. Neuroimmun.*, **32**, 11–18.

FISHMAN, W.H. (1974). β-Glucuronidase. In *Methods of Enzymatic Analysis*. ed. Bergmeyer, H.U. vol. 2, pp. 929–943, New York: Verlag Chemie, Academic Press.

FROSSARD, N. & ADVENIER, C. (1991). Tachykinin receptors and the airways. *Life Sci.*, **49**, 1941–1953.

GARCEZ DO CARMO, L., CORDEIRO, R., LAGENTE, V., LEFORT, J., RANDON, J. & VARGAFTIG, B.B. (1986). Failure of a combined anti-histamine and anti-leukotriene treatment to suppress passive anaphylaxis in the guinea-pig. *Int. J. Immunopharmacol.*, **8**, 985–995.

HARTUNG, H.P. & TOYKA, K.V. (1983). Activation of macrophages by substance P: induction of oxidative burst and thromboxane release. *Eur. J. Pharmacol.*, **89**, 301–305.

HARTUNG, H.P., WOLTERS, K. & TOYKA, K.V. (1986). Substance P: binding properties and studies on cellular responses in guinea-pig macrophages. *J. Immunol.*, **136**, 3856–3863.

JOSEPH, M., TONNEL, A.B., TORPIER, G., CAPRON, A., ARNOUX, B. & BENVENISTE, J. (1983). Involvement of immunoglobulin E in the secretory processes of alveolar macrophages from asthmatic patients. *J. Clin. Invest.*, **71**, 221–230.

KADIRI, C., CHERQUI, G., MASLIAH, J., RYBKYNE, T., ETIENNE, J. & BEREZIAT, G. (1990). Mechanism of N-formyl-methionyl-leucyl-phenylalanine- and Platelet-Activating Factor-induced arachidonic acid release in guinea-pig alveolar macrophages: involvement of a GTP-binding protein and role of protein kinase A and protein kinase C. *Mol. Pharmacol.*, **38**, 418–425.

KINDT, G.C., VAN DE WINKEL, J.G.J., MOORE, S.A. & ANDERSON, C.L. (1991). Identification and structural characterization of Fc γ-receptors on pulmonary alveolar macrophages. *Am. J. Physiol.*, **260**, L403–L411.

MATSUSE, T., THOMSON, R.J., CHEN, X., SALARI, H. & SCHELLENBERG, R.R. (1991). Capsaicin inhibits airway hyperresponsiveness but not lipoxygenase activity or eosinophilia after repeated aerosolized antigen in guinea-pigs. *Am. Rev. Respir. Dis.*, **144**, 368–372.

NISHIZUKA, Y. (1984). Role of protein kinase C in cell surface signal transduction and tumour production. *Nature*, **308**, 693–698.

NOHR, D. & WEIHE, E. (1991). Tachykinin-, calcitonin gene-related peptide-, and protein gene product 9.5-immunoreactive nerve fibers in alveolar walls of mammals. *Neurosci. Lett.*, **134**, 17–20.

PRETOLANI, M., LEFORT, J., DUMAREY, C. & VARGAFTIG, B.B. (1989). Role of lipoxygenase metabolites for the hyper-responsiveness to Platelet-Activating Factor of lungs from actively sensitized guinea-pigs. *J. Pharmacol. Exp. Ther.*, **248**, 353–359.

RANKIN, J.A. (1989). The contribution of alveolar macrophages to hyperreactive airway disease. *J. Allergy Clin. Immunol.*, **83**, 722–729.

ROSSMAN, M.D., CHIEN, P., CASSIZZI-CPREK, A., ELIAS, J.A., HOLIAN, A. & SCHREIBER, A.D. (1986). The binding of monomeric IgG to human blood monocytes and alveolar macrophages. *Am. Rev. Respir. Dis.*, **133**, 292–297.

ROVERO, P., PESTELLINI, V., PATACCHINI, R., GIULIANI, S., SANTICIOLI, P., MAGGI, C.A., MELI, A. & GIACHETTI, A. (1989). A potent and selective agonist for NK_2 tachykinin receptor. *Peptides*, **10**, 593–595.

SAKATA, A., IDA, E., TOMINAGA, M. & ONOUE, K. (1987). Arachidonic acid acts as an intracellular activator on NADPH-oxidase in Fc gamma receptor-mediated superoxide generation in macrophages. *J. Immunol.*, **138**, 4353–4359.

SARIA, A., LUNDBERG, J.M., SKOFITSCH, G. & LEMBECK, F. (1983). Vascular protein leakage in various tissues induced by substance P, capsaicin, bradykinin, serotonin, histamine and by antigen challenge. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **324**, 212–218.

SERRA, M.C., BAZZONI, F., DELLA BIANCA, V., GRESKOWIAK, M. & ROSSI, F. (1988). Activation of human neutrophils by substance P. Effect on oxidative metabolism, exocytosis, cytosolic Ca^{2+} concentration and inositol phosphate formation. *J. Immunol.*, **141**, 2118–2124.

SIBILLE, Y. & REYNOLDS, H.Y. (1990). Macrophages and polymorphonuclear neutrophils in lung defense and injury. *Am. Rev. Respir. Dis.*, **141**, 471–501.

SPIEGELBERG, H.L., BOLTZ-NITULESCU, G., PLUMMER, J.M. & MELEWICZ, F.M. (1983). Characterization of the IgE Fc receptors on monocytes and macrophages. *Fed. Proc.*, **42**, 124–128.

TAMOAKI, J., SAKAI, N., KANEMURA, T., YAMAWAKI, I. & TAKIZAWA, T. (1991). IgE-dependent activation of alveolar macrophages augments neurally mediated contraction of small airways. *Br. J. Pharmacol.*, **103**, 1458–1462.

(Received April 30, 1992
Revised July 7, 1992
Accepted July 28, 1992)

Br. J. Pharmacol. (1992), **107**, 970–975

Ramipril prevents left ventricular hypertrophy with myocardial fibrosis without blood pressure reduction: a one year study in rats

[1]Wolfgang Linz, *Jutta Schaper, Gabriele Wiemer, Udo Albus & Bernward A. Schölkens

Department of Pharmacology, Hoechst AG, D-6230 Frankfurt/Main 80, Germany and *Max-Planck-Institute-Experimental Cardiology, D-6350 Bad Nauheim, Germany

1 Angiotensin converting enzyme (ACE)-inhibitors have been demonstrated to be effective in the treatment of cardiac hypertrophy when used in antihypertensive doses. The aim of our one year study with an ACE-inhibitor in rats was to separate local cardiac effects produced by a non-antihypertensive dose from those on systemic blood pressure when an antihypertensive dose was used.

2 Rats made hypertensive by aortic banding were subjected to chronic oral treatment for one year with an antihypertensive dose of the ACE inhibitor, ramipril 1 mg kg^{-1} daily, (RA 1 mg) or received a low dose of 10 $\mu g\ kg^{-1}$ daily (RA 10 μg) which did not affect high blood pressure.

3 Chronic treatment with the ACE-inhibitor prevented left ventricular hypertrophy in the antihypertensive rats as did the low dose which had no effects on blood pressure. Similar effects were observed on myocardial fibrosis. Plasma ACE activity was inhibited in the RA 1 mg but not in the RA 10 μg group although conversion of angiotensin (Ang) I to Ang II in isolated aortic strips was suppressed in both treated groups. Plasma catecholamines were increased in the untreated control group, but treatment with either dose of ramipril normalized the values. The myocardial phosphocreatine to ATP ratio (an indicator of the energy state in the heart) was reduced in the vehicle control group whereas the hearts from treated animals showed a normal ratio comparable to hearts from sham-operated animals.

4 After one year, five animals were separated from each group, treatment withdrawn, and housed for additional six months. In the RA 1 mg group, blood pressure did not reach the value of the control vehicle group and surprisingly, left ventricular hypertrophy and myocardial fibrosis did not recur in animals during withdrawal of treatment.

5 These data show that long term ACE inhibitor treatment with ramipril in antihypertensive and non-antihypertensive doses prevented cardiac hypertrophy and myocardial fibrosis. This protective effect was still present after 6 months treatment withdrawal.

Keywords: Aortic banding; cardiac hypertrophy; myocardial fibrosis; long term treatment; angiotensin converting enzyme inhibition; low dose ramipril

Introduction

Left ventricular hypertrophy (LVH) is regarded as an independent risk factor in hypertensive patients. It is associated with increased incidence of cardiac failure, myocardial infarction, severe arrhythmias and sudden death (Messerli & Ketelhut, 1991). Clinical data indicate, that reversal of LVH may improve the prognosis of hypertensive patients (Dahlöf *et al.*, 1992). It is also known, that therapeutic approaches differ in the regression of this disorder despite application of equihypotensive doses of such antihypertensives (Hill *et al.*, 1979).

The renin angiotensin system (RAS) is involved in the development and maintenance of hypertension and cardiac hypertrophy (Hall & Karlberg, 1986), and angiotensin converting enzyme (ACE) gene expression in the heart is induced in LVH (Schunkert *et al.*, 1990). Furthermore in hypertrophied myocardium following left ventricular infarction, increased ACE activity was measured (Johnston *et al.*, 1991). A direct role of angiotensin II (Ang II) as a myocardial growth factor seems probable (Schelling *et al.*, 1991).

In experimental and clinical studies ACE inhibitors have been demonstrated to be effective in the treatment of cardiac hypertrophy when used in antihypertensive doses. The aim of our one year study in rats made hypertensive by aortic banding was to separate cardiac effects of the ACE inhibitor, ramipril, from those on systemic blood pressure by use of doses with and without effect on hypertension.

[1] Author for correspondence: c/o Hoechst AG, SBU Cardiovascular Agents (H 821), P.O.B. 800320, D-6230 Frankfurt am Main 80, Germany.

Methods

Adult male Sprague Dawley rats weighing 270–280 g (Möllegaard, Skensved, Denmark) were fasted for 12 h before surgery. Anaesthesia was induced by i.p. injection of 200 mg kg^{-1} hexobarbitone (Evipan). The abdomen was opened by a parallel cut to the linea alba. The abdominal aorta was exposed above the left renal artery and a silk thread was passed under it. A cannula no. 1 (0.9 × 40 mm) was placed longitudinally to the aorta and both aorta and cannula were tied. The cannula was then removed, leaving an aortic lumen determined by the diameter of the cannula. Before the abdomen was closed with catgut, the animals received 5.5 mg rolitetracycline (Reverin, Hoechst AG, Frankfurt, Germany). The skin was closed by clipping and covered with tar spray. Sham-operated animals were subjected to the same procedure, but without aortic banding.

During the first 5 days following the operation the animals received rolitetracycline (1 g 350 ml^{-1}) in the drinking water.

The animals were alloted to four groups of 30 rats each as follows: Group I, sham-operated; Group II, aortic banding without treatment; Group III, aortic banding, and ramipril treatment with 1 mg kg^{-1} day^{-1}, and antihypertensive dose; Group IV, aortic banding, and ramipril treatment with 10 μg kg^{-1} day^{-1}, a non-antihypertensive dose. Ramipril treatment

started the day after the operation and continued for one year by daily oral gavage. The animals were weighed weekly.

Final examinations

At the end of the experiments after one year the animals were anaesthetized with hexobarbitone (200 mg kg^{-1} i.p.) and blood pressure measured via catheters in the left carotid artery. Blood pressure measurements in concious rats by conventional tail-cuff methods were not possible, since there was a large drop of blood pressure distal to the ligature; hence, we had only one measurement at the end of the study. However, we have observed in normal rats that blood pressure values measured under hexobarbitone anaesthesia are not different from those measured in concious rats with a photoelectric tail-cuff pulse detector. This was also reported by other groups when sodium-pentobarbitone anaesthesia was used (Owen & Reidy, 1985).

The hearts were excised, cleaned of blood with saline, gently blotted to dryness, and the total cardiac mass, left ventricular weight (LVW) including the septum as well as the remaining cardiac tissue representing the right ventricle (RVW) were determined (to the nearest 0.1 mg). Weights are given per 100 g body weight.

Biochemistry

In the thoracic aorta, basal guanosine 3':5'-cyclic monophosphate (cyclic GMP) content was determined by radioimmunoassay (New England Nuclear, Dreieich, Germany). Cyclic GMP content was expressed as pmol mg^{-1} protein. Hearts for measurements of ATP, phosphocreatine and glycogen (5 to 8 hearts per group) were quickly removed and placed in liquid nitrogen. Thereafter in the left ventricular tissue, phosphocreatine (PCr), ATP and glycogen were determined (Linz *et al.*, 1989). From PCr and ATP values the PCr to ATP ratio was calculated.

Plasma renin-activity was measured by incubation of 25 µl of rat plasma with an excess of renin substrate. Plasma ACE-activity was determined radioenzymatically with [^{3}H]-Hip-Gly-Gly as substrate (Waeber *et al.*, 1989). Plasma catecholamine content of noradrenaline and adrenaline was measured by high performance liquid chromatography (h.p.l.c.).

Morphological studies

Left ventricular tissue was stained for fibronectin by a specific monoclonal antibody (ICN Biologicals, Lisle, United Kingdom).

Frozen sections of 4 µm thickness were fixed for 10 min at −20°C in cold acetone, rinsed and incubated with the first antibody during 1 h in a moist chamber. After rinsing with buffer, the biotinylated antirabbit IgG (Amersham, United Kingdom) was added to the sections. Fluorisothiocyanat labelled spectravidin was finally added after rinsing and the sections were mounted with Mowiol. The sections were viewed in an Olympus Vanox T microscope equipped for fluorescence microscopy and connected to an automatic videorecording system (VIPER, Gesotec, Darmstadt, Germany). The pictures were digitized and the degree of fibrosis automatically determined as a percentage of the entire tissue section. According to morphometric principles, these percentage values are representative for the entire left ventricle (Weibel, 1969).

Functional studies

In the isolated aorta the contractile response to Ang I or Ang II and the relaxing effect of bradykinin (BK-endothelium dependent relaxation) was tested. Intact proximal parts of the thoracic aorta were sectioned into 2 mm wide rings, cut off, and suspended at 1 g tension in 25 ml organ chambers filled with a buffer solution at 37°C of the following composition (mM): NaCl 113.8, $NaHCO_3$ 22, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.1, $CaCl_2$ 2.5 and glucose 5.5, gassed with a 95% O_2:5% CO_2 mixture to give pH 7.4. After 1 h, when a stable contractile tone had been established, Ang I (1×10^{-8} to 1×10^{-6} mol l^{-1}) was added to test the ability of the tissue ACE to convert Ang I into Ang II. Contraction of aortic strips was registered in mN. After a wash-out period of 10 min, noradrenaline was added for a final concentration of 1×10^{-8} mol l^{-1}, which produced a stable submaximum isotonic contraction. Then BK was added to give final concentrations of 1×10^{-8} to 1×10^{-6} mol l^{-1}. Relaxation of aortic strips was assessed as percentage decrease in contraction. The maximal response to Ang I was observed in aortic strips from sham-operated animals at 1×10^{-7} mol l^{-1} and the maximal relaxation we found in these aortic strips at 1×10^{-7} mol l^{-1} BK.

Withdrawal

After one year five animals were separated from each group and treatment was stopped. After an additional six months blood pressure, heart weight, left ventricular weight and myocardial fibrosis were determined; in addition functional and biochemical studies were carried out (see above).

Ramipril was synthetized in the Pharma Synthesis, Hoechst AG, and dissolved in saline. Angiotensin I and bradykinin were purchased from Sigma Chemicals, München, Germany.

Statistical analysis

Statistical analysis of the data was performed with ANOVA and Bonferroni Test when appropriate. Differences were considered significant if $P < 0.05$. Results are given as mean ± s.d.

Results

At the end of the year, plasma ACE activity and renin activity were measured. After one year of aortic banding and ramipril treatment, plasma renin activities were not changed in the animals (Table 1). Plasma ACE activity was not different in sham-operated animals and in control vehicle animals. However, plasma ACE activity was inhibited in the group which received the 1 mg kg^{-1} dose of ramipril whereas plasma ACE activity was not reduced by the lower dose of 10 µg kg^{-1} (Table 1).

To estimate tissue ACE activity, isolated aortae from all groups were exposed to 1×10^{-7} mol l^{-1} Ang I to measure the Ang II-induced contraction of the blood vessels after conversion of Ang I to Ang II by tissue ACE. In both ramipril groups the contraction of the aortae after Ang I application was significantly reduced in comparison to the sham and control vehicle group (Figure 1).

After contraction with noradrenaline, BK induced a relaxation of 45 ± 5% on aortic strips from sham-operated animals. Subjecting rats to aortic banding reduced the endothelium-dependent relaxation of these blood vessels to 12 ± 3% when exposed to BK. However, this effect was prevented by treating the animals with either 1 mg kg^{-1} or 10 µg kg^{-1} ramipril, which preserved the BK-induced relaxation at 40 ± 4% and 38 ± 5% respectively.

In sham-operated animals, aortic cyclic GMP tissue content was 58.2 ± 6.9 fmol mg^{-1} protein. Cyclic GMP was significantly lowered in the control vehicle group (37.6 ± 2.7 fmol mg^{-1} protein); however, ramipril treatment in both doses increased cyclic GMP to values above those in sham-operated animals (RA 1 mg: 95.6 ± 17.6 fmol mg^{-1} protein; RA 10 µg 72.4 ± 15.3 fmol mg^{-1} protein).

Mean arterial blood pressure (MAP) was increased after aortic banding. This effect was completely abolished by the higher dose of 1 mg kg^{-1} ramipril known to have blood

Table 1 Oral treatment for one year with ramipril 1 mg or 10 μg kg^{-1} day^{-1} in rats with aortic banding

	Sham	*Vehic con*	*RA 1 mg*	*RA 10 μg*
PRA (ng AI ml^{-1} h^{-1})	7.3 ± 1.4	5.3 ± 1.2	7.2 ± 1.5	5.4 ± 1.1
PACEA (nmol min^{-1} ml^{-1})	148 ± 9	166 ± 9	17 ± 7*	132 ± 8
HW (mg 100 g^{-1} b.wt.)	300 ± 3	393 ± 5*	294 ± 5	309 ± 6
PAd (nmol l^{-1})	9.1 ± 1.7	19.9 ± 2.8*	8.9 ± 1.5	10.2 ± 2.1
ATP (μmol g^{-1} wet wt.)	5.8 ± 0.5	5.0 ± 0.3*	5.7 ± 0.4	5.6 ± 0.4
PCr (μmol g^{-1} wet wt.)	7.9 ± 0.5	4.2 ± 0.4*	7.6 ± 0.4	7.7 ± 0.3
Glycogen (μmol g^{-1} wet wt.)	26.6 ± 3.3	27.5 ± 3.5	25.8 ± 3.7	25.9 ± 4

Plasma renin activity (PRA), plasma ACE activity (PACEA), heart weight (HW), plasma adrenaline (PAd), heart-ATP, -phosphocreatine (PCr), and -glycogen content. Vehic con: vehicle control group.
*$P<0.05$ versus sham-operated group.

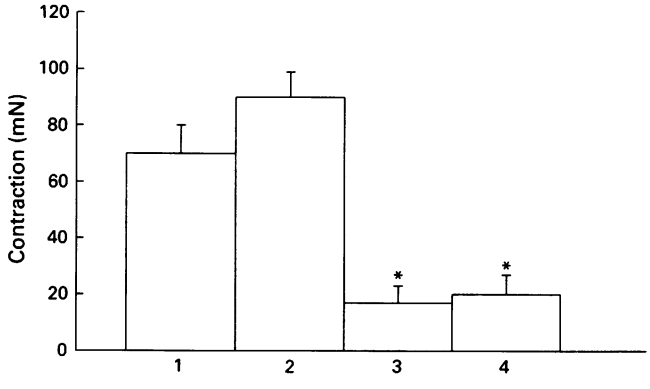

Figure 1 Tissue angiotensin-converting enzyme activity reflected as conversion of angiotensin (Ang) I (1×10^{-7} mol l^{-1}) to Ang II in isolated aortic strips (contraction in mN) from rats treated for one year with ramipril 1 mg (3) or 10 μg kg^{-1} day^{-1} (4). *n*: 15–22 per group. *$P<0.05$ vs sham (1). Vehicle control (2).

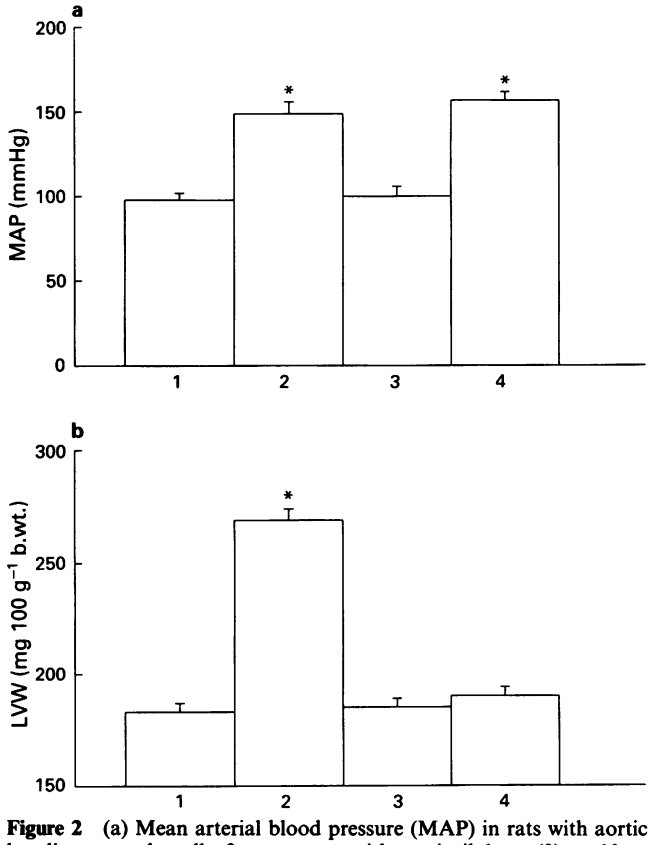

Figure 2 (a) Mean arterial blood pressure (MAP) in rats with aortic banding treated orally for one year with ramipril 1 mg (3) or 10 μg kg^{-1} day^{-1} (4). *n*: 15–22 per group. (b) Left ventricular weight (LVW) in rats with aortic banding treated orally for one year with ramipril 1 mg (3) or 10 μg kg^{-1} day^{-1} (4). *n*: 15–22 per group. *$P<0.05$ vs sham (1). vehicle control (2).

pressure lowering effects, whereas the lower dose of 10 μg kg^{-1} ramipril did not decrease blood pressure (Figure 2a).

A clear dissociation between blood pressure and left ventricular weight was found in the low dose RA group which showed no ventricular hypertrophy although hypertension was not prevented. Left ventricular weight in the high dose RA group did not differ from sham-operated matching controls, whereas hearts from control vehicle-treated animals showed ventricular hypertrophy (Figure 2b). Right ventricular weight (44–54 mg 100 g^{-1} b.wt.) and body weight (490–520 g) were not different in all four groups.

In line with the values for left ventricular hypertrophy are the observations on the occurrence of myocardial fibrosis: this was not seen in hearts from animals treated with the higher as well as the lower dose of ramipril, whereas in hearts from rats with aortic banding and treated with vehicle, myocardial fibrosis occurred (Figure 3).

Plasma noradrenaline increased from 2.3 ± 0.9 nmol l^{-1} in sham-operated animals to 5.4 ± 1.2 nmol l^{-1} following aortic banding, whereas the values were not significantly different from the sham group (2.8 ± 1.0 and 2.2 ± 0.8 nmol l^{-1}, respectively) for 1 mg kg^{-1} and 10 μg kg^{-1} ramipril. Plasma adrenaline values were comparable (Table 1).

The phosphocreatine to ATP ratio (Table 1) was reduced in hypertrophied hearts from rats of the vehicle control group (0.84), whereas ACE inhibitor treatment improved the ratio (RA 1 mg: 1.34 and RA 10 μg: 1.37) compared to the ratio found in hearts from sham-operated animals (1.34).

Withdrawal of the treatment did not change left ventricular weight to body ratio in the different groups (Figure 4b), and in the earlier RA 1 mg group (with prevention of hypertension) blood pressure did not reach the value of the stenosis vehicle group (Figure 4a).

Myocardial fibrosis did not occur after six months' withdrawal of ACE inhibitor treatment (Table 2).

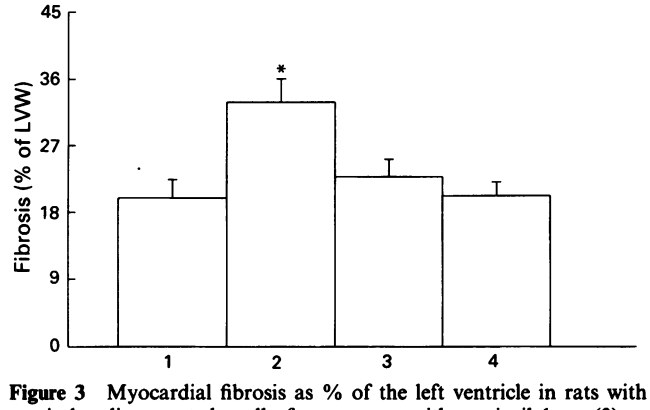

Figure 3 Myocardial fibrosis as % of the left ventricle in rats with aortic banding treated orally for one year with ramipril 1 mg (3) or 10 μg kg^{-1} day^{-1} (4). *n*: 15–22 per group; *$P<0.05$ vs sham (1). vehicle control (2).

Table 2 Withdrawal of ramipril treatment after one year for six months

	Sham	*Vehic con*	*RA 1 mg*	*RA 10 μg*
Fib % LVW	22.9 ± 1.5	36 ± 2*	23.5 ± 1.8	24.9 ± 2
Ang I to Ang II (contraction in mN)	75 ± 5	86 ± 8	61 ± 6	66 ± 5
BK (relaxation in %)	48 ± 4	9 ± 3*	46 ± 5	45 ± 4
cGMP (fmol mg^{-1} prot)	64 ± 6	36 ± 4*	83 ± 7	78 ± 6

Fibrosis % of left ventricular weight (Fib % LVW), conversion of angiotensin (Ang) I to Ang II in isolated aortic rings, relaxing effects by bradykinin (BK), basal cyclic GMP (cGMP) content in rings of rat aorta. Vehic con: vehicle control group. *$P<0.05$ versus sham operated group.

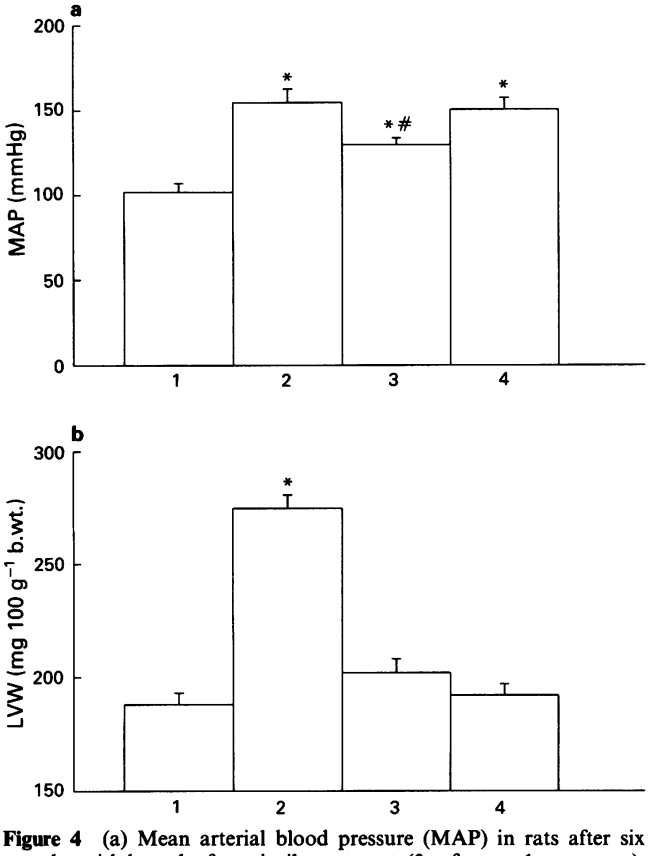

Figure 4 (a) Mean arterial blood pressure (MAP) in rats after six months withdrawal of ramipril treatment (3 = former 1 mg group), (4 = former 10 μg group). *n*: 5 per group. *$P<0.05$ vs sham (1); #$P<0.05$ vs vehicle control (2). (b) Left ventricular weight (LVW) in rats after six months withdrawal of ramipril treatment (3 = former 1 mg group), (4 = former 10 μg group) *n*: 5 per group. *$P<0.05$ vs sham (1). Vehicle control (2).

The conversion of Ang I to Ang II was not different between the groups, but the impaired relaxing effect in the former untreated control group was still present whereas aortic strips of both the earlier treated groups relaxed normally (Table 2). These relaxations were accompanied by elevated basal cyclic GMP levels in strips from previously treated animals (Table 2).

Discussion

The present study shows that long term ACE inhibitor treatment with ramipril over one year in antihypertensive and non-antihypertensive doses prevents LVH and myocardial fibrosis. Earlier experimental studies had shown that ACE inhibitors significantly prevent or reduce LVH due to aortic banding when antihypertensive doses were used (Clozel & Hefti, 1988; Kromer & Riegger, 1988). In spontaneously hypertensive rats (SHR) eleven months treatment with enalapril resulted in attenuation of blood pressure, limitation of cardiac hypertrophy and myocardial fibrosis (Pahor *et al.*, 1991).

Our observation that treatment with ramipril effectively prevents myocardial fibrosis and cardiac hypertrophy even in the absence of a fall in blood pressure implies tissue specific autocrine/paracrine mechanisms influenced by this ACE inhibitor. Such an antihypertrophic effect of ramipril without blood pressure reduction was also seen in remodelling of vascular structure in SHR (Friberg *et al.*, 1991). Furthermore depressed endothelium-mediated dilatation in renal hypertension recovered with treatment with the low dose of ACE inhibitor despite maintained hypertension (Goetz *et al.*, 1991).

Recently we demonstrated that a non-antihypertensive dose of ramipril in rats subjected to banding of the abdominal aorta caused regression of the cardiac mass, strongly supporting the hypothesis that Ang II itself is a tropic growth-promoting factor, independent of its haemodynamic effects (Linz *et al.*, 1989). Therefore haemodynamic changes alone could not account for the effect on cardiac hypertrophy.

In the rat heart overloaded in terms of pressure by constriction of the abdominal aorta, the adaptive increase in mass is characterized as concentric hypertrophy in which wall thickness increases without chamber enlargement (Rossi & Carillo, 1991). The stimulus for the appearance seems to be dependent on Ang II (Giacomelli *et al.*, 1976). Furthermore it was shown that Ang II stimulates collagen synthesis in vascular smooth muscle cells (Kato *et al.*, 1991).

On the other hand ACE inhibitors caused regression of cardiac hypertrophy and reduced myocardial tissue Ang II in SHR (Nagano *et al.*, 1991). This corresponds with our functional findings on isolated aortic strips of treated animals where the conversion of Ang I to Ang II was inhibited by either treatment regimen, indicating tissue ACE inhibition in the target organs. In contrast, plasma ACE activity was only inhibited in the high dose group in our one year study. Aortic strips from animals treated with high as well as low dose ramipril showed a normal relaxation after BK comparable to the relaxation of strips from sham-operated animals, whereas the endothelium-dependent relaxation in the aorta from stenosis vehicle treated animals was impaired. Reduced responses to Ang I and enhanced BK action were also observed in a one year study in rats with congestive heart failure treated with enalapril in a blood pressure lowering dose (Sweet *et al.*, 1987).

The effect of ramipril without blood pressure reduction could be explained by the interaction with tissue specific autocrine/paracrine mechanisms activated by local ACE inhibition.

Since inhibition of ACE, besides reducing Ang II formation, also increases BK concentrations, it is conceivable that enhanced endotheluim-derived BK with subsequent generation of nitric oxide (NO) (Wiemer *et al.*, 1991) contributes to the prevention of the hypertrophic response by ACE inhibitors. In the same model the Ang II receptor antagonist, Losartan (DuP 753), given in a blood pressure lowering dose was less effective on cardiac hypertrophy than ramipril in a

dose without effect on blood pressure (Linz et al., 1991). Further underlining the importance of BK is the recent observation that this antihypertrophic effect of ramipril was abolished by the BK_2 kinin receptor antagonist HOE 140 (Linz & Schölkens, 1992). A comparable sequence of events has been demonstrated in a model of neointimal proliferation in response to endothelial injury in the rat carotid artery, where the marked antiproliferative effect of ramipril was significantly reduced by the coadministration of the BK_2 kinin antagonist HOE 140 (Farhy et al., 1992). The possible participation of BK in the myocardial antihypertrophic as well as antifibrotic action of ACE inhibitors is also supported by the observation that the phosphocreatine to ATP ratio was low in hypertrophied hearts whereas hearts from treated animals showed an improved normal ratio as seen in sham-operated animals. This reduction of phosphocreatine to ATP ratio is characteristic for myocardial hypertrophy as shown for failing hypertrophied human myocardium using ^{31}P magnetic resonance spectroscopy (Conway et al., 1991). The normalization of phosphocreatine to ATP ratio by ACE inhibitor treatment might be explained by favourable metabolic effects of BK optimizing nutritional flow across the capillary wall which in turn leads to an elevated glucose uptake in the heart (Rösen et al., 1983), improving the energy situation in the hypertrophied myocardium.

As well as Ang II, the sympathetic nervous system needs also to be considered. Noradrenaline infused subcutaneously by use of miniosmotic pumps produced a concentric myocardial hypertrophy in concious rats (Newling et al., 1989). Stimulatory effects of Ang II on the facilitation of the peripheral sympathetic neurotransmission are known (Clough et al., 1981). Sen & Bumpus (1979) have shown that α-methyldopa and reserpine can each inhibit enhanced myocardial collagen synthesis in rats with genetic hypertension. The normalized plasma catecholamine content in our ramipril-treated rats supports the hypothesis that besides Ang II, catecholamines are also involved in the genesis of LVH and myocardial fibrosis.

Early observations with ACE inhibitors have shown that it is not necessary to suppress plasma ACE activity continously to keep the blood pressure of hypertensive animals as well as patients, normalized throughout the day during long-term treatment (Unger et al., 1985; Waeber et al., 1989). These observations have been taken as evidence for an antihypertensive action of ACE inhibitors not directly mediated by the blockade of the circulating RAS. Our withdrawal study confirms these observations concerning blood pressure changes in the ealier high dose RA 1 mg group and shows for the first time this phenomenon in LVH. ACE activity was normal in all groups; however, the antihypertrophic effect on LVH was still present probably via a signal set by long term ACE inhibition.

Conclusion

Long term ACE inhibition with ramipril effectively prevents cardiac hypertrophy and myocardial fibrosis even in the absence of a fall in blood pressure. This protective effect is still present after 6 months withdrawal of treatment. Interactions with autocrine-paracrine mechanisms involving decreased Ang II formation and increased BK generation with an attenuation of sympathetic activities should be considered as contributors to these beneficial cardiac effects of ACE inhibitors.

References

CLOUGH, D.P., COLLINS, M.G., CONWAY, J., HATTON, R. & KEDDI, J.R. (1982). Interaction of angiotensin-converting enzyme inhibitors with the function of the sympathetic nervous system. *Am. J. Cardiol.*, **49**, 1410–1414.

CLOZEL, J.P. & HEFTI, F. (1988). Cilazapril prevents the development of cardiac hypertrophy and the decrease of coronary vascular reserve in spontaneously hypertensive rats. *J. Cardiovasc. Pharmacol.*, **11**, 568–572.

CONWAY, M.A., ALLIS, J., OUWERKERK, R., NIIOKA, T., RAJAGOPALAN, B. & RADDA, G.K. (1991). Detection of low phosphocreatine to ATP ratio in failing hypertrophied human myocardium by ^{31}P magnetic resonance spectroscopy. *Lancet*, **338**, 973–976.

DAHLÖF, B., PENNERT, K. & HANSSON, L. (1992). Reversal of left ventricular hypertrophy in hypertensive patients. A metaanalysis of 109 treatment studies. *Am. J. Hypertens.*, **5**, 95–110.

FARHY, R.D., HO, K.-L., CARRETERO, O.A. & SCICLI, A.G. (1992). Kinins mediate the antiproliferative effect of ramipril in rat carotid artery. *Biochem. Biophys. Res. Commun.*, **182**, 283–288.

FRIBERG, P., ADAMS, A.A., WÅHLANDER, H. & SOHTELL, M. (1991). Effects of chronic low-dose ACE-inhibition on blood pressure and vascular structure in SHR. *7th International Symposium on SHR and Related Studies*, Lyon, October 28–30, 1991.

GIACOMELLI, F., ANVERSA, P. & WIENER, J. (1976). Effect of angiotensin-induced hypertension on rat coronary arteries and myocardium. *Am. J. Pathol.*, **84**, 111–138.

GOETZ, R.M., ALLGEIER, J., BUSSE, R. & HOLTZ, J. (1991). Depressed endothelium-mediated dilation in renal hypertension: recovery under treatment by low dose angiotensin-converting enzyme (ACE) inhibition in spite of maintained hypertension. *Eur. Heart J.*, **12**, Abstract Supplement, August 1991, XIIIth Congress of the European Society of Cardiology.

HALL, C. & KARLBERG, B.E. (1986). Plasma concentration of angiotensin II and aldosterone during acute left ventricular failure in the dog. Effect of converting enzyme inhibition. *Res. Exp. Med.*, **186**, 387–395.

HILL, L.S., MONOGHAN, M. & RICHARDSON, P.J. (1979). Regression of left ventricular hypertrophy during treatment with antihypertensive agents. *Br. J. Clin. Pharmacol.*, **7**, 255s–260s.

JOHNSTON, C.I., MOOSER, V., SUN, Y. & FABRIS, B. (1991). Changes in cardiac angiotensin converting enzyme after myocardial infarction and hypertrophy in rats. *Clin. Exp. Pharmacol. Physiol.*, **18**, 107–110.

KATO, H., SUZUKI, H., TAJIMA, S., OGATA, Y., TOMINAGA, T., SATO, A. & SARUTA, T. (1991). Angiotensin II stimulates collagen synthesis in cultured vascular smooth muscle cells. *J. Hypertens.*, **9**, 17–22.

KROMER, E.P. & RIEGGER, G.A.J. (1988). Effects of long-term angiotensin converting enzyme inhibition on myocardial hypertrophy in experimental aortic stenosis in the rat. *Am. J. Cardiol.*, **62**, 161–163.

LINZ, W., HENNING, R. & SCHÖLKENS, B.A. (1991). Role of angiotensin II receptor antagonism and converting enzyme inhibition in the progression and regression of cardiac hypertrophy in rats. *J. Hypertens.*, **9** (Suppl 6), S400–S401.

LINZ, W. & SCHÖLKENS, B.A. (1992). A specific B_2-bradykinin receptor antagonist HOE 140 abolishes the antihypertrophic effect of ramipril. *Br. J. Pharmacol.*, **105**, 771–772.

LINZ, W., SCHÖLKENS, B.A. & GANTEN, D. (1989). Converting enzyme inhibition specifically prevents the development and induces regression of cardiac hypertrophy in rats. *Clin. Exp. Hypertens.*, **A11(7)**, 1325–1350.

MESSERLI, F.H. & KETELHUT, R. (1991). Left ventricular hypertrophy: an independent risk factor. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 4), S59–S67.

NAGANO, M., HIGAKI, J., MIKAMI, H., NAKAMURA, M., HIGASHIMORI, K., KATAHIRA, K., TABUCHI, Y., MORIGUCHI, A., NAKAMURA, F. & OGIHARA, T. (1991). Converting enzyme inhibitors regressed cardiac hypertrophy and reduced tissue angiotensin II in spontaneously hypertensive rats. *J. Hypertens.*, **9**, 595–599.

NEWLING, R.P., FLETCHER, P.J., CONTIS, M. & SHAW, J. (1989). Noradrenaline and cardiac hypertrophy in the rat: changes in morphology, blood pressure and ventricular performance. *J. Hypertens.*, **7**, 561–567.

OWEN, G.K & REIDY, M.A. (1985). Hyperplastic growth response of vascular smooth muscle cells following induction of acute hypertension in rats by aortic coarctation. *Circ. Res.*, **57**, 695–705.

PAHOR, M., BERNABEI, R., SGADARI, B., GAMBASSI, G. Jr., LO GUIDICE, R., PACIFICI, L., RAMACCI, M.T., LAGRASTA, C., OLIVETTI, G. & CARBONIN, P. (1991). Enalapril prevents cardiac fibrosis and arrhythmias in hypertensive rats. *Hypertension*, **18**, 148–157.

RÖSEN, P., ECKEL, J. & REINAUER, H. (1983). Influence of bradykinin on glucose uptake and metabolism, studies in isolated cardiac myocytes and isolated perfused rat hearts. *Hoppe Seylers Z. Physiol. Chem.*, **364**, 1431–1438.

ROSSI, M.A. & CARILLO, S.V. (1991). Cardiac hypertrophy due to pressure and volume overload: distinctly different biological phenomena? *Int. J. Cardiol.*, **31**, 133–141.

SCHELLING, P., FISCHER, H. & GANTEN, D. (1991). Angiotensin and cell growth: a link to cardiovascular hypertrophy? *J. Hypertens.*, **9**, 3–15.

SCHUNKERT, H., DZAU, V.J., TANG, S.S., HIRSCH, A.T., APSTEIN, C.S. & LORELL, B.H. (1990). Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. *J. Clin. Invest.*, **86**, 1913–1920.

SEN, S. & BUMPUS, F.M. (1979). Collagen synthesis in development and reversal of cardiac hypertrophy in spontaneously hypertensive rats. *Am. J. Cardiol.*, **44**, 954–958.

SWEET, C.S., EMMERT, S.E., STABILITO, I.I. & RIBEIRO, L.G.T. (1987). Increased survival in rats with congestive heart failure treated with enalapril. *J. Cardiovasc. Pharmacol.*, **10**, 636–642.

UNGER, TH., GANTEN, D., LANG, R.E. & SCHÖLKENS, B.A. (1985). Persistent tissue converting enzyme inhibition following chronic treatment with HOE 498 and MK 421 in spontaneously hypertensive rats. *J. Cardiovasc. Pharmacol.*, **7**, 36–41.

WAEBER, B., NUSSBERGER, J., JUILLERAT, L. & BRUNNER, H.R. (1989). Angiotensin converting enzyme inhibition: discrepancy between antihypertensive effect and suppression of enzyme activity. *J. Cardiovasc. Pharmacol.*, **14** (suppl. 4), S53–S59.

WEIBEL, E.R. (1969). Stereological principles for morphometry in electron microscopy. *Int. Rev. Cytol.*, **26**, 235–302.

WIEMER, G., SCHÖLKENS, B.A., BECKER, R.H.A. & BUSSE, R. (1991). Ramiprilat enhances endothelial autacoid formation by inhibiting breakdown of endothelium-derived bradykinin. *Hypertension*, **18**, 558–563.

(Received March 18, 1992
Revised July 3, 1992
Accepted July 29, 1992)

Br. J. Pharmacol. (1992), **107**, 976–982

Role of the L-arginine-NO pathway and of cyclic GMP in electrical field-induced noradrenaline release and vasoconstriction in the rat tail artery

[1]Bernard Bucher, Sylvin Ouedraogo, Martin Tschöpl, Dominique Paya & Jean-Claude Stoclet

Laboratoire de Pharmacologie Cellulaire et Moléculaire, C.N.R.S. URA 600, Université Louis Pasteur Strasbourg, B.P. 24, 67401 Illkirch, France

1 The possible roles of the L-arginine-NO pathway and of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in regulating the prejunctional release of noradrenaline and neurogenic vasoconstriction were investigated in the perfused rat tail artery.

2 In the presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME; 30 μM), an inhibitor of NO formation, the vasoconstrictor responses to perivascular nerve stimulation (24 pulses at 0.4 Hz, 0.3 ms, 200 mA) and to exogenous noradrenaline (1 μM) were significantly enhanced, whereas the stimulation-evoked tritium overflow from [^{3}H]-noradrenaline preloaded arteries was not modified. The vasoconstriction enhancing effect of L-NAME was prevented by L-arginine (1 mM) but not D-arginine (1 mM) and was abolished by removal of the endothelium.

3 The NO donor, 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1; 0.1-30 μM), and the cyclic GMP phosphodiesterase inhibitor, zaprinast (0.1-30 μM) both induced a concentration-dependent inhibition of the electrical field stimulation-induced vasoconstriction, while atrial natriuretic peptide (ANP; 100 nM) produced only a slight decrease of the vasoconstrictor response. Methylene blue (3 μM), a known inhibitor of soluble guanylate cyclase increased the electrical field stimulation-induced vasoconstriction. SIN-1 and methylene blue when administered simultaneously, antagonized each others effect. None of the compounds tested (SIN-1, zaprinast, ANP or methylene blue) had any significant effect on the stimulation-evoked [^{3}H]-noradrenaline overflow.

4 8-Bromo-cyclic GMP, a potent activator of cyclic GMP-dependent protein kinase, markedly and concentration-dependently (3–300 μM) increased [^{3}H]-noradrenaline overflow but decreased field stimulation-induced vasoconstriction. Dibutyryl-cyclic GMP (100 μM), a weak activator of cyclic GMP-dependent protein kinase, affected neither the pre- nor the postjunctional response to electrical field stimulation.

5 These data show that an NO-like substance of endothelial origin, derived from L-arginine, attenuates vasoconstriction in the rat tail artery, whether neurally-induced or evoked by exogenous noradrenaline. Since noradrenaline release was unaltered by compounds modifying NO production, this NO-like compound acted through a postjunctional mechanism. The lack of prejunctional effects of both soluble and membrane-associated guanylate cyclase activators, despite a large effect of 8-bromo-cyclic GMP, suggests that endogenous cyclic GMP production, if present in sympathetic nerves, may not be involved in the regulation of noradrenaline release in the rat tail artery.

Keywords: Neurogenic vasoconstriction; noradrenaline release; nitric oxide (NO); N^{ω}-nitro-L-arginine methyl ester (L-NAME); cyclic GMP; rat tail artery

Introduction

In blood vessels, nitric oxide (NO) produced from L-arginine (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; 1988; Sakuma *et al.*, 1988), or possibly a molecule such as S-nitroso-L-cysteine (Myers *et al.*, 1990), accounts for the biological actions of the endothelium-derived relaxing factor (EDRF) originally described by Furchgott & Zawadzki in 1980. The metabolic pathway for the formation of NO is present in a variety of cells where it serves as a transduction mechanism for the stimulation of soluble guanylate cyclase (for reviews see Ignarro, 1990; Moncada *et al.*, 1991). Recent *in vitro* studies have suggested that NO or a related product of the L-arginine-NO pathway is also involved in non-adrenergic, non-cholinergic (NANC) neurogenic relaxant responses in blood vessels (Toda *et al.*, 1990b; Toda & Okamura, 1990; Ahlner *et al.*, 1991) and in other tissues (Gillespie *et al.*, 1989; Bult *et al.*, 1990; Li & Rand, 1990; Toda *et al.*, 1990a; Tucker *et al.*, 1990; Maggi *et al.*, 1991). Moreover, it has been reported that the presence of the endothelium decreased neurogenic vasoconstriction in the rabbit carotid artery (Tesfamarian *et al.*, 1987) and noradrenaline release from sympathetic nerves in rabbit and canine blood vessels (Cohen & Weisbrod, 1988; Greenberg *et al.*, 1989; 1990). Thus, NO production may diminish noradrenaline release. However, an inhibitor of NO production did not alter noradrenaline release in the dog isolated temporal artery (Toda *et al.*, 1991), and it even reduced *in vivo* adrenaline release in the anaesthetized rabbit (Halbrügge *et al.*, 1991).

The aim of the present study was to investigate whether or not NO, from endothelial or extra-endothelial origin, and guanosine 3':5'-cyclic monophosphate (cyclic GMP) modulate noradrenaline release and neurogenic vasoconstriction in a vascular preparation, the rat tail artery. The release of [^{3}H]-noradrenaline from postganglionic sympathetic nerve endings and vasoconstrictor responses to nerve stimulation and to exogenously applied noradrenaline were assessed in arteries with and without endothelium. The experiments were performed in the presence or in the absence of various

[1] Author for correspondence.

reagents including L-arginine, the substrate of NO synthase, its D-enantiomer and N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO production (Hobbs & Gibson, 1990; Rees *et al.*, 1990). The effect of an NO donor, SIN-1, the active metabolite of molsidonine (Feelisch & Noack, 1987), which activates soluble guanylate cyclase (Kukowetz & Holzmann, 1986), and of atrial natriuretic peptide (ANP), which stimulates the plasma membrane guanylate cyclase (Waldman *et al.*, 1984), were also examined and compared with those of membrane permeating cyclic GMP analogues, and of drugs which inhibit either cyclic GMP degradation (zaprinast; Lugnier *et al.*, 1986) or cyclic GMP production (methylene blue; Gruetter *et al.*, 1981; Martin *et al.*, 1985).

Methods

Measurement of vasoconstriction elicited by exogenous noradrenaline

Male Wistar rats (12 weeks old) were killed by cervical dislocation and exsanguinated. A segment of about 2–2.5 cm of the proximal part of the ventral tail artery was dissected out as previously described (Bucher *et al.*, 1987; Illes *et al.*, 1987) and kept in oxygenated (95% O_2; 5% CO_2) physiological saline solution which contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. The two latter compounds were added to prevent the spontaneous oxidation of catecholamines. The arteries were cannulated at both ends and suspended vertically in an organ bath containing 4 ml of medium and perfused via their proximal ends with medium by means of a roller pump. The perfusion rate was gradually increased from 0 to 2.2 ml min^{-1} during the first 10 min after suspension of the arterial segment in the bath and kept constant thereafter. The intraluminal perfusion pressure was determined with a pressure transducer and recorded on a pen recorder. Changes in perfusion pressure reflected changes in the resistance to flow, i.e. the degree of vasoconstriction. The arteries were allowed to equilibrate for 1 h in the bathing medium, during which time the fluid was replaced every 15 min. The presence of functional endothelium was assessed by the vasodilator action of acetylcholine (10 μM) in noradrenaline (1 μM) precontracted arteries. The relaxations thus produced were found to be about 50% of the contraction to noradrenaline. During the equilibration period the endothelium was removed in some arteries by passing a stream of 95% O_2:5%CO_2 through the lumen of the arterial segment at a pressure of 40–50 mmHg (Spokas & Folco, 1984). After 2 min gas injury, perfusion with medium was resumed. The failure of acetylcholine to induce relaxation in noradrenaline precontracted arteries was taken as an indication of endothelium removal. This was also confirmed in some experiments by scanning electron microscopy. All compounds were administered extraluminally to the bath fluid. Vasoconstriction was elicited by 1 μM noradrenaline which was administered every 20 min and washed out 1 min later. L-Arg (1 mM) or D-Arg (1 mM) were added 20 min before the first (S_1) vasoconstriction elicited by exogenous noradrenaline and maintained throughout. After the second vasoconstriction S_2, (chosen as a reference since the response was stable thereafter in control preparations), L-NAME (30 μM) was added to the organ bath for the duration of the experiment and noradrenaline addition was repeated twice (S_3 and S_4). The solvent for the compounds was added in control experiments.

Measurement of [^{3}H]-noradrenaline release and vasoconstriction

Rat tail arteries were dissected out, cannulated at one end and preincubated for 1 h in 1.5 ml of medium of the same composition as above containing in addition 2.2 μM(−)-[^{3}H]-noradrenaline (specific activity 4.4 Ci $mmol^{-1}$). The arteries were washed 3 times with 20 ml of [^{3}H]-noradrenaline-free medium, suspended vertically, distal end uppermost, between two platinum wire electrodes and perfused via a peristaltic pump at a rate of 2.2 ml min^{-1} with physiological saline solution containing 10 μM cocaine in order to block the re-uptake of released [^{3}H]-noradrenaline. After having passed through the lumen, the perfusate was allowed to superfuse the advential surface of the vessel. The endothelium was removed in some arteries by the procedure indicated above.

Each artery was subjected to 6 stimulation periods (24 pulses at 0.4 Hz; 0.3 ms; 200 mA). The first stimulation period was applied after 96 min of perfusion and others followed at intervals of 16 min. Collection of the perfusate/superfusate started after 124 min of perfusion in 1, 2 or 6 min fractions. The stimulation period beginning at 128 min was termed S_1 and subsequent ones S_2-S_n. S_2 served as a control of stimulation-evoked tritium overflow (since the response was stable thereafter). The compounds were infused with a syringe pump into the perfusion stream at a rate of 17 μl min^{-1} for 8 min before either S_1 or S_3 and this was maintained until the end of the experiment when the arteries were solubilized in 1 ml Soluene 100 (Packard Instrument, Paris, France). Tritium in the superfusate samples and arteries was measured by liquid scintillation spectrometry.

Tritium outflow was calculated as a fraction of the amount of tritium present in the tissue at the onset of the respective collection period (fractional rate of outflow). For evaluation of stimulation-evoked tritium overflow, the difference between the overall tritium outflow during stimulation plus the following 4 min and the estimated basal outflow was calculated. The latter was assumed to change linearly from the 1 min collection period before the beginning of stimulation to that 5 to 6 min after the start of stimulation. The evoked tritium overflow was calculated as a percentage of the amount of tissue tritium at the start of the respective stimulation period.

In some experiments the endothelium morphology was examined to determine whether or not electrical field stimulation produced any endothelial damage. Scanning electron microscopy confirmed that, at the end of electrical field stimulation experiments, the endothelium was morphologically intact in non-denuded arteries, whereas control denuded arteries were devoid of a homogeneous intact endothelial cell layer.

Data and statistical analysis

In order to quantify the drug effects on the evoked overflow or on the induced vasoconstriction, the ratio of the overflow or vasoconstriction evoked by the stimulation period in the presence of the drug at the fourth stimulation period (S_4) over the last evoked overflow or vasoconstriction preceding application of the drug (S_2) was determined. S_4 was chosen because in preliminary experiments the maximal effect of each drug concentration was reached in these conditions.

Results are given as mean ± s.e.mean where n is the number of experiments. Comparisons were made by the Mann-Whitney test if the Kruskall-Wallis analysis indicated a significant difference between multiple groups. A probability level of 0.05 or less was considered significant. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni (Wallenstein *et al.*, 1980).

Drugs

The following compounds were used: (−)-noradrenaline hydrochloride, N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), D-arginine hydrochloride, 8-bromoguanosine 3′:5′-cyclic monophosphate (sodium salt; 8-bromo-cyclic GMP), N^2-2′-O-dibutyryl-guanosine 3′:5′-cyclic monophosphate (sodium salt; dibutyryl cyclic GMP), acetyl-

choline chloride (Sigma, L'Isle d'Abeau Chesnes, France); L-arginine hydrochloride (Calbiochem, LaJolla, CA, USA); atrial natriuretic peptide (rat), ANP (1-28), (Bachem, Bubendorf, Switzerland); SIN-1 (3-morpholinosydnonimine-N-ethylcarbamide; kindly donated by Hoechst, France); zaprinast (M&B 22948; kindly donated by May and Baker, Dagenham, UK). Noradrenaline was prepared daily in the physiological saline solution. Stock solutions of all other substances were prepared with Milli-Q water (Millipore) and diluted as required, with the exception of SIN-1 which was initially dissolved in 5% glucose and of zaprinast which was prepared as a 100 μM stock solution in 1 M NaOH. The final concentration of NaOH in the medium was 2 mM or less and did not significantly alter the basal outflow or the electrically-evoked overflow of tritium or the stimulation-induced vasoconstriction. Experiments with SIN-1 were conducted in a darkened room. (−)-[Ring-2,5,6-^{3}H]-noradrenaline, specific activity 40.8-43.7 Ci mmol^{-1} (New England Nuclear, Dreieich, Germany) was diluted with unlabelled (−)-noradrenaline hydrochloride in order to obtain a specific activity of 4.4 Ci mmol^{-1}.

Results

Vasoconstriction evoked by noradrenaline

In arteries with endothelium, the increase in perfusion pressure produced by the second addition of noradrenaline amounted to 51.7 ± 3.5 mmHg ($n = 24$), whereas that observed in arteries without endothelium was significantly greater (79.6 ± 11.3 mmHg; $n = 6$; $P < 0.01$). Incubation of the arteries with L-NAME, or with L- or D-arginine, did not cause any significant change in the basal perfusion pressure. The presence of 30 μM L-NAME enhanced the vasoconstrictor responses of arteries with endothelium (Table 1) and this effect was prevented by the presence of 1 mM of L-but not of D-arginine. In endothelium-denuded arteries L-NAME had no effect on noradrenaline induced vasoconstriction (Table 1).

Table 1 Effect of N$^\omega$-nitro-L-arginine methyl ester (L-NAME) alone or in combination with either L- or D-arginine on the vasoconstrictor response elicited by noradrenaline in rat tail arteries with functional endothelium (unless otherwise indicated)

Treatment	n	*S_4/S_2 ratio Vasoconstriction*
Control	6	1.09 ± 0.03
L-NAME 30 μM	6	1.55 ± 0.07[a,b]
L-NAME 30 μM + L-arginine 1 mM	6	1.18 ± 0.04
L-NAME 30 μM + D-arginine 1 mM	6	1.72 ± 0.08[a,b]
Endothelium-denuded + L-NAME 30 μM	6	1.08 ± 0.01[c]

L- or D-arginine was added 20 min before the first stimulation (S_1) elicited by exogenous 1 μM noradrenaline and maintained throughout. L-NAME was added 20 min before the third (S_3) stimulation elicited by noradrenaline and maintained in the medium for the duration of the experiment. S_4/S_2 values represent the ratio of the vasoconstriction elicited by the fourth stimulation period (S_4) to that evoked preceding the application of L-NAME (S_2). Mean ± s.e.mean of n experiments. [a]$P < 0.01$; significant differences from drug-free control experiments. [b]$P < 0.01$; significant differences from experiments in the presence of L-NAME + L-arginine. [c]$P < 0.01$; significant difference from L-NAME experiments in arteries with intact endothelium.

[^{3}H]-noradrenaline overflow and vasoconstriction evoked by electrical field stimulation

In control arteries with endothelium, the tritium overflow evoked by the stimulation period S_2 was 0.211 ± 0.022% of tissue tritium ($n = 14$) and this did not change significantly on subsequent stimulations so that the S_4/S_2 ratio was close to unity (Table 2). L-Arginine or D-arginine when added 8 min before S_1 did not significantly modify the responses to nerve stimulation: the tritium overflow evoked at the stimulation period S_2 was 0.236 ± 0.022% of tissue tritium ($n = 6$) in the presence of L-arginine and 0.212 ± 0.015% of tissue tritium ($n = 6$) in the presence of D-arginine. In the absence of functional endothelium, the tritium overflow evoked by the stimulation period S_2 was 0.178 ± 0.012% of tissue tritium ($n = 6$) and was not significantly different from that observed in arteries with endothelium.

Table 2 Effect of N$^\omega$-nitro-L-arginine methyl ester (L-NAME) alone or in combination with either L- or D-arginine on field stimulation evoked [^{3}H]-noradrenaline overflow and vasoconstriction in rat tail arteries with functional endothelium

Treatment	n	*S_4/S_2 ratio Tritium overflow*	*Vasoconstriction*
Control	14	1.04 ± 0.03	1.05 ± 0.02
L-NAME 30 μM	6	1.09 ± 0.13	1.30 ± 0.06[a,b]
L-NAME 30 μM + L-arginine 1 mM	6	1.08 ± 0.04	1.06 ± 0.01
L-NAME 30 μM + D-arginine 1 mM	6	1.10 ± 0.06	1.39 ± 0.07[a,b]

Rat tail arteries were preincubated with [^{3}H]-noradrenaline. Four periods (S_1–S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). L- or D-arginine was added 8 min before the first field stimulation (S_1). L-NAME was added 8 min before S_3 in the continued presence of L- or D-arginine. All compounds were maintained in the medium for the duration of the experiment. Their effects were evaluated as the ratios of tritium overflow or vasoconstriction evoked by S_4 over that evoked preceding the application of L-NAME (S_2). Mean ± s.e.mean of n experiments.
[a]$P < 0.01$; significant differences from drug-free control experiments.
[b]$P < 0.01$; significant differences from experiments in the presence of L-NAME + L-arginine.

Because the absolute values of the electrically-evoked vasoconstriction varied considerably from day to day, the effects of the drugs were expressed only as the ratio of the response before the addition of the drugs to that obtained in the presence of the drug. In arteries with endothelium, the presence of 30 μM L-NAME enhanced the vasoconstrictor responses to periarterial nerve stimulation, but was without effect on the evoked tritium overflow (Table 2). In the presence of L- but not D-arginine, the vasoconstrictor effect of L-NAME was prevented and again no effect could be detected on the evoked tritium overflow (Table 2).

In arteries with endothelium, the NO donor SIN-1 (Figure 1), the soluble guanylate cyclase inhibitor methylene blue (Figure 2), the membrane guanylate cyclase activator ANP (10 and 100 nM; S_4/S_2: 0.98 ± 0.05; $n = 7$ and S_4/S_2: 1.00 ± 0.04; $n = 7$; respectively) and the selective cyclic GMP phosphodiesterase inhibitor, zaprinast (Figure 3) had no significant effect on the electrically-evoked tritium overflow. However, the stimulation-induced vasoconstriction was concentration-dependently inhibited by both SIN-1 (Figure 1) and zaprinast (Figure 3). It was practically unaffected by ANP (10 nM; S_4/S_2: 0.97 ± 0.03; $n = 7$), which had only a slight effect at the highest concentration investigated (100 nM; S_4/S_2: 0.95 ± 0.01; $n = 6$; $P < 0.05$ vs control). Methylene blue enhanced the electrical field-induced vasoconstriction (Figure 2). After simultaneous addition of methylene blue and SIN-1 the vasoconstrictor response to noradrenaline was not significantly different from control response, showing that the two compounds inhibited each others effects. Moreover, in endothelium-denuded arteries the electrical field-elicited tritium overflow and vasoconstriction was unchanged by L-NAME, methylene blue and zaprinast (Table 3).

8-Bromo-cyclic GMP (3-300 μM) increased the stimulation-evoked tritium overflow in a concentration-dependent manner. In spite of this effect, however, 8-bromo-cyclic GMP significantly decreased the electrical field-induced vasoconstrictor response, over the same concentration-range (Figure 4). By contrast, another permeant analogue of cyclic GMP, dibutyryl cyclic GMP (100 μM) was without effect on either stimulation-induced vasoconstriction (S_4/S_2: 1.00 ± 0.06; $n = 6$) or tritium overflow (S_4/S_2: 0.99 ± 0.02; $n = 6$).

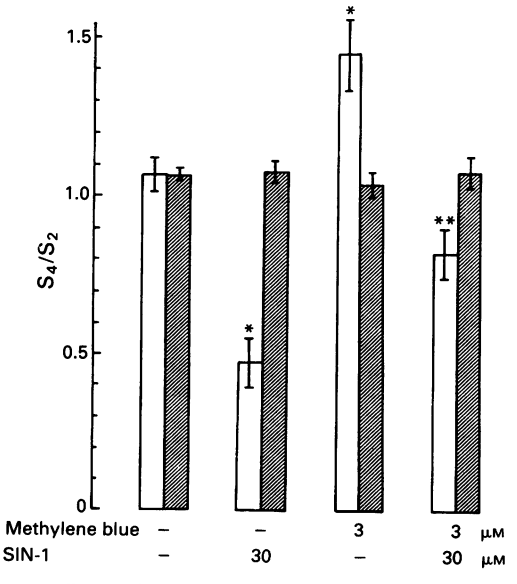

Figure 2 Effect of 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1) and methylene blue applied alone or in combination on the electrically-evoked change in perfusion pressure (open columns) and tritium overflow (hatched columns) in rat tail arteries with functional endothelium preincubated with [^{3}H]-noradrenaline. Four periods (S_1–S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). SIN-1, methylene blue or their solvents (control group) were added 8 min before S_3. All compounds were maintained in the medium for the duration of the experiment. The effects of the drugs are presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2. Each column represents the mean ± s.e.mean from 6 arteries; s.e.mean shown by vertical bars. *$P < 0.05$ in comparison with control arteries; **$P < 0.05$ in comparison with SIN-1-treated arteries.

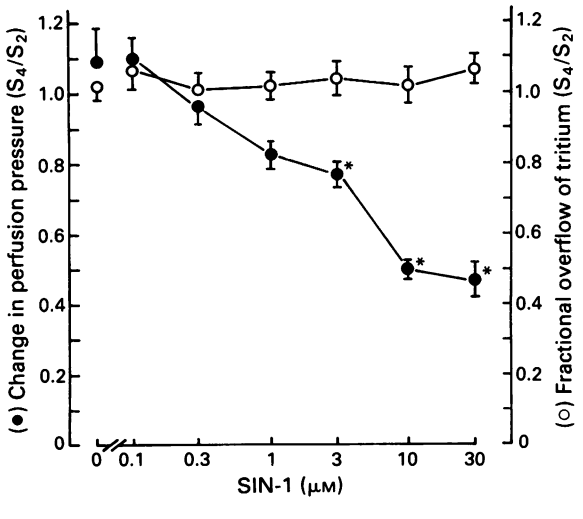

Figure 1 Effect of 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1) on the electrically-evoked overflow of tritium (○) and change in perfusion pressure (●) in rat tail arteries with functional endothelium preincubated with [^{3}H]-noradrenaline. Four periods (S_1–S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). Each concentration of SIN-1 or its solvent (control group) was added 8 min before S3 and maintained in the medium for the duration of the experiment. The effect of the drug is presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2. Each point represents the mean ± s.e.mean from 6 arteries; s.e.mean shown by vertical bars. *$P < 0.05$ in comparison with control arteries.

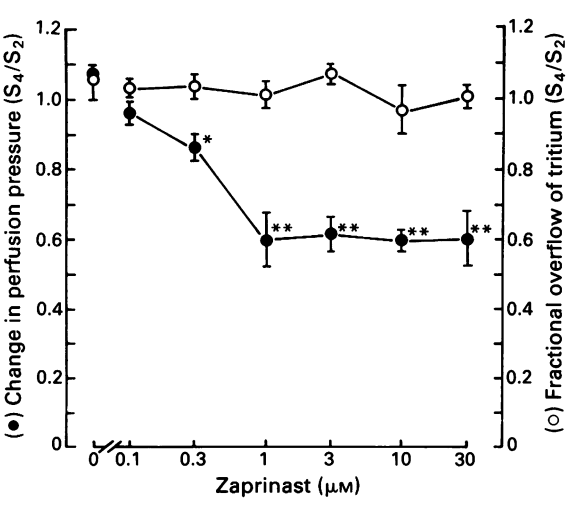

Figure 3 Effect of zaprinast on the electrically-evoked overflow of tritium (○) and change in perfusion pressure (●) in rat tail arteries with functional endothelium preincubated with [^{3}H]-noradrenaline. Four periods (S_1–S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). Each concentration of zaprinast or its solvent (control group) was added 8 min before S_3 and maintained in the medium for the duration of the experiment. The effect of the drug is presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2. Each point represents the mean ± s.e.mean from 6 arteries; s.e.mean shown by vertical bars. Significant difference from control: *$P < 0.05$, *$P < 0.01$.

Table 3 Effect of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), methylene blue and zaprinast on field stimulation evoked [^{3}H]-noradrenaline overflow and vasoconstriction in endothelium-denuded rat tail arteries

Treatment	n	S_4/S_2 *ratio*	
		Tritium overflow	*Vasoconstriction*
Rubbed endothelium control	6	1.03 ± 0.03	1.02 ± 0.02
Rubbed endothelium + L-NAME 30 μM	6	1.12 ± 0.05	1.02 ± 0.03
Rubbed endothelium + methylene blue 3 μM	6	1.04 ± 0.04	1.00 ± 0.03
Rubbed endothelium + zaprinast 1 μM	6	1.07 ± 0.04	1.02 ± 0.03

Rat tail arteries were preincubated with [^{3}H]-noradrenaline. Four periods (S_1-S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). L-NAME, methylene blue or zaprinast were added 8 min before S_3 and maintained in the medium for the duration of the experiment. The effect of the drugs is presented as the ratio of tritium overflow or vasoconstriction evoked by S_4 over that evoked preceding the application of the drugs (S_2). Mean ± s.e.mean of *n* experiments. There was no statistically significant difference between the control and treated arteries.

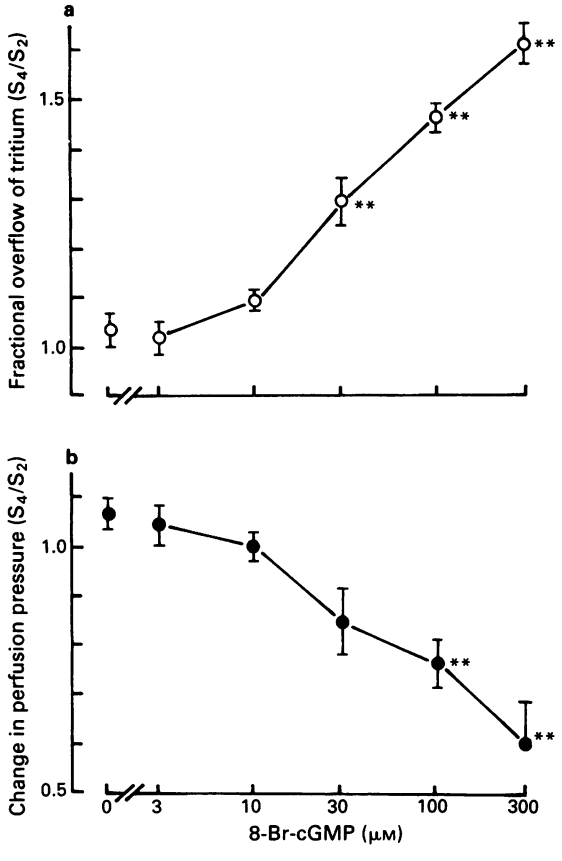

Figure 4 Effect of 8-bromo-cyclic GMP (8-Br-cGMP) on the electrically-evoked overflow of tritium (○) in (a) and change in perfusion pressure (●) in (b), in rat tail arteries with functional endothelium preincubated with [^{3}H]-noradrenaline. Four periods (S_1-S_4) of field stimulation were delivered at intervals of 16 min (24 pulses at 0.4 Hz, 200 mA, 0.3 ms duration). Each concentration of 8-Br-cGMP or its solvent (control group) was added 8 min before S_3 and maintained in the medium for the duration of the experiment. The effect of the drug is presented as the ratio of tritium overflow or change in perfusion pressure evoked by S_4 over that evoked by S_2. Each point represents the means ± s.e.mean from 6 arteries; s.e.mean shown by vertical bars. $^{**}P<0.01$ in comparison with control arteries.

Discussion

The present investigation analyses the role of the L-arginine-NO pathway and of cyclic GMP in the rat tail artery. A variety of pharmacological agents were used which either modulate NO production (L-NAME and L-arginine), activate guanylate cyclase indirectly (SIN-1, a NO donor) or directly (ANP), or inhibit soluble guanylate cyclase (methylene blue). A lack of specificity of the drugs used either to modulate cyclic GMP metabolism or to mimic its effect (cyclic GMP analogues) may partially explain conflicting conclusions which have been published regarding the involvement of cyclic GMP in neurogenic vasoconstriction. Noradrenaline release was monitored here simultaneously with the vasoconstrictor response. The results concern the regulation of neurogenic vasoconstriction and noradrenaline overflow and suggest differential effects of 8-bromo-cyclic GMP on the one hand, and drugs that increase endogenous cyclic GMP levels on the other hand, on electrical field-stimulated noradrenaline release.

The data reported here clearly indicate by direct overflow measurement that in the rat tail artery, the enhancing effect of L-NAME on the vasoconstrictor response to perivascular nerve stimulation is not due to a prejunctional alteration of noradrenaline release. Rather, this potentiation is produced by the inhibition of the endothelial L-arginine-NO pathway. These results are in line with recent data in the same tissue (Vo *et al.*, 1991), showing that endothelial-derived NO inhibits vasoconstriction elicited by nerve stimulation. In this preparation, the release of NO from inhibitory non-adrenergic, non-cholinergic nerve endings is unlikely since we were unable to demonstrate any neurogenic relaxation in tail arteries when adrenergic responses were blocked with guanethidine and tone was increased with phenylephrine (unpublished data).

Results obtained in other tissues also agree with those obtained in the rat tail artery. Toda *et al.* (1991) recently reported that the potentiating action of an inhibitor of NO synthase on neurogenic contraction of the dog temporal artery did not involve any modification in noradrenaline release. In addition, in the perfused rabbit heart, coronary flow-induced formation of EDRF did not affect the stimulation-evoked liberation of sympathetic transmitter (Wennmalm *et al.*, 1991). Taken together, these data support the view that neither the endothelium nor the L-arginine-NO

pathway are implicated in the modulation of noradrenaline release from sympathetic nerves. However, some conflicting results have also been reported. The endothelium was found to inhibit noradrenaline release in the rabbit carotid artery, although methylene blue was unable to counteract this effect (Cohen & Weisbrod, 1988). Furthermore, the presence of the endothelium has been reported to decrease noradrenaline release in canine mesenteric and pulmonary arteries and veins (Greenberg *et al.*, 1989; 1990). Moreover, both endothelial and neuronal NO production has been implicated in the inhibition of noradrenaline release induced by bradykinin in canine mesenteric and pulmonary arteries (Greenberg *et al.*, 1991). Perhaps there are tissues or species differences in the effect of endothelium-derived compounds, in particular of NO, on the release of noradrenaline.

Although the compounds tested here did not alter noradrenaline release, with the exception of cyclic GMP analogues, they produced their expected inhibitory or enhancing effect on field stimulation-induced vasoconstriction, presumably by modulating cellular cyclic GMP levels and thus the degree of activation of the cyclic GMP-dependent protein kinase in smooth muscle cells (Cornwell & Lincoln, 1989). Even the very slight effect of ANP on the vasoconstrictor response was not entirely unexpected, since despite the activation of membrane-bound guanylate cyclase, ANP causes only partial or no relaxation in some arteries, for an unknown reason (Cohen & Schenck, 1985; Faison *et al.*, 1985).

In view of the lack of effect of the compounds which are able to increase the intracellular cyclic GMP concentration, the pronounced action of 8-bromo-cyclic GMP at the prejunctional level seems surprising. Previous studies on the effects of cyclic GMP permeable analogues on field stimulated-evoked release of noradrenaline in a variety of tissues and experimental conditions have shown either no effect, e.g. in guinea-pig ileum, heart and vas deferens and rat cerebral cortex (for review see Starke, 1987; Axelsson *et al.*, 1980) or a small facilitatory effect, e.g. cat spleen, mouse atria (Cubeddu *et al.*, 1975, Johnston *et al.*, 1987) or an inhibitory effect, e.g. rat pineal (Pelayo *et al.*, 1978).

There are several possible reasons for the effect of 8-bromo-cyclic GMP, as opposed to the lack of effect of agents increasing endogenous cyclic GMP levels, in the rat tail artery. First, there are differences between the relative affinities of cyclic GMP and its 8-bromo derivative for different cyclic GMP-dependent protein kinase isoforms (Wolfe *et al.*, 1989), the dibutyryl derivative being a weak activator and the 8-bromo derivative a potent activator (Francis *et al.*, 1988). Whether such cyclic GMP-dependent protein kinase isoforms exist in sympathetic nerve endings is, however, unknown. Second, cyclic AMP-dependent protein kinase, which is also differentially activated by cyclic GMP and its analogues (Francis *et al.*, 1988) may be involved, since cyclic AMP enhances noradrenaline release in the rat tail artery (Bucher *et al.*, 1990). Finally, both cytosolic and membrane-bound guanylate cyclases may not be present, or have an activity too low to permit cyclic GMP to reach a sufficient level to activate cyclic GMP-dependent protein kinase in the postganglionic sympathetic nerve endings of the rat tail artery.

In conclusion, the current study shows that, in the rat tail artery, neurogenic vasoconstriction is modulated by NO, or an NO-like substance of endothelial origin. The data provide no evidence for either an extra-endothelial production of NO or the existence of prejunctional actions of NO or of endogenous cyclic GMP on electrical field-induced noradrenaline release in this tissue, in spite of a pronounced effect of 8-bromo-cyclic GMP. In addition, they suggest differential effects of cyclic GMP and its permeant analogues on noradrenaline release.

M.T. is supported by a research fellowship provided by the Ministère de la Recherche et de la Technologie (Réseaux Européens de Laboratoires, n° 89R0306).

References

AHLNER, J., LJUSEGREN, M.E., GRUNDSTRÖM, N. & AXELSSON, K.L. (1991). Role of nitric oxide and cyclic GMP as mediators of endothelium-independent neurogenic relaxation in bovine mesenteric artery. *Circ. Res.*, **68**, 756–762.

AXELSSON, K.L., ANDERSSON, R.G.G. & WIKBERG, J.E.S. (1980). Effect of cGMP derivatives on contraction relaxation cycle, release of norepinephrine and protein kinase activity in guinea pig vas deferens. *Acta Pharmacol. Toxicol.*, **47**, 328–334.

BUCHER, B., BETTERMANN, R. & ILLES, P. (1987). Plasma concentration and vascular effect of β-endorphin in spontaneously hypertensive and Wistar-Kyoto rats. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 428–432.

BUCHER, B., PAIN, L., STOCLET, J.-C. & ILLES, P. (1990). Role of cyclic AMP in the prejunctional α_2-adrenoceptor modulation of noradrenaline release from the rat tail artery. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **342**, 640–649.

BULT, H., BOECKXSTAENS, G.E., PELCKMANS, P.A., JORDAENS, F.H., VANMAERCKE, Y.M. & HERMANN, A.G. (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature*, **345**, 346–347.

COHEN, M.L. & SCHENCK K.W. (1985). Atriopeptin II: differential sensitivity of arteries and veins from the rat. *Eur. J. Pharmacol.*, **108**, 103–104.

COHEN, R.A. & WEISBROD, R.M. (1988). Endothelium inhibit norepinephrine release from adrenergic nerves of rabbit carotid artery. *Am. J. Physiol.*, **254**, H871–H878.

CORNWELL, T.L. & LINCOLN, T.M. (1989). Regulation of intracellular Ca^{2+} levels in cultured vascular smooth muscle cells: reduction of Ca^{2+} by atriopeptin and 8-bromo-cyclic GMP is mediated by cyclic GMP-dependent protein kinase. *J. Biol. Chem.*, **264**, 1146–1155.

CUBEDDU, L., BARNES, X.E. & WEINER, N. (1975). Release of norepinephrine and dopamine-β-hydroxylase by nerve stimulation. IV. An evaluation of a role for cyclic adenosine monophosphate. *J. Pharmacol. Exp. Ther.*, **193**, 105–127.

FAISON, E.P., SIEGL, P.K.S., MORGAN, G. & WINQUIST, R.J. (1985). Regional vasorelaxant selectivity of natriuretic factor in isolated rabbit vessels. *Life Sci.*, **37**, 1073–1079.

FEELISCH, M. & NOACK, E. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.*, **139**, 19–30.

FRANCIS, S.H., NOBLETT, B.D., TODD, B.W., WELLS, J.N. & CORBIN, J.D. (1988). Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogs that preferentially activate purified cGMP-dependent protein kinase. *Mol. Pharmacol.*, **34**, 506–517.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GILLESPIE, J.S., LIU, X.R. & MARTIN, W. (1989). The effects of L-arginine and N^G-mono-methyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br. J. Pharmacol.*, **98**, 1080–1082.

GREENBERG, S.S., DIECKE, F.P.J., PEEVY, K. & TANAKA, T.P. (1989). The endothelium modulates adrenergic neurotransmission to canine pulmonary arteries and veins. *Eur. J. Pharmacol.*, **162**, 67–80.

GREENBERG, S.S., DIECKE, F.P.J., PEEVY, K. & TANAKA, T.P. (1990). Release of norepinephrine from adrenergic nerve endings of blood vessels is modulated by endothelium-derived relaxing factor. *Am. J. Hypertens.*, **3**, 211–218.

GREENBERG, S.S., PEEVY, K. & TANAKA, T.P. (1991). Endothelium-derived and intraneuronal nitric oxide-dependent inhibition of norepinephrine efflux from sympathetic nerves by bradykinin. *Am. J. Hypertens.*, **4**, 464–467.

GRUETTER, C.A., KADOWITZ, P.J. & IGNARRO, L.J. (1981). Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerin, sodium nitrate and amyl nitrate. *Can. J. Physiol. Pharmacol.*, **59**, 150–156.

HALBRÜGGE, T., LÜTSCH, K., THYEN, A. & GRAEFE, K.-H. (1991). Role of nitric oxide formation in the regulation of haemodynamics and the release of noradrenaline and adrenaline. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **344**, 720–727.

HOBBS, A.J. & GIBSON, A. (1990). L-N^G-nitro-arginine and its methyl ester are potent inhibitors of non-adrenergic, non-cholinergic transmission in the rat anococcygeus. *Br. J. Pharmacol.*, **100**, 749–752.

IGNARRO, L.J. (1990). Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension*, **16**, 477–483.

IGNARRO, L.J., BYRNS, R.E., BUGA, G.M. & WOOD, K.S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ. Res.*, **61**, 866–879.

ILLES, P., BETTERMANN, R., BROD. I. & BUCHER, B. (1987). β-Endorphin-sensitive opioid receptors in the rat tail artery. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 420–427.

JOHNSTON, H., MAJEWSKI, H. & MUSGRAVE, I.F. (1987). Involvement of cyclic nucleotides in prejunctional modulation of noradrenaline release in mouse atria. *Br. J. Pharmacol.*, **91**, 773–781.

KUKOWETZ, W.R. & HOLZMANN, S. (1986). Cyclic GMP as the mediator of molsidomine-induced vasodilation. *Eur. J. Pharmacol.*, **122**, 103–109.

LI, C.G. & RAND, M.J. (1990). Nitric oxide and vasoactive intestinal polypeptide mediate non-adrenergic, non-cholinergic inhibitory transmission to smooth muscle of the rat gastric fundus. *Eur. J. Pharmacol.*, **191**, 303–309.

LUGNIER C., SCHOEFFTER, P., LEBEC, A., STROUTHOU, E. & STOCLET, J.C. (1986). Selective inhibition of cyclic nucleotide phosphodiesterases of human, bovine and rat aorta. *Biochem. Pharmacol.*, **37**, 1743–1751.

MAGGI, C.A., BARBANTI, G., TURINI, D. & GIULIANI, S. (1991). Effect of N^G-monomethyl L-arginine (L-NMMA) and N^G-nitro L-arginine (L-NOARG) on non-adrenergic non-cholinergic relaxation in the circular muscle of the human ileum. *Br. J. Pharmacol.*, **103**, 1970–1972.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MYERS, P.R., MINOR, R.L. Jr., GUERRA, Jr. R., BATES, J.N. & HARRISON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature*, **345**, 161–163.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PELAYO, F., DUBOCOVICH, M.L. & LANGER, S.Z. (1978). Possible role of cyclic nucleotides in regulation of noradrenaline release from rat pineal through presynaptic adrenoceptors. *Nature*, **274**, 76–78.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

SAKUMA, I., STUEHR, D.J., GROSS, S.S., NATHAN, C. & LEVI, R. (1988). Identification of arginine as a precursor of endothelium-derived relaxing factor (EDRF). *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8664–8667.

SPOKAS, E.G. & FOLCO, G.C. (1984). Intima-related vasodilatation of the perfused rat caudal artery. *Eur. J. Pharmacol.*, **100**, 211–217.

STARKE, K. (1987). Presynaptic α-autoreceptors. *Rev. Physiol. Biochem. Pharmacol.*, **107**, 73–146.

TESFAMARIAN, B., WEISBROD, R.M. & COHEN, R.A. (1987). Endothelium inhibits responses of rabbit carotid artery to adrenergic nerve stimulation. *Am. J. Physiol.*, **253**, H792–H798.

TODA, N., BABA, H. & OKAMURA, T. (1990a). Role of nitric oxide in non-adrenergic, non-cholinergic nerve-mediated relaxation in dog duodenal longitudinal muscle strips. *Jpn. J. Pharmacol.*, **53**, 281–285.

TODA, N., MINAMI, Y. & OKAMURA, T. (1990b). Inhibitory effects of L-N^G-nitro-arginine on the synthesis of EDRF and the cerebroarterial response to vasodilator nerve stimulation. *Life Sci.*, **47**, 345–351.

TODA, N. & OKAMURA, T. (1990). Possible role of nitric oxide in transmitting information from vasodilator nerve to cerebrospinal muscle. *Biochem. Biophys. Res. Commun.*, **170**, 308–313.

TODA, N., YOSHIDA, K. & OKAMURA, T. (1991). Analysis of the potentiating action of N^G-nitro-L-arginine on the contraction of the dog temporal artery elicited by transmural stimulation of noradrenergic nerves. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **343**, 221–224.

TUCKER, J.F., BRAVE, S.R., CHARALAMBOUS, L., HOBBS, A.J. & GIBSON, A. (1990). L-N^G-nitro arginine inhibits non-adrenergic, non-cholinergic relaxations of guinea-pig isolated tracheal smooth muscle. *Br. J. Pharmacol.*, **100**, 663–664.

VO, P.A., REID, J.J. & RAND, M.J. (1991). Endothelial nitric oxide attenuates vasoconstrictor responses to nerve stimulation and noradrenaline in the rat tail artery. *Eur. J. Pharmacol.*, **199**, 123–125.

WALDMAN, S.A., PAPOPORT, R.M. & MURAD, F. (1984). Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. *J. Biol. Chem.*, **259**, 14332–14334.

WALLENSTEIN, S., ZUCKER, C.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47**, 1–9.

WENNMALM, A., BENTHIN, G., KARWATOWSKA-PROKOPCZUK, E., LUNDBERG, J. & PETERSSON, A.S. (1991). Release of endothelial mediators and sympathetic transmitters at different coronary flow rates in rabbit hearts. *J. Physiol.*, **435**, 163–173.

WOLFE, L., CORBIN, J.D. & FRANCIS, S.H. (1989). Characterization of a novel isoenzyme of cGMP-dependent protein kinase from bovine aorta. *J. Biol. Chem.*, **264**, 7734–7741.

(Received May 28, 1992
Revised July 27, 1992
Accepted July 29, 1992)

Br. J. Pharmacol. (1992), **107**, 983–990

Endothelium-dependent increase in vascular sensitivity to phenylephrine in long-term streptozotocin diabetic rat aorta

[1] Kyoung S.K. Chang & Wendell C. Stevens

Department of Anesthesiology, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97201 and Veterans Administration Medical Center, Anesthesiology Service, 3710 S.W. US Veterans Hospital Road, Portland, Oregon 97201, U.S.A.

1 The effect of short- and long-term streptozotocin (STZ)-induced diabetes (12 and 52 weeks) on the vascular response to phenylephrine was examined in the isolated thoracic aorta with and without intact endothelium from diabetic, age matched control rats and diabetic rats treated with insulin.

2 Twelve weeks after induction of diabetes, aortae with intact endothelium demonstrated no changes either in sensitivity (defined as pD_2) or contractility (defined as the maximal developed tension per aortic tissue wet weight) to phenylephrine.

3 In contrast, 52 weeks after induction of diabetes, aortae with intact endothelium demonstrated an increased sensitivity to phenylephrine while contractility to phenylephrine was not changed. Insulin treatment partially corrected the increased sensitivity to phenylephrine observed in diabetic rat aorta.

4 Removal of endothelium abolished the difference in phenylephrine sensitivity between diabetic and control aortae at 52 weeks.

5 Pretreatment of intact aortae with methylene blue, an inhibitor of endothelium-derived relaxing factor (EDRF), abolished the difference in phenylephrine sensitivity between control and diabetic rat aortae at 52 weeks, while pretreatment with indomethacin, an inhibitor of cyclo-oxygenase, had no effect. These results suggest that decreases in production or release of EDRF might be responsible for the increased vascular sensitivity to phenylephrine observed in long-term STZ diabetic rats.

6 Acetylcholine-induced relaxation, which is EDRF-dependent, was less in diabetic rat aortae with intact endothelium at 52 weeks, but not at 12 weeks. These results further support the theory that decreases in capacity of the endothelium to synthesize or release EDRF may occur in long-term STZ diabetic rats.

Keywords: Streptozotocin; diabetes; phenylephrine; endothelium; blood vessel; endothelium-derived relaxing factor; insulin; methylene blue

Introduction

Hypertension frequently accompanies diabetes mellitus (Christlieb, 1973). However, the mechanism of its development is not known. Augmented vascular reactivity to vasoconstrictors or impairment of the relaxation process may be the functional changes which lead to hypertension which occurs with diabetes.

Recent studies indicate that vascular endothelium mediates the relaxant responses to a wide range of vasodilators (Furchgott, 1984), and modulates the constrictor responses to many agonists, including α-adrenoceptor agonists such as noradrenaline (Cocks & Angus, 1983), phenylephrine (Martin *et al.*, 1986), and clonidine (Eglème *et al.*, 1984; Carrier & White, 1985). Removal of vascular endothelium enhanced the constrictor responses to these agents (Cocks & Angus, 1983; Malta *et al.*, 1986; Martin *et al.*, 1986). Suppression of the constrictor response to these agonists by intact vascular endothelium is thought to be due to the continuous, basal release of endothelium-derived relaxing factor (EDRF)(s) from intact endothelium (Griffith *et al.*, 1984; Martin *et al.*, 1986).

Since widespread endothelial cell layer abnormalities are important morphological features of diabetes mellitus (Arbogast *et al.*, 1984), diabetes-induced endothelial cell damage may play a role in the enhancement of the constrictor response to neurotransmitters and circulating hormones, and thus contribute to development of hypertension.

The streptozotocin (STZ) diabetic rat model has been employed widely to study changes in the vascular reactivity to α-adrenoceptor agonists. However, results of studies of the vascular response to noradrenaline have been inconsistent in this model; enhanced (White & Carrier, 1988), unchanged (MacLeod & McNeil, 1985) or attenuated responses (Pfaffman *et al.*, 1982; Head *et al.*, 1987) have all been described. Since most of these studies were performed within 12 weeks after onset of diabetes, conflicting data suggest the need for further study beyond this time period. Three studies which extended the diabetic duration up to 43–52 weeks found increased response to noradrenaline (Jackson & Carrier, 1981; MacLeod & McNeil, 1985; Tanz *et al.*, 1991). Furthermore, since the severity of endothelial cell damage progresses as duration of diabetes increases (Dolgov *et al.*, 1982), and hypertension develops in the long-term but not in the short-term STZ diabetic rat model (Jackson & Carrier, 1981; Chang & Stevens, 1988), the aim of our study was to investigate: (1) whether the changes in the vascular reactivity to the α-adrenoceptor agonist, phenylephrine is related to duration of diabetes; (2) whether endothelium plays a role in altering the vascular response to phenylephrine; (3) whether treatment with insulin prevents this abnormality.

We studied the effects of short- and long-term STZ diabetes on the reactivity of phenylephrine, a specific α_1-adrenoceptor agonist using isolated thoracic aortic rings with and without endothelium. The phenylephrine effect was studied in preparations obtained from three groups of animals: controls, rats made diabetic with STZ and insulin-treated diabetic rats. We chose phenylephrine since rat aorta has predominantly α_1-postjunctional receptors (Alosachie &

[1] Author for correspondence at first address.

Godfraind, 1988). Our results demonstrated an increase in vascular sensitivity to phenylephrine in long-term STZ diabetic rats. Since removal of endothelium abolished the difference in phenylephrine sensitivity, diabetes-related-endothelial damage may play a role in the changes in vascular sensitivity to phenylephrine.

Methods

Animals

Male Sprague-Dawley rats (Simenson, 200 to 250 g) were randomly divided into three groups: diabetic, control, and insulin-treated diabetic. The diabetic group received a single tail vein injection of STZ, 55 mg kg^{-1}, dissolved in a citrate buffer (0.01 M solution, pH 4.5) (Rerup, 1970). Age-matched control rats were injected with the buffer solution alone. Randomly selected diabetic rats received insulin subcutaneously daily (2 u per day of protamine zinc insulin) after the development of diabetes had been confirmed 48 h after the injection of STZ (insulin-treated diabetic). All animals were provided with food and water *ad libitum*. A blood sample was taken from the tail vein at 48 h and at the time of study (12 or 52 weeks). The plasma glucose level was determined with a Beckman glucose analyzer.

Preparation of tissue

Twelve and 52 weeks after induction of diabetes, the thoracic aorta from the diaphragm to the heart was removed under ether anaesthesia, placed in oxygenated Krebs-Henseleit solution and dissected free of fat and connective tissue, care being taken not to damage the endothelial cell layers or stretch the vessels. Rings approximately 3 mm in width were then cut, mounted between two stainless steel wires, and placed in a 20 ml muscle bath containing a modified Krebs-Henseleit solution of the following composition in mM: KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, $CaCl_2$ 2.54, NaCl 119, $NaHCO_3$ 25 and glucose 11, pH 7.4. The solution was continuously aerated with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Tissues were equilibrated for 2 h under a resting tension of 2 g with bath fluids changed every 30 min. In preliminary length-tension experiments, optimal resting tension was determined to be 2 g for both control and diabetic aortic rings. Isometric tensions were recorded with a Grass FT-03 force-displacement transducer and recorded on a Grass polygraph. In some rings, endothelium was removed by gently rubbing the intimal surface with a wooden stick for 30–60 s (Furchgott & Zawadzki, 1980).

Concentration-response curve

After a 2 h equilibration period, rings were contracted with phenylephrine (3×10^{-7} M) to induce submaximal contraction (50–70% of maximal contraction). Acetylcholine (10^{-5} M) was then added to the bath to assess the integrity of the endothelium. No relaxation to acetylcholine in the denuded preparation indicated effective denuding. After acetylcholine testing, the rings were washed and left to re-equilibrate for 60 min.

After a 60 min equilibration period, concentration-response curves for phenylephrine (10^{-9}–10^{-5} M) were obtained by cumulative addition of phenylephrine. The maximal response to phenylephrine was then relaxed by adding acetylcholine (10^{-5} M). To obtain a full concentration-response curve for acetylcholine, separate aortic preparations were used. After precontracting the aortic rings submaximally with phenylephrine (3×10^{-7} M), acetylcholine was added cumulatively. To determine the effects of methylene blue or indomethacin on phenylephrine contractility, the tissue was incubated with either methylene blue (10^{-5} M) for 10 min or indomethacin (5×10^{-6} M) for 45 min before obtaining a subsequent phenylephrine response.

At the end of the experiments, the aortic rings were blotted and weighed. Contractile response was expressed either as a percentage of the maximal phenylephrine contraction or as milligrams (mg) of tension developed per mg of tissue wet weight. The EC_{50} value was calculated from individual log concentration-response curves by regression analysis (over the range of 10–90% of the maximum response). The sensitivity (potency) to phenylephrine was expressed as pD_2 ($-\log EC_{50}$) according to the method of Fleming *et al.* (1972). Vascular smooth muscle contractility was evaluated by the maximally developed tension per unit tissue weight.

Drugs

Acetylcholine chloride, phenylephrine hydrochloride, methylene blue, indomethacin and streptozotocin were obtained from Sigma Chemical Company. Protamine zinc insulin was from Eli Lilly. Solutions were prepared in distilled water just before use except for indomethacin which was dissolved in ethanol.

Statistical analysis

Data are expressed as mean ± s.e.mean. Statistical analysis of the data was performed by Student's unpaired t test for comparison of two groups and one-way analysis of variance for comparison of three or more groups. When F was significant, differences between individual groups were calculated with Tukey's test. A $P<0.05$ was considered statistically significant.

Results

Increase in blood glucose and decrease in body weight were observed in the diabetic rats at both 12 and 52 weeks after STZ injection (Table 1). Blood glucose and body weight of diabetic rats treated with insulin were similar to those of controls at 12 weeks. At 52 weeks, they were intermediate between control and diabetic rats (Table 1).

Aorta

Aortic wet weight was significantly lower in the diabetic rats at 12 weeks compared to controls. There was no difference between control and diabetic aorta at 52 weeks despite the lower body weight of diabetic rats. Aortic weight of diabetic rats treated with insulin was similar to controls at both time periods (Table 1).

Phenylephrine response

Twelve weeks after induction of STZ diabetes, in aortic rings with intact endothelium, there was no difference in sensitivity (pD_2) (Figure 1a, Table 2) or maximal contractility (mg of developed tension per mg tissue) in response to phenylephrine between control, diabetic and diabetic rats treated with insulin. In contrast, 52 weeks after induction of STZ diabetes, phenylephrine dose-response curves of the diabetic rat aortae with endothelium were shifted to the left of those in control aortae with endothelium, thus demonstrating an increased sensitivity (pD_2) to phenylephrine (Figure 1b, Table 2). Maximum contractile response to phenylephrine remained unchanged (Table 3). The insulin treatment partially attenuated the increased sensitivity to phenylephrine (Table 2).

When endothelium was removed, there was no difference in sensitivity to phenylephrine among the groups (Figure 1b, Table 2). Endothelium removal shifted the phenylephrine dose-response curves to the left of those with intact endothelium in all three groups of animals. However, the curve shift depicting the response to phenylephrine in the

Table 1 Mean blood glucose, and body and aortic weights in groups of experimental rats

Time (Weeks)	*Experimental group*	*Blood glucose* (mg dl^{-1})	*Body wt.* (g)	*Aortic wet wt.* (mg)
12	Control (8)	131 ± 25	460 ± 19	4.70 ± 0.44
	Diabetic (5)	415 ± 27†††***	193 ± 14†††***	2.80 ± 0.30††**
	Diabetic + insulin (6)	142 ± 29	429 ± 10	5.33 ± 0.08
52	Control (7)	139 ± 18	546 ± 35	6.71 ± 0.99
	Diabetic (7)	438 ± 41†††***	247 ± 23†††***	6.86 ± 0.83
	Diabetic + insulin (11)	188 ± 13*	465 ± 20*	7.91 ± 0.91

Numbers in parentheses are the numbers of animals studied. Values are mean ± s.e.mean.
*$P<0.05$; **$P<0.01$; ***$P<0.001$: denotes significant difference from corresponding control.
††$P<0.01$; †††$P<0.001$: denotes significant difference from insulin-treated diabetic.

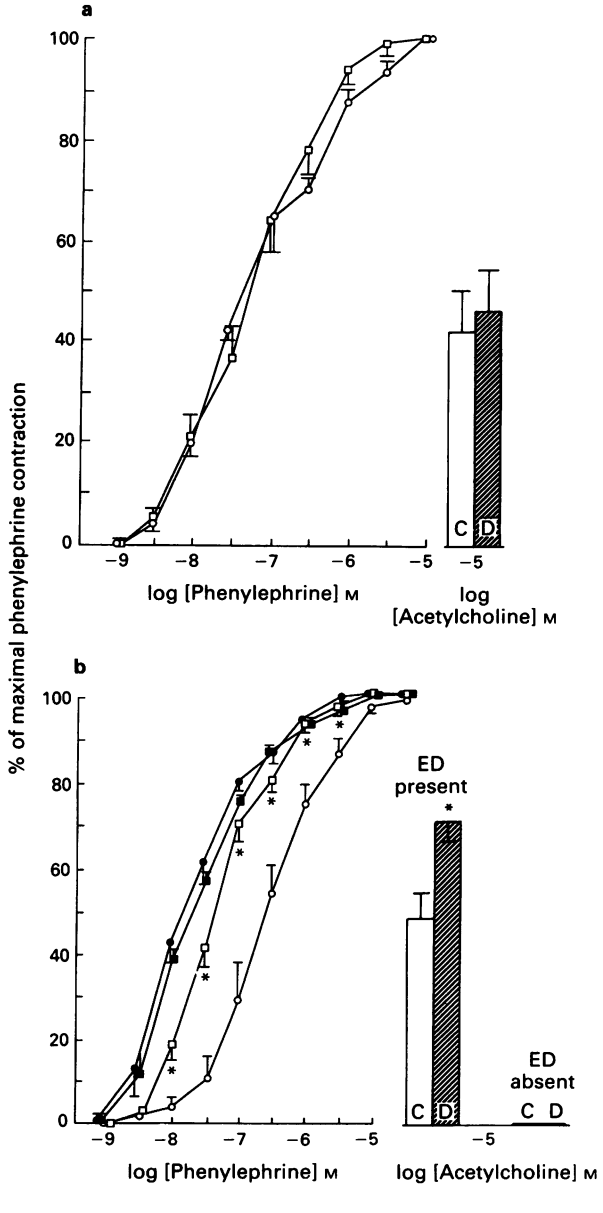

Figure 1 Cumulative log concentration-response curves for phenylephrine in aortic rings with and without endothelium from control and diabetic rats at 12 (a) and 52 (b) weeks after induction of diabetes. At 12 weeks, phenylephrine response was studied only in the endothelium (ED) intact rings. Columns on the right indicate relaxation response to acetylcholine (10^{-5} M) in the rings contracted maximally with phenylephrine (10^{-5} M) at the end of the cumulative phenylephrine concentration-response. The responses to phenylephrine and acetylcholine are depicted as % of maximal phenylephrine contraction. In (a): control (○), $n = 8$, and diabetic (□), $n = 5$ aortic rings. In (b): rings with endothelium, control (○), $n = 7$ and diabetic (□), $n = 7$; rings without endothelium: control (●), $n = 7$ and diabetic (■), $n = 7$. C, control; D, diabetic. Each point represents the mean with s.e.mean shown by vertical bars. *$P<0.05$ when compared to their respective controls with and without endothelium.

Table 2 Phenylephrine pD_2 ($-\log EC_{50}$) in the presence and absence of endothelium

Time (weeks)	*Experimental group*	*Endothelium present*	*Endothelium absent*
12	Control (8)	7.18 ± 0.08	–
	Diabetic (5)	7.24 ± 0.16	–
	Diabetic + insulin (6)	7.10 ± 0.17	–
52	Control (7)	6.57 ± 0.15[1]	7.85 ± 0.13‡‡‡
	Diabetic (7)	7.33 ± 0.10††**	7.71 ± 0.05‡
	Diabetic + insulin (11)	7.02 ± 0.07**	7.63 ± 0.16‡‡‡

Numbers in parentheses are the numbers of animals studied. Values are mean ± s.e.mean.
**$P<0.01$: denotes significant difference from corresponding control.
††$P<0.01$: denotes significant difference from insulin-treated diabetic.
‡$P<0.05$; ‡‡‡$P<0.001$: denotes significant difference from endothelium present.
[1]$P<0.05$: denotes significant difference from corresponding group at 12 weeks.

Table 3 Maximal phenylephrine contraction (mg of developed tension/mg of aortic tissue) in the presence and absence of endothelium

Time (weeks)	*Experimental group*	*Endothelium present*	*Endothelium absent*
12	Control (8)	750 ± 70	–
	Diabetic (5)	720 ± 21	–
	Diabetic + insulin (6)	650 ± 124	–
52	Control (7)	299 ± 46†	285 ± 47
	Diabetic (7)	253 ± 33†	328 ± 47
	Diabetic + insulin (11)	205 ± 35†	384 ± 78

Numbers in parentheses are the numbers of animals studied. Values are mean ± s.e.mean.
†$P<0.01$: denotes significant difference from corresponding group at 12 weeks.

presence or absence of endothelium was significantly greater in control than in diabetic rat aortae, while that of insulin-treated diabetic aortae was intermediate [shift of pD_2: control 1.20 ± 0.09 ($n = 7$); diabetic 0.38 ± 0.09 ($n = 7$)*; diabetic + insulin 0.62 ± 0.03 ($n = 11$) *$P < 0.05$ vs. control]. As a result, after removal of endothelium, there was no difference in the phenylephrine sensitivity between aortic rings from control, diabetic and diabetic treated with insulin.

The maximal contraction elicited by phenylephrine was not affected by the removal of endothelium in any group of animals (Table 3).

Effects of methylene blue and indomethacin on the phenylephrine response

Endothelium releases two major vasodilators, EDRF and prostacyclin. To determine which vasodilator is responsible for the increased sensitivity to phenylephrine in the diabetic aorta, the aortic rings with intact endothelium were treated with either an inhibitor of EDRF, methylene blue (10^{-5} M), or an inhibitor of cyclo-oxygenase, indomethacin (5.0×10^{-6} M). Exposure of aortic rings to methylene blue caused slight contractions in both control and diabetic rats. The contractions elicited by methylene blue tended to be smaller in diabetic as compared with control rat aortae [mg of developed tension per mg of tissue: control, 35 ± 10 ($n = 13$); diabetic, 22 ± 3 ($n = 11$)].

After methylene blue-treatment the response to phenylephrine (3×10^{-7} M) was similar in control and diabetic aortae (Figure 2). Methylene blue treatment enhanced phenylephrine-induced contraction both in control and diabetic aortae. However, the magnitude of enhancement was significantly greater in the control group than in the diabetic group (Figure 2). As a result, after methylene blue treatment, there was no difference in the phenylephrine response between the aortic rings of control and diabetic rats. Indomethacin treatment did not have any effect on the aortic response to phenylephrine in either control or diabetic groups. The response of insulin-treated diabetic aortae to phenylephrine was similar to that of controls in the presence of both methylene blue and indomethacin (Figure 2).

Acetylcholine-mediated relaxations

In the aortic rings with intact endothelium, the relaxation elicited by acetylcholine was similar between control and diabetic rats at 12 weeks, whether tested in the same aortic rings in which phenylephrine dose-responses were obtained or in the separate aortic rings in which acetylcholine dose-responses were obtained (Figures 1a,3,4a, Table 4). However, at 52 weeks, acetylcholine-induced relaxation, which is endothelium-dependent, was significantly less in the diabetic aortae (Figures 1b,3,4b, Table 4). The maximum relaxation response to acetylcholine was impaired without change in the sensitivity (Table 4). Treatment of aortic rings with indomethacin (5×10^{-6} M) had no effect on the attenuated acetylcholine response observed in these diabetic aortae [% relaxation to acetylcholine (10^{-5} M); control, 65 ± 6 ($n = 4$); diabetic, 40 ± 5 ($n = 4$)]. Insulin treatment partially reversed the decreased relaxation observed in the 52-week diabetic rat aortae (Figure 3, Table 4). Removal of endothelium abolished acetylcholine-induced relaxation in all three groups of rat aortae (Figures 1b and 4).

Discussion

The present results demonstrate that vascular sensitivity (pD_2) to phenylephrine is enhanced in the long-term (52 weeks) but not in short-term STZ diabetic rat aortae (12 weeks). Our results suggest that damage of endothelium, but not of underlying smooth muscle, contributes to the increased phenylephrine sensitivity since removal of endothelium abolished the difference in the phenylephrine sensitivity between control and diabetic rats. Previous studies from this laboratory (Tanz *et al.*, 1991) also support the view that endothelium but not underlying smooth muscle is affected by STZ diabetes. In the latter study, we found that nitroglycerin-induced vasodilatation which is endothelium-independent, was not altered in the aortae from 52-week diabetic rats while endothelium-dependent vasodilatation by histamine was impaired.

Diabetes induces both structural (Arbogast *et al.*, 1984)

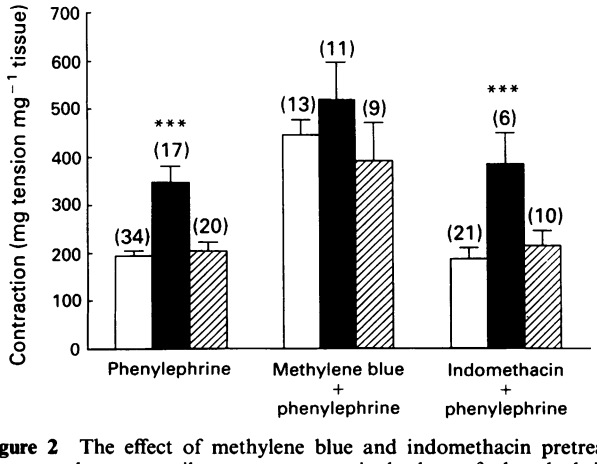

Figure 2 The effect of methylene blue and indomethacin pretreatment on the contractile response to a single dose of phenylephrine (3×10^{-7} M) in the aortic rings with endothelium from control (open columns), diabetic (solid columns) and insulin-treated diabetic rats (hatched columns) at 52 weeks after induction of diabetes. The aortic rings were incubated with methylene blue (10^{-5} M for 10 min) or indomethacin (5×10^{-6} M for 45 min) before phenylephrine response was obtained. The response to phenylephrine is expressed as mg of tension developed per mg of aortic tissue. The number of aortic rings in each group is given in parentheses. Each column represents the mean with s.e.mean shown by vertical bars. ***$P < 0.001$ when compared to control group.

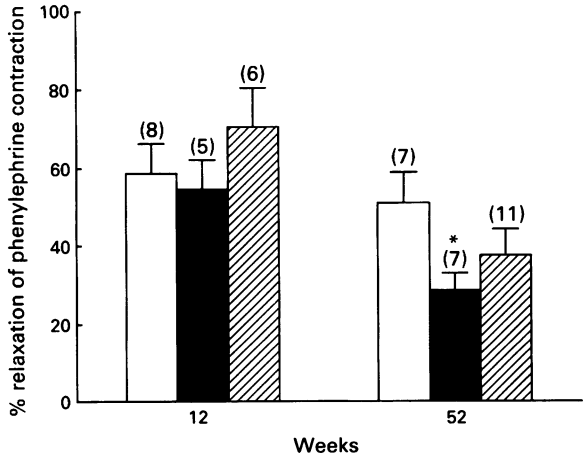

Figure 3 The relaxation response to acetylcholine (10^{-5} M) in the aortic rings with endothelium maximally contracted with phenylephrine (10^{-5} M) at the end of the cumulative phenylephrine concentration-response from control (open columns) diabetic (solid columns) and insulin-treated diabetic rats (hatched columns) at 12 and 52 weeks after induction of diabetes. The response is expressed as % relaxation of maximal phenylephrine contraction (10^{-5} M). The number of animals in each group is given in parentheses. Each column represents the mean with s.e.mean shown by vertical bars. *$P < 0.05$ when compared to control group.

Table 4 Acetylcholine pD_2 and maximal relaxation in the aortic rings with endothelium precontracted submaximally with phenylephrine (3×10^{-7} M)

Time (weeks)	*Experimental group*	*pD_2*	*Maximal relaxation* (%)
12	Control (8)	6.95 ± 0.08	62.4 ± 7.5
	Diabetic (5)	6.91 ± 0.12††	50.8 ± 4.5††
	Diabetic + insulin (6)	7.43 ± 0.10**	70.4 ± 5.3
52	Control (7)	7.16 ± 0.20	59.0 ± 2.7
	Diabetic (7)	6.78 ± 0.15	32.8 ± 4.3***†^{tt}
	Diabetic + insulin (11)	6.90 ± 0.25	46.9 ± 2.0*^{ttt}

Numbers in parentheses are the numbers of animals studied. Values are mean ± s.e.mean.
*$P<0.05$; **$P<0.01$; ***$P<0.001$: denotes significant difference from corresponding control.
†$P<0.05$; ††$P<0.01$: denotes significant difference from insulin-treated diabetic.
$^{tt}P<0.01$; $^{ttt}P<0.001$: denotes significant difference from corresponding group at 12 weeks.

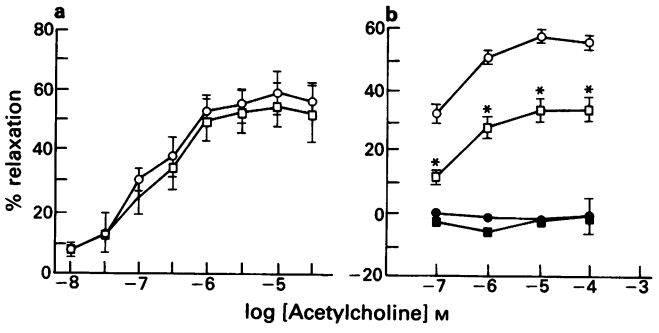

Figure 4 Cumulative log concentration-relaxation curves for acetylcholine in the aortic rings with (open symbols) and without (closed symbols) endothelium from control (○, ●) and diabetic (□, ■) at 12 (a) and 52 (b) weeks after induction of diabetes. Each group represents 6–8 animals. The acetylcholine concentration-relaxation curves are depicted as % relaxation of phenylephrine contraction (3×10^{-7} M). Each point represents the mean with s.e.mean shown by vertical bars. *$P<0.05$ when compared to control group.

and functional (Colwell *et al.*, 1981) abnormalities in the endothelial cells. Possible mechanism(s) for the enhanced phenylephrine sensitivity in the long-term diabetic rats include reductions in the release of vasodilators or increase in the release of vasoconstrictors from diabetic endothelium. Although evidence indicates that the synthesis of vasoconstrictor, thromboxane A_2 is increased in the diabetic rat blood vessels (Roth *et al.*, 1983; Sterin-Borda *et al.*, 1984), it is not likely that increases in the vasoconstricting substance are responsible for the increased phenylephrine sensitivity. If that were the case, removal of endothelium should decrease the phenylephrine sensitivity. The present findings that the removal of endothelium enhanced phenylephrine sensitivity (more in control than in diabetic aortae) suggests that release and/or synthesis of vasodilator substance is decreased from diabetic endothelium. Two potent vasodilators which are released from endothelium are EDRF (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987) and prostacyclin (Moncada *et al.*, 1976).

The present results with methylene blue, an inhibitor of soluble guanylate cyclase (EDRF) and indomethacin indicate that reductions in the release and/or production of EDRF but not prostacyclin from diabetic endothelium is responsible for the increased vascular sensitivity to phenylephrine seen in the long-term diabetic rats. Methylene blue pretreatment abolished the difference in the phenylephrine response between control and diabetic rat aorta while indomethacin pretreatment had no effect on it (Figure 2). This view was further supported by the observation that acetylcholine-induced relaxation, which is EDRF dependent, was impaired in the long-term, but not in short-term STZ diabetic rat aortae. In the latter the phenylephrine response was normal.

Although there is substantial evidence for decreases in the prostacyclin synthesis from vessels of diabetic patients (Johnson *et al.*, 1979) and STZ-induced diabetic rats (Harrison *et al.*, 1978; Valentovic & Lubawy, 1983), it is not likely that decreases in the prostacyclin production or release are responsible for the enhanced phenylephrine sensitivity of long-term STZ diabetic rat aortae. The present observation that the concentration of indomethacin (5.0×10^{-6} M) which is known to inhibit cyclo-oxygenase (Flower, 1974) did not increase the phenylephrine response in the normal control aortae suggests that basal or stimulated release of prostacyclin has a little effect on the phenylephrine-induced contraction. This view is consistent with the postulations that vascular biosynthesis of prostacyclin does not play a major role in the pathogenesis of hypertension (Gryglewski *et al.*, 1988). On the other hand, recent evidence strongly suggests a role of EDRF in the regulation of vascular tone, vasospasm and hypertension (Vane *et al.*, 1990).

It is possible that endothelial damage can decrease the release and/or synthesis of EDRF without affecting prostacyclin levels since prostacyclin is synthesized by both endothelial and smooth muscle cells (Weksler *et al.*, 1978) while EDRF is produced primarily by endothelial cells. EDRF has recently been identified as nitric oxide (NO) (Palmer *et al.*, 1987; Furchgott, 1988). It is currently thought that EDRF is released spontaneously from intact vascular endothelium and dilates underlying smooth muscles by increasing guanosine 3':5'-cyclic monophosphate (cyclic GMP). The associated tonic elevation of cyclic GMP inhibits the contractile response to various vasoconstrictors including α-adrenoceptor agonists. The evidence in favour of this view is that removal of endothelium or exposure of rat aortae to the guanylate cyclase inhibitor, methylene blue, decreased basal levels of cyclic GMP with concomitant contractions (Miller *et al.*, 1984). Our results support this view because the aortic rings with endothelium from control as well as diabetic rats contracted slightly to exposure to methylene blue and the contractile response to methylene blue tended to be lower in diabetic as compared with control rat aortae. It is well documented that modulation of contractile responses by endothelium is different for α_1- and α_2-adrenoceptor agonists. For α_1-adrenoceptor agonists such as noradrenaline and phenylephrine, the presence of endothelium reduces vascular sensitivity with little change in the maximal response (Eglème *et al.*, 1984; Malta *et al.*, 1986; Martin *et al.*, 1986). Consequently, removal of endothelium increases vascular sensitivity to an α_1-adrenoceptor agonist with little or no increase in the maximal contraction (Malta *et al.*, 1986; Martin *et al.*, 1986; Alosachie & Godfraind, 1988). In the present study, similar results were observed. While removal of endothelium enhanced phenylephrine sensitivity both in control and diabetic rat aortae at 52 weeks (more in control than diabetic), removal of endothelium had a minimum effect on the maximal response induced by phenylephrine. The latter observation is consistent with the concept that EDRF acts as an endogenous functional antagonist to reduce the efficacy of α-adrenoceptor agonists (Alosochie & Godfraind, 1988).

The role of endothelium in diabetes-induced changes in vascular response to α-adrenoceptor agonists had been studied with conflicting results during early stages of diabetes. Fortes *et al.* (1983) and Abiru *et al.* (1990) demonstrated that differences in the noradrenaline response disappeared after removal of endothelium of aortae from 10- and

24-week alloxan diabetic rabbits (Abiru *et al.*, 1990) and rats (Fortes *et al.*, 1983). In contrast, Harris & MacLeod (1988) and White & Carrier (1988) found that removal of endothelium had no effect on the increased response to phenylephrine in the short-term (12-week) STZ diabetic rat aorta and mesenteric arteries. Further, Gebremedhin *et al.* (1987, 1989) demonstrated that in blood vessels (femoral and renal arteries) from 12-week alloxan diabetic dogs, response to phenylephrine was increased in preparations with denuded but not with intact endothelium.

Gebremedhin *et al.* (1987, 1989) postulated that during early stage of diabetes the hyperresponsiveness of diabetic smooth muscle to phenylephrine was masked by increased endothelial release of EDRF. Alternatively, it is possible that during the early stage of diabetes, endothelial function is not changed and that the alterations in the underlying smooth muscle may be responsible for the increased phenylephrine response. However, we have not observed any evidence of increased phenylephrine responses in the aorta with intact endothelium at early stages of STZ-diabetes. Neither sensitivity nor contractility to phenylephrine was changed in the 12-week diabetic rat aortae with intact endothelium (Tables 2 and 3). Although most studies agree that the sensitivity to phenylephrine was not changed during early stage of diabetes (up to 12 weeks in the STZ diabetic rats), they disagree in the maximal contraction developed in the response to phenylephrine: increase (Agrawal & McNeil, 1987), decrease (Pfaffman *et al.*, 1982), no change (Scarborough & Carrier, 1984; White & Carrier, 1988) all have been reported.

In contrast, studies which have extended the diabetic duration up to 43–52 weeks have demonstrated a consistent increase in sensitivity to noradrenaline in rat aortae (MacLeod & McNeil, 1985) and mesenteric arteries (Jackson & Carrier, 1981) from STZ diabetic rats which is consistent with our present findings with phenylephrine.

Diabetes decreased both body and aortic weights during the early stage of diabetes. This may be due to the caloric deficiency associated with the diabetic state. In contrast, 52 weeks after induction of diabetes, there was no difference in the aortic weights between control and diabetic rats, even though the body weights of 52-week diabetic rats were significantly lower than those of age-matched control rats. This suggests that aortic weight of diabetic rats was disproportionately large in relation to their body weights.

In view of recent evidence that nitric oxide (EDRF) inhibits mitogenesis and proliferation of smooth muscle cells (Garg & Hassid, 1989a) and mesangial cells (Garg & Hassid, 1989b), the decrease in the EDRF release (and probably also prostacyclin) as a result of diabetic endothelial damage might contribute to the increase in the aortic weight of long-term diabetic rats. In addition, lipid accumulation in the wall of the diabetic artery (extracellular and intracellular) (Colwell *et al.*, 1981) may further increase the weight of the diabetic aorta.

Our study demonstrates that the aging process affects the responses to phenylephrine and acetylcholine differently in normal as well as diabetic aortae. In normal rat aortae, phenylephrine response (sensitivity as well as maximum contractility) was decreased significantly with increasing age while acetylcholine-induced relaxation was well maintained (Table 2–4). On the other hand, in diabetic aortae, while the maximum response to phenylephrine was similarly decreased with increasing age, phenylephrine sensitivity was not affected by aging. It is not known whether the relative increase in the phenylephrine sensitivity in diabetic aortae compared to controls in the 52-week STZ diabetic rats is due to the lack of normal aging process or to another mechanism producing supersensitivity in diabetes. In view of decreased acetylcholine-induced relaxation in the aged diabetic aortae (52 weeks), we hypothesize that diabetic aortae undergo a normal aging process with superimposed endothelial damage which causes increased sensitivity to phenylephrine. Similar lack of progressive changes in sensitivity to noradrenaline with increasing duration of diabetes was also reported in aortae from 52-week diabetic rats (MacLeod & McNeil, 1985).

As with the contractile response to phenylephrine, during early stage of diabetes (up to 12 weeks), conflicting results have been reported in the literature with regard to the endothelium-dependent relaxation by acetylcholine in diabetic blood vessels: no impairment (Gebremedhin *et al.*, 1987; Head *et al.*, 1987; Wakabayashi *et al.*, 1987; Harris & MacLeod, 1988), enhanced relaxation (Gebremedhin *et al.*, 1989) or a decreased relaxation (Oyama *et al.*, 1986; Meraji *et al.*, 1987; Durante & Sunahara, 1988) all have been reported.

In contrast, no study of the response to acetylcholine in rats that have been diabetic for more than 6 months was reported. Fortes *et al.* (1983) demonstrated no impairment in the endothelium-dependent relaxation by acetylcholine in the 6-month alloxan diabetic rat aortae. To our knowledge, ours is the first study which demonstrates impaired endothelium-dependent relaxation by acetylcholine in long-term STZ diabetic animals. In a previous study from this laboratory (Tanz *et al.*, 1991), endothelium-dependent relaxation by histamine was also found to be impaired in the STZ diabetic rat aortae (52 weeks), thus suggesting nonspecific impairment of endothelium-dependent relaxation.

The increased vascular sensitivity to phenylephrine seen in long-term STZ diabetic rats (52 weeks) is not likely to be due to the toxic effect of STZ. There was no change in the phenylephrine sensitivity in the 12-week diabetic rat aortae. Daily insulin treatment corrected partially the increased phenylephrine sensitivity. In other words, alterations in the phenylephrine sensitivity were related to metabolic abnormalities.

The latter observations are consistent with findings of others (Pfaffman *et al.*, 1982; Head *et al.*, 1987; Tanz *et al.*, 1991) which demonstrated reversal of the abnormalities with insulin treatment regardless of whether vascular responses to noradrenaline or phenylephrine were decreased (Pfaffman *et al.*, 1982; Head *et al.*, 1987) or increased (Tanz *et al.*, 1991). While daily insulin treatment initiated as soon as diabetes developed partially prevented the development of the increased phenylephrine sensitivity as shown in the present study, it is not known if the increased phenylephrine sensitivity observed at 52 weeks would be reversed by insulin treatment initiated once the abnormality is established.

In conclusion, we found that vascular sensitivity to phenylephrine was increased in the long-term (52 weeks) but not in short-term STZ diabetic rat aortae. Enhanced sensitivity to phenylephrine is likely to be due to the diabetes-related endothelial damage or dysfunction since the removal of endothelium abolished the difference in the phenylephrine sensitivity between control and diabetic rat aortae. Reduction in the release of EDRF, but not prostacyclin is responsible for the enhanced sensitivity to phenylephrine since pretreatment with methylene blue, an inhibitor of EDRF, but not with indomethacin abolished the difference in the phenylephrine sensitivity. Impaired endothelium-dependent relaxation by acetylcholine in the aortae from the 52-week diabetic rat but not from 12-week diabetic aortae further support the concept that decrease in EDRF is responsible for the enhanced phenylephrine sensitivity. Insulin treatment partially corrected all the abnormalities observed in the diabetic rat aortae.

We thank Miriam Lacy for her technical assistance and Becki Stephenson for her secretarial assistance. This work was supported by the Veterans Administration and American Diabetes Association, Oregon Affiliate, Inc.

References

ABIRU, T., KAMATA, K., MIYATA, N. & KASUYA, Y. (1990). Differences in vascular responses to vasoactive agents of basilar artery and aorta from rabbits with alloxan-induced diabetes. *Can. J. Physiol. Pharmacol.*, **68**, 882–888.

AGRAWAL, D.K. & MCNEIL, J.H. (1987). Vascular responses to agonists in rat mesenteric artery from diabetic rats. *Can. J. Physiol. Pharmacol.*, **65**, 1484–1490.

ALOSACHIE, I. & GODFRAIND, T. (1988). The modulatory role of vascular endothelium in the interaction of agonists and antagonists with α-adrenoceptors in the rat aorta. *Br. J. Pharmacol.*, **95**, 619–629.

ARBOGAST, B.W., BERRY, D.L. & NEWELL, C.L. (1984). Injury of arterial endothelial cells in diabetic, sucrose-fed and aged rats. *Atherosclerosis*, **51**, 31–45.

CARRIER, G.O. & WHITE, R.E. (1985). Enhancement of alpha-1 and alpha-2 adrenergic agonist-induced vasoconstriction by removal of endothelium in rat aorta. *J. Pharmacol. Exp. Ther.*, **232**, 682–687.

CHANG, K.S.K. & STEVENS, W.C. (1988). Heart rate and blood pressure response to methacholine in long-term streptozotocin diabetic rats. *Proc. West. Pharmacol. Soc.*, **31**, 173–176.

CHRISTLIEB, A.R. (1973). Diabetes and hypertensive vascular disease. Mechanisms and treatment. *Am. J. Cardiol.*, **32**, 592–606.

COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305**, 627–630.

COLWELL, J.A., HALUSHKA, P.V., SARJI, K.E., LOPES-VIRELLA, M.F. & SAGEL, J. (1979). Vascular disease in diabetes: pathophysiological mechanisms and therapy. *Arch. Intern. Med.*, **139**, 225–230.

DOLGOV, V.V., ZAIKINA, O.E., BONDARENKO, M.F. & REPIN, V.S. (1982). Aortic endothelium of alloxan diabetic rabbits: a quantitative study using scanning electronmicroscopy. *Diabetologia*, **22**, 338–343.

DURANTE, W., SEN, A.K. & SUNAHARA, F.A. (1988). Impairment of endothelium-dependent relaxation in aortae from spontaneously diabetic rats. *Br. J. Pharmacol.*, **94**, 463–468.

EGLÈME, C., GODFRAIND, T & MILLER, R.C. (1984). Enhanced responsiveness of rat isolated aorta to clonidine after removal of endothelial cells. *Br. J. Pharmacol.*, **81**, 16–18.

FLEMING, W.W., WESTFALL, D.P., DE LA LANDE, I.S. & JELLETT, L.B. (1972). Log-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. *J. Pharmacol. Exp. Ther.*, **181**, 339–345.

FLOWER, R.J. (1974). Drugs which inhibit prostaglandin synthesis. *Pharmacol. Rev.*, **26**, 33–67.

FORTES, Z.B., LEME, J.G. & SCIVOLETTO, R. (1983). Vascular reactivity in diabetes mellitus: role of the endothelial cell. *Br. J. Pharmacol.*, **79**, 771–781.

FURCHGOTT, R.F. (1984). The role of endothelium in the responses of vascular smooth muscle to drugs. *Annu. Rev. Pharmacol. Toxicol.*, **24**, 175–197.

FURCHGOTT, R.F. (1988). Studies on the relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In *Vasodilation: Vascular Smooth Muscle, Peptides, Autonomic Nerves*. ed. Vanhoutte, P.M. pp. 401–404. New York: Raven Press.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GARG, U.C. & HASSID, A. (1989a). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.*, **83**, 1774–1777.

GARG, U.C. & HASSID, A. (1989b). Inhibition of rat mesangial cell mitogenesis by nitric oxide generating vasodilators. *Am. J. Physiol.*, **257**, F60–F66.

GEBREMEDHIN, D., KOLTAI, M.Z., POGÁSTA, G., MAGYAR, K. & HADHÁZY, P. (1987). Differential contractile responsiveness of femoral arteries from healthy and diabetic dogs: role of endothelium. *Arch. Int. Pharmacodyn.*, **288**, 100–108.

GEBREMEDHIN, D., KOLTAI, M.Z., POGÁSTA, G., MAGYAR, K. & HADHÁZY, P. (1989). Altered responsiveness of diabetic dog renal arteries to acetylcholine and phenylephrine: role of endothelium. *Pharmacology*, **38**, 177–184.

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J., NEWBY, A.C. & HENDERSON, A.H. (1984). The nature of endothelium-derived vascular relaxant factor. *Nature*, **308**, 645–647.

GRYGLEWSKI, R.J., BOTTING, R.M. & VANE, J.R. (1988). Mediators produced by the endothelial cell. *Hypertension*, **12**, 530–548.

HARRIS, K.H. & MACLEOD, K.M. (1988). Influence of the endothelium on contractile responses of arteries from diabetic rats. *Eur. J. Pharmacol.*, **153**, 55–64.

HARRISON, H.E., REECE, A.H. & JOHNSON, M. (1978). Decreased vascular prostacyclin in experimental diabetes. *Life Sci.*, **23**, 351–356.

HEAD, R.J., LONGHURST, P.A., PANEK, R.L. & STITZEL, R.E. (1987). A contrasting effect of the diabetic state upon the contractile responses of aortic preparations from the rat and rabbit. *Br. J. Pharmacol.*, **91**, 275–286.

JACKSON, C.V. & CARRIER, G.O. (1981). Supersensitivity of isolated mesentenic arteries to noradrenaline in the long-term experimental diabetic rat. *J. Auton. Pharmacol.*, **1**, 399–405.

JOHNSON, M., HARRISON, H.E., RAFTERY, A.T. & ELDER, J.B. (1979). Vascular prostacyclin may be reduced in diabetes in man (Letter). *Lancet*, **i**, 325–326.

MACLEOD, K.M. & MCNEIL, J.H. (1985). The influence of chronic experimental diabetes on contractile responses of rat isolated blood vessel. *Can. J. Physiol. Pharmacol.*, **63**, 52–57.

MALTA, E., SCHINI, V. & MILLER, R.C. (1986). Role of efficacy in the assessment of the actions of α-adrenoceptor agonists in rat aorta with endothelium. *J. Pharm. Pharmacol.*, **38**, 209–213.

MARTIN, W., FURCHGOTT, R.F., VILLANI, G.M. et al. (1986). Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.*, **237**, 529–538.

MERAJI, S., JAYAKODY, L., SENARATNE, M.P.J., THOMSON, A.B.R. & KAPPAGODA, T. (1987). Endothelium-dependent relaxation in aorta of BB rat. *Diabetes*, **36**, 978–981.

MILLER, R.C., MONY, M., SCHINI, V., SCHOEFFTER, P. & STOCLET, J.C. (1984). Endothelial mediated inhibition of contraction and increase in cyclic GMP levels evoked by the α-adrenoceptor agonist B-HT 920 in rat isolated aorta. *Br. J. Pharmacol.*, **83**, 903–908.

MONCADA, S., GRYGLEWSKI, R., BUNTING, S. & VANE, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263**, 663–665.

OYAMA, Y., KAWASAKI, H., HATTORI, Y. & KANNO, M. (1986). Attenuation of endothelium-dependent relaxation in aorta from diabetic rats. *Eur. J. Pharmacol.*, **131**, 75–78.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PFAFFMAN, M.A., BALL, C.R., DARBY, A. & HILMAN, R. (1982). Insulin reversal of diabetes-induced inhibition of vascular contractility in the rat. *Am. J. Physiol.*, **242**, H490–H495.

RERUP, C.C. (1970). Drugs producing diabetes through damage of the insulin secretory cells. *Pharmacol. Rev.*, **22**, 485–518.

ROTH, D.M., REIBEL, D.K. & LEFER, A.M. (1983). Vascular responsiveness and eicosanoid production in diabetic rats. *Diabetologia*, **24**, 372–376.

SCARBOROUGH, N.L. & CARRIER, G.O. (1984). Nifedipine and alpha adrenoceptors in rat aorta. II. Role of extracellular calcium in enhanced alpha-2 adrenoceptor mediated contraction in diabetes. *J. Pharmacol. Exp. Ther.*, **231**, 603–609.

STERIN-BORDA, L., FRANCHI, A.M., BORDA, E.S., DEL CASTILLO, E., GIMENO, M.F. & GIMENO, A.L. (1984). Augmented thromboxane generation by mesenteric arteries from pancreatectomized diabetic dogs is coincident with the vascular tone enhancement evoked by Na arachidonate and prostacyclin. *Eur. J. Pharmacol.*, **103**, 211–221.

TANZ, R.D., LI, X., WELLER, T.S. & CHANG, K.S.K. (1991). Pharmacological studies on the responsiveness of cardiovascular tissues from diabetic rats. In *The Diabetic Heart*. ed. Nagano, M. & Dhalla, N.S. pp. 71–86. New York: Raven Press.

VALENTOVIC, M.A. & LUBAWY, W.C. (1983). Impact of insulin or tolbutamide treatment on ^{14}C-arachidonic acid conversion to prostacyclin and/or thromboxane in lungs, aortas, and platelets of streptozotocin-induced diabetic rats. *Diabetics*, **32**, 846–851.

VANE, J.R., ÄNGGÅRD, E.E. & BOTTING, R.M. (1990). Regulatory functions of the vascular endothelium. *N. Engl. J. Med.*, **323**, 27–36.

WAKABAYASHI, I., HATAKE, K., KIMURA, N., KAKISHITA, E. & NAGAI, K. (1987). Modulation of vascular tonus by the endothelium in experimental diabetes. *Life Sci.*, **40**, 643–648.

WEKSLER, B.B., LEY, C.W. & JAFFE, E.A. (1978). Stimulation of endothelial cell prostacyclin production by thrombin, trypsin, and ionophore A 23187. *J. Clin. Invest.*, **62**, 923–930.

WHITE, R.E. & CARRIER, G.O. (1988). Enhanced vascular α-adrenergic neuroeffector system in diabetes: importance of calcium. *Am. J Physiol.*, **255**, H1036–H1042.

(Received March 9, 1992
Revised July 20, 1992
Accepted July 30, 1992)

Br. J. Pharmacol. (1992), **107**, 991–995

BK_1 and BK_2 bradykinin receptors in the rat duodenum smooth muscle

Teresa Feres, Antonio C.M. Paiva & [1]Therezinha B. Paiva

Department of Biophysics, Escola Paulista de Medicina, 04034 São Paulo, SP, Brazil

1 The dual action of bradykinin (relaxation and contraction) on the rat duodenum was investigated by studying its effect on adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in cultured duodenal smooth muscle cells, and the effects of apamin on the isolated muscle responses to agonists and antagonists of BK_1 and BK_2 receptors.

2 No change was observed in the cyclic AMP content of cultured cells incubated with up to 100 nM bradykinin.

3 Apamin (100–500 nM) inhibited the relaxant component and enhanced the contractile component of the responses to bradykinin and to the BK_2-specific analogue [$Thi^{5,8}$,D-Phe^7]-bradykinin.

4 Apamin (100–500 nM) did not affect the contractile response of stretched duodenum preparations to the BK_1-specific agonist des-Arg^9-bradykinin.

5 The BK_2 antagonist, [D-$Arg^0Hyp^3Thi^{5,8}$,D-Phe^7]-bradykinin, at a concentration which completely inhibited the relaxant response to bradykinin and to [$Thi^{5,8}$,D-Phe^7]-bradykinin, also prevented the contraction in response to either agonist in the presence of apamin.

6 Our results demonstrate two populations of bradykinin receptors in rat duodenum: a BK_2 subtype responsible for the biphasic response of the non-stretched duodenum, and a BK_1 subtype responsible for the contractile effect on the stretched tissue.

Keywords: Apamin; bradykinin; potassium channels; rat duodenum; receptors; smooth muscle

Introduction

Previous studies demonstrated that bradykinin produces hyperpolarization followed by depolarization in guinea-pig taenia caeci smooth muscle (Den Hertog *et al.*, 1988) and in neuroblastoma cultured cells (Brown & Higashida, 1988). The hyperpolarization is due to activation of a Ca^{2+}-dependent K^+ current by an increase in intracellular Ca^{2+}, mediated by the release of inositol 1,4,5-trisphosphate (Brown & Higashida, 1988; Lang *et al.*, 1991). The subsequent depolarization is associated with a fall in membrane conductance, primarily due to the inhibition of a voltage-dependent K^+ current (Brown & Higashida, 1988). As a consequence of these changes in membrane conductance, the response of isolated smooth muscles to bradykinin may have a biphasic character, consisting of a relaxation followed by contraction. However, the relaxant component of the response is observed only in smooth muscle preparations that maintain a naturally high tonus in the resting state, such as the guinea-pig taenia caeci and the rat duodenum (Carter *et al.*, 1986).

In the case of the rat duodenum, bradykinin has a predominantly relaxant effect but under conditions that decrease its resting tonus, such as low Ca^{2+} concentration in the medium or exposure to stretching, a distinct contractile component is also evident (Antonio, 1968; Boschcov *et al.*, 1984). Under these conditions, bradykinin concentrations ranging from 0.1 to 10 nM induce only relaxation, whereas higher concentrations induce relaxation followed by contraction. Two subtypes of bradykinin BK_1 and BK_2 receptors were proposed to mediate, respectively, the contractile and the relaxant components of the biphasic response (Boschcov *et al.*, 1984; Paiva *et al.*, 1989). The BK_2 receptor subtype was characterized by a distinct behaviour towards two bradykinin analogues that were shown to be BK_2 antagonists in other systems: [$Thi^{5,8}$,D-Phe^7]-bradykinin and [D-$Arg^0Hyp^3Thi^{5,8}$,D-Phe^7]-bradykinin (Stewart & Vavrek, 1986; Schachter *et al.*, 1987). In the rat duodenum the former is an agonist and the latter an antagonist for the relaxant effect. Furthermore, Hall & Morton (1991) showed competitive antagonism of relaxant responses to bradykinin with another BK_2 receptor antagonist, Lys, Lys-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin, with a pK_B estimate similar to that reported in other BK_2 receptor tissues. As for the BK_1 receptor subtype responsible for the contractile response of the duodenum, it was characterized (Paiva *et al.*, 1989) as being activated by both des-Arg^9-bradykinin (which is a BK_1 agonist in other systems) and des-Arg^9-[Leu^8]-bradykinin (which is a BK_1 antagonist in other systems) (Regoli & Barabe, 1980; Regoli *et al.*, 1986).

Two different mechanisms have been proposed for the relaxant response in the rat duodenum. The finding that apamin, a toxin that specifically blocks Ca^{2+}-dependent K^+ channels (Hugues *et al.*, 1982), inhibited both the relaxation and the opening of K^+ channels evoked by bradykinin in smooth muscle preparations indicates that the relaxation is caused by hyperpolarization due to activation of these channels (Hall & Morton, 1991). However, Liebmann *et al.* (1987) detected increased adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in duodenum strips treated with bradykinin and proposed that the relaxant response is due to stimulation of adenylyl cyclase activity.

The aim of this work was to investigate further the mechanism of the dual action of bradykinin (relaxation and contraction) on the rat duodenum by studying its effect on the cyclic AMP levels in cultured duodenal smooth muscle cells, and by examining the effects of apamin on the responses to agonists or antagonists of BK_1 and BK_2 receptors in the stretched and non-stretched rat duodenum.

Methods

Cyclic AMP determination

Duodenal smooth muscle cells were obtained from Wistar rats by enzymic dispersion and primary cultures were grown

[1] Author for correspondence.

as previously described (Shimuta et al., 1990). The culture medium of confluent cells (10^6 cells) was removed and the cells were rinsed at 37°C with assay buffer (composition, mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.49, NaH_2PO_4 0.36, $NaHCO_3$ 11.9 and glucose 5.0) and then 1 ml of the assay buffer was added in the presence or in the absence of 1.0 mM theophylline. After equilibration for 30 min, the cells were incubated for the desired times with various test reagents. The reaction was terminated by aspirating off the assay medium and the cells in culture dishes were immediately frozen in an acetone-dry ice bath. The cyclic AMP formed was extracted with 0.3% ice-cold perchloric acid and the extract was neutralized with 30% $KHCO_3$. The cyclic AMP in the extract was measured by radioimmunossay, as described by Gilman (1970) and Tovey et al. (1974).

Isolated duodenum preparation

Wistar rats of either sex, weighing between 190 and 220 g, were killed by a blow to the head. After bleeding, the abdomen was opened and the duodenum removed. The preparation was suspended in a 5 ml chamber containing Tyrode solution kept at 37°C and bubbled with a mixture of CO_2 (5%) and O_2 (95%). The composition of the Tyrode solution was (mM): NaCl 137, KCl 12.7, $CaCl_2$ 1.36, $MgCl_2$ 0.49, NaH_2PO_4 0.36, $NaHCO_3$ 11.9, glucose 5.0. Isotonic recordings were made, under 1 g load, on smoked drums with frontal levers having a 6 fold amplification, after a 60 min equilibration period.

The concentration-response curves to bradykinin were obtained within the first 90 min after the end of the equilibration period. The drugs, in volumes not exceeding 0.2 ml, were added directly to the organ bath and the preparation was washed after 90 s contact. The interval between additions was 15 min for the lower bradykinin concentrations (which produced only relaxation), and 30 min when higher concentrations were used to elicit contractile responses (to avoid the tachyphylaxis observed for this component of the response). The relaxant component of the response was measured from the baseline to the lowest point, and the contractile component from the baseline to the peak of the recorded response. The dose-response curves were analyzed by linear regression of their double reciprocal plots. Maximum responses and EC_{50} values were estimated from the ordinate intercept and slope of the straight lines obtained. For the experiments with des-Arg^9-bradykinin, the duodenum preparations were left under 1 g load for 4 h before the responses were recorded. This stretching of the preparation is important to enhance the contraction produced by that analogue (Boschcov et al., 1984).

Materials

The peptides used in this study were synthetic products made in this laboratory, with the exception of [Thi^{5,8},D-Phe^7]-bradykinin and [D-Arg^{0}Hyp^{3}Thi^{5,8}D-Phe^7]-bradykinin, which were kind gifts from Prof. J.M. Stewart and Dr R. Vavrek. The inorganic salts were products of the highest analytical grade from Merck Darmstadt. Apamin, phorbol-12,13-dibutyrate (PDBu) and isoprenaline were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The [^{3}H]-cyclic AMP assay kit was obtained from Diagnostic Products Corporation, Los Angeles, CA, U.S.A. Culture medium, supplements and foetal bovine serum were obtained from Gibco, N.Y., U.S.A.

All tracings presented in the figures are representative of at least 4 independent experiments and data are expressed as means ± standard errors and were analyzed by Student's t test.

Results

Cyclic AMP levels in cultured duodenal smooth muscle cells after bradykinin and isoprenaline stimulation

Liebmann et al. (1987), based on their finding of an increased cyclic AMP concentration in bradykinin-treated rat duodenum strips, proposed that this could be the mechanism of the relaxation produced by bradykinin in that tissue. However, these authors found that the bradykinin-evoked cyclic AMP production was increased in calcium-free medium, contrasting with the inhibition of the duodenum relaxation in this condition (Antonio, 1968). Since the results obtained with duodenum strips might be affected by the presence of the mucosa, we determined the effect of bradykinin on the cyclic AMP content of cultured duodenal smooth muscle cells.

Bradykinin, at a concentration which caused maximum relaxation in the rat isolated duodenum (10 nM), was added to the cultured cells, and the cyclic AMP content of the cells was determined after incubation times corresponding to the maximum amplitude of the relaxation observed in the tissue (30 s) and to the full return to the basal tonus (2 min). Figure 1a shows that no changes were observed in the cyclic AMP content with either incubation time. No changes in cyclic AMP content were also found when the cells were incubated for 2 min with a higher bradykinin concentration (100 nM, not shown), which in the isolated organ caused a biphasic response (relaxation followed by contraction).

As a control for the responsiveness of the cells to agents evoking cyclic AMP increases, we determined the effect of the β-adrenoceptor agonist, isoprenaline (2 μM), which caused a significant time-dependent increase in the level of cyclic AMP (Figure 1b). This increase was also observed in the presence of the phosphodiesterase inhibitor, theophylline (1 mM, not shown).

Since phorbol esters enhance agonist-induced cyclic AMP accumulation in other cells (Nabika et al., 1985), we determined the effect of bradykinin and isoprenaline on cyclic AMP levels of cells treated with 100 nM phorbol dibutyrate for 10 min. A significant increase in cyclic AMP accumu-

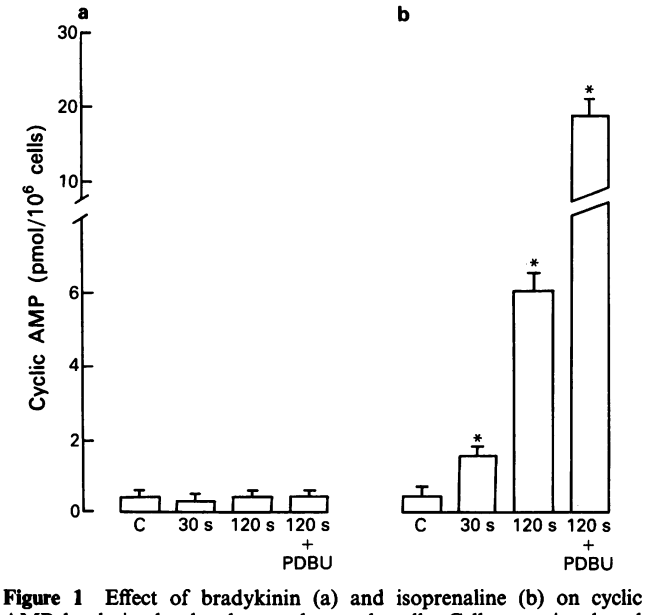

Figure 1 Effect of bradykinin (a) and isoprenaline (b) on cyclic AMP levels in duodenal smooth muscle cells. Cells were incubated with 10 nM bradykinin or 2 μM isoprenaline for 30 s or 120 s, in the presence or in the absence of 100 nM phorbol dibutyrate (PDBU) for 10 min. The values are mean (± s.e.mean, vertical bars) of four experiments done in triplicate. *Significantly different ($P<0.05$) from the respective controls.

lation was observed in the cultured cells incubated with isoprenaline, but not with bradykinin, either at 10 nM (Figure 1) or 100 nM (not shown).

Effect of apamin on the responses to bradykinin and analogue peptides

The relaxant effect of a low concentration of bradykinin (1 nM) on the rat duodenum was shown to be blocked by apamin (Hall & Morton, 1991). We have extended this observation to include higher bradykinin concentrations, in which the biphasic response becomes evident, in order to determine the effect of apamin on the contractile component of the response. In agreement with Hall & Morton's (1991) finding, we found that 100 nM apamin completely abolished the relaxation elicited by 1 nM bradykinin but at agonist concentrations of 10 nM or higher, the inhibition consisted mainly of a reduction of the amplitude and duration of the relaxation. This effect was dependent on the apamin concentration, being most pronounced at the highest concentration used, i.e. 500 nM (Figure 2). At this apamin concentration, the relaxation elicited by 100 nM bradykinin had its amplitude reduced from 31 ± 6 mm to 18 ± 3 mm and its duration reduced from 60 ± 4 s to 8 ± 1 s ($P<0.001$). The inhibition of the relaxation was accompanied by an increase of the concentration-dependent contractile component, the pD_2 for which was significantly greater (7.2 ± 0.05, $n = 8$) in the presence of 500 nM apamin than in its absence (6.6 ± 0.08, $n = 12$).

The response to the BK_1-specific agonist, des-Arg^9-bradykinin, which in the stretched rat duodenum causes only a contraction (Paiva *et al.*, 1989), was not affected by apamin in the concentration-rage, 100–500 nM (Figure 3). In contrast, the response to a maximally effective concentration (800 nM) of the BK_2-specific analogue, [Thi^{5,8},D-Phe^7]-bradykinin, which in the non-stretched rat duodenum produces only relaxation, was affected by apamin in a very similar way to that observed in the case of bradykinin (Figure 4): the relaxation was inhibited and a contractile component appeared which is absent in the normal response to that analogue.

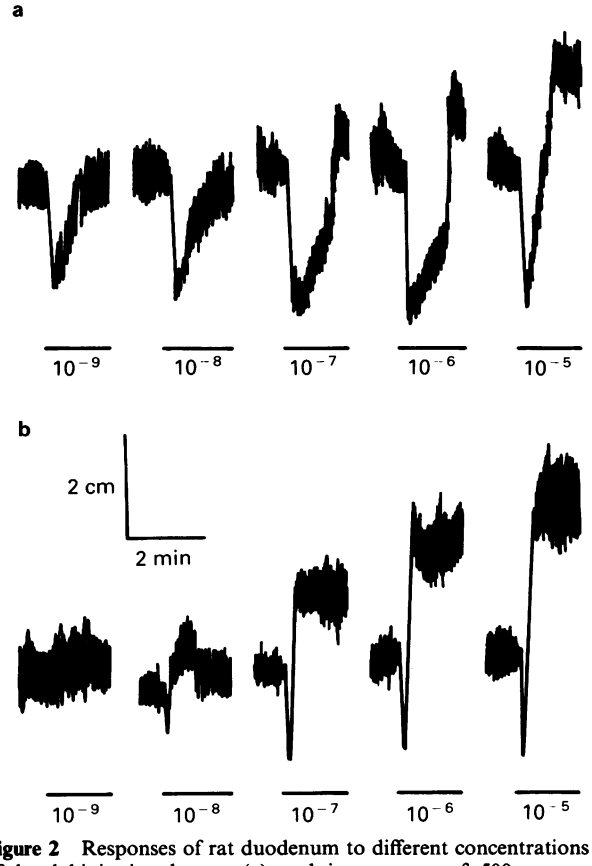

Figure 2 Responses of rat duodenum to different concentrations (M) of bradykinin in absence (a) and in presence of 500 nM apamin added 5 min before the addition of bradykinin (b). Each treatment with bradykinin lasted for 90 s (horizontal bars) and was followed by washing and a rest period (indicated by the interruptions in the tracings). The interval between bradykinin additions was 15 min for the concentrations 1 and 10 nM and 30 min for the higher doses. These results are representative of those obtained in 8 experiments.

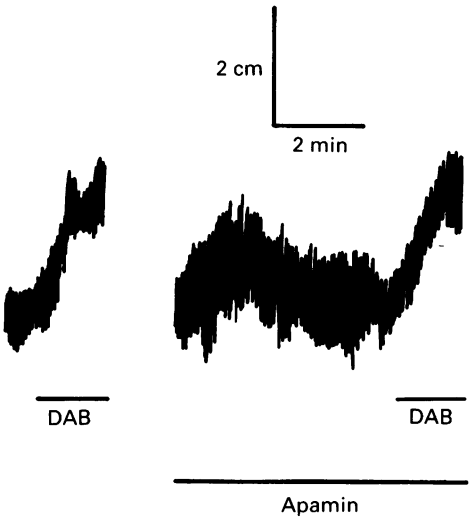

Figure 3 Responses to 20 nM des-Arg^9-bradykinin (DAB) in the absence and in the presence of 500 nM apamin, added 5 min before the addition of the agonist. The duodenum preparation was equilibrated for 4 h under 1 g load before the experiment. The tracing shown is representative of 4 experiments.

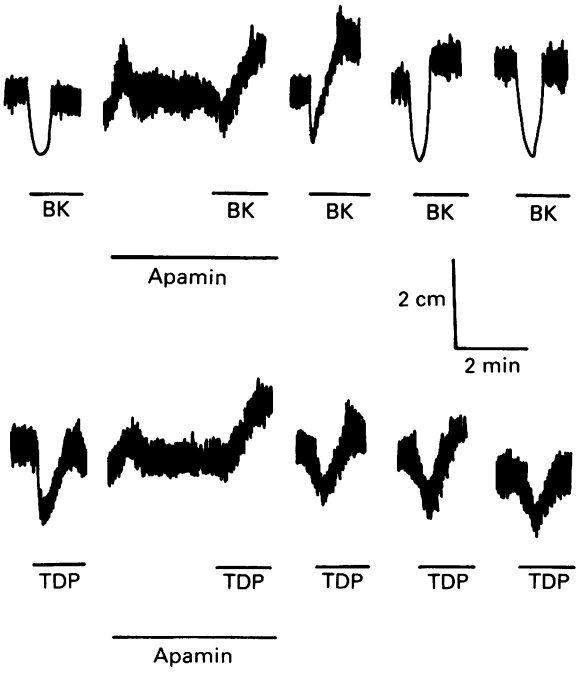

Figure 4 Effect of apamin (500 nM) on the relaxant responses (a) to 2 nM bradykinin (BK) and (b) to 800 nM BK_2 agonist [Thi^{5,8},D-Phe^7]-bradykinin (TDP). The interval between successive additions of the agonist was 30 min and the preparation was washed 90 s after each addition. Apamin was added (both panels) 3 min before the next addition of the agonists. These results are representative of 6 experiments.

After removal of apamin, the normal responses to new additions of bradykinin and to [Thi[5,8],D-Phe[7]]-bradykinin slowly recovered, the amplitude and duration of the relaxant component gradually increasing, and the contractile component disappearing after approx. 90 min (Figure 4).

In order to determine whether the enhancement of the contractile responses by apamin is a consequence of the inhibition of the relaxant component of the response, we studied the effect of apamin when the responses to bradykinin and [Thi[5,8],D-Phe[7]]-bradykinin were inhibited by an antangonist of the BK_2 receptor. It was previously shown that the specific BK_2 antagonist, [D-Arg[0]Hyp[3]Thi[5,8],D-Phe[7]]-bradykinin, inhibits the relaxant but not the contractile component of the response of the duodenum to bradykinin (Pereira & Paiva, 1989). Figure 5 shows that this antagonist, at a concentration (150 nM) which completely inhibited the relaxant response to bradykinin and to [Thi[5,8],D-Phe[7]]-bradykinin, also prevented the contraction in response to either agonist in the presence of apamin. After washout of the preparation, new additions of either of the two agonists yielded responses consisting of a contractile component that gradually disappeared during the 1 h recovery period. This indicates that, whereas [Thi[5,8],D-Phe[7]]-bradykinin is quickly washed out, the effect of apamin is only slowly reversed, as also seen in the experiment depicted in Figure 4.

Discussion

Since the classification of bradykinin receptors into BK_1 and BK_2 types (Regoli & Barabé, 1980), the great majority of the responses to that peptide have been attributed to BK_2 receptors, BK_1 receptors having been found in few tissues and under special conditions. An interesting system is the rat duodenum, in which the two components of the biphasic response were attributed to subtypes of the BK_1 and BK_2 receptors (Paiva *et al.*, 1989).

The relaxant component of the response of the duodenum to bradykinin has been ascribed to two different mechanisms. Whereas Liebmann *et al.* (1987) postulate the mediation by adenylyl cyclase stimulation, based on their finding of increased cyclic AMP in duodenum strips treated with bradykinin, Hall & Morton (1991) interpret the inhibition of the relaxation by apamin as evidence that it is due to activation of Ca^{2+}-dependent K^+ channels, as also postulated for the taenia caeci (Carter *et al.*, 1986; Den Hertog *et al.*, 1988).

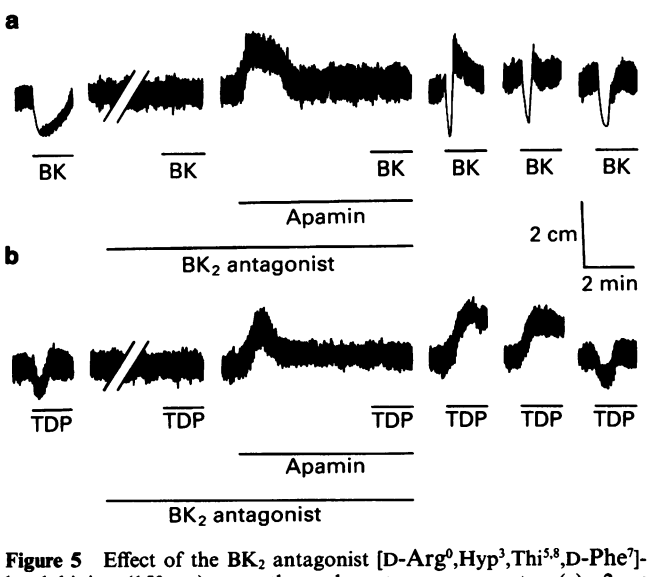

Figure 5 Effect of the BK_2 antagonist [D-Arg[0],Hyp[3],Thi[5,8],D-Phe[7]]-bradykinin (150 nM) on the relaxant responses to (a) 2 nM bradykinin (BK), and (b) 800 nM [Thi[5,8],D-Phe[7]]-bradykinin (TDP) in the absence and in the presence of 500 nM apamin. The BK_2 antagonist and the apamin were added 10 and 5 min respectively before the next addition of the agonists. The intervals between additions were 30 min, and the preparation was washed 90 s after each addition. These results are representative of 6 experiments.

In duodenal cultured smooth muscle cells, we did not detect cyclic AMP accumulation in response to bradykinin concentrations above those known to cause maximum relaxant responses of the duodenum. An increase in cyclic AMP levels was not observed even in the presence of phorbol ester, under conditions which enhance agonist-induced cyclic AMP accumulation in other tissues, including vascular smooth muscle cells (Nabika *et al.*, 1985). The difference between our results and those of Liebmann *et al.* (1987) may be due to the fact that we used only smooth muscle cells, whereas these authors used whole duodenum strips, where cells other than smooth muscle are present. The mucosa was shown to accumulate cyclic AMP under other stimuli (Karlstrom, 1986), and the presence of these cells might influence the results obtained with the strips.

Our results are in agreement with those of Hall & Morton (1991), who found that the relaxant effect induced by 1 nM bradykinin in the rat isolated duodenum was totally inhibited by apamin. In addition, we further observed that, in the presence of higher bradykinin concentrations (but within a range where only relaxant responses are normally observed), apamin significantly modified these responses: the relaxant component was present, but with smaller amplitude and very short duration, and a dose-dependent contraction became evident, with a pD_2 value (7.2 ± 0.05) significantly higher than that seen in the absence of apamin (6.6 ± 0.05).

These results support the conclusion that the relaxant response of the duodenum to bradykinin is due to the activation of Ca^{2+}-dependent K^+ channels and they further indicate that the biphasic response reflects a balance between hyperpolarization (relaxation) and depolarization (contraction). The appearance of the contractile component at low bradykinin concentrations in the presence of apamin would, therefore, result from the inhibition of hyperpolarization, allowing the predominance of the contractile effect due to membrane depolarization. A similar balance between the two components of the biphasic response of the duodenum was described in spontaneously hypertensive rats (Miasiro *et al.*, 1985), in which the predominantly contractile response was ascribed to a reduced relaxant component, probably because low calmodulin levels impair Ca^{2+}-dependent K^+ channels in these animals (Feres *et al.*, 1992).

The finding that the contractile component of the response may be present at low bradykinin concentrations, being masked by the relaxant component, and that this also occurs with [Thi[5,8],D-Phe[7]]-bradykinin, previously thought to be a pure BK_2 relaxant agonist in the rat duodenum (Paiva *et al.*, 1989), suggests a revision of the proposal that different receptor subtypes are responsible for the two components of the response of the non-stretched duodenum. This is also suggested by the finding that BK_2 antagonist [D-Arg[0],Hyp[3],Thi[5,8],D-Phe[7]]-bradykinin, which was previously shown to inhibit the relaxation, also inhibits the contractions induced by bradykinin and [Thi[5,8],D-Phe[7]]-bradykinin in the presence of apamin. It is possible that stimulation of the same population of BK_2 receptors by bradykinin could activate the phosphoinositide pathway, as has been shown in other tissues, such as the guinea-pig ileum (Ransom *et al.*, 1992). This could give rise to a dual effect, as proposed for other systems (Brown & Higashida, 1988): mobilization of calcium ions through inositol trisphosphate could produce hyperpolarization through Ca^{2+}-dependent K^+ channels, and diacylglycerol could cause depolarization through inhibition of voltage-dependent K^+ channels.

Our finding that the contractile response of the stretched duodenum to des-Arg[9]-bradykinin is not affected by apamin is in line with previous results showing that this response is due to activation of a subtype of BK_1 receptor that is distinct from the BK_2 subtype involved in the biphasic response of the non-stretched tissue.

In conclusion, our results, indicate that the rat duodenum

possesses two distinct populations of bradykinin receptors: (1) a BK_2 subtype, which is activated by [Thi^{5,8},D-Phe^7]-bradykinin and inhibited by [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, and is responsible for the dual effect resulting in the biphasic response of the non-stretched duodenum; and (2) a BK_1 subtype, activated by both des-Arg^9-bradykinin and des-Arg^9-[Leu^8]-bradykinin, which is responsible for the contractile response of the stretched duodenum.

This work was supported by grants from the Brazilian National Research Council (CNPq) and the São Paulo State Research Foundation, and had the technical assistance of Nelson A. Mora.

References

ANTONIO, A. (1968). The relaxing effect of bradykinin on intestinal smooth muscle. *Br. J. Pharmacol. Chemother.* **32,** 78–86.

BOSCHCOV, P., PAIVA, A.C.M., PAIVA, T.B. & SHIMUTA, S.I. (1984). Further evidence for the existence of two receptor sites for bradykinin responsible for the diphasic effect in the rat isolated duodenum. *Br. J. Pharmacol.*, **83,** 591–600.

BROWN, D.A. & HIGASHIDA, H. (1988). Inositol 1,4,5-trisphosphate and diacylglycerol mimic bradykinin effects on mouse neuroblastoma × rat glioma hybrid cells. *J. Physiol.*, **397,** 185–207.

CARTER, T.D., HALL, J.M., McCABE, D.V., MORTON, I.K.M. & SCHACHTER, M. (1986). Biphasic actions of bradykinin in the guinea-pig taenia caeci preparation. *Br. J. Pharmacol.*, **90,** 137P.

DEN HERTOG, A., NELEMANS, A. & AKKER, J.V. (1988). The multiple action of bradykinin on smooth muscle of guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **151,** 357–363.

FERES, T., VIANNA, L.M., PAIVA, A.C.M. & PAIVA, T.B. (1992). Effect of treatment with vitamin D_3 on the responses of the duodenum of spontaneously hypertensive rats to bradykinin and to potassium. *Br. J. Pharmacol.*, **105,** 881–884.

GILMAN, A.G. (1970). A protein binding assay for adenosine 3'5' cyclic monophosphate. *Proc. Natl. Acad. Sci. U.S.A.*, **67,** 305–312.

HALL, J.M. & MORTON, I.K.M. (1991). Bradykinin B_2 receptor evoked K^+ permeability increase mediates relaxation in the rat duodenum. *Eur. J. Pharmacol.*, **193,** 231–238.

HUGUES, M., ROMEY, G., DUVAL, D., VINCENT, J.P. & LAZDUNSKI, M. (1982). Apamin as a selective blocker of the calcium-dependent potassium channel in neuroblastoma cells: Voltage-clamp and biochemical characterization of the toxin receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **79,** 1308–1312.

KARLSTROM, L. (1986). Mechanisms in bile-salt induced secretion in the small intestine. *Acta Physiol. Scand.*, **126,** Suppl. 549, 1–48.

LANG, F., PAULMICHL, M., PFEILSCHIFFER, J., FRIEDRICH, F., WÖLL, E., WALDEGGERS, S., RITTER, M. & TSCHERNKO, E. (1991). Cellular mechanisms of bradykinin-induced hyperpolarization in renal epitheloid MDCK-cells. *Biochim. Biophys. Acta.*, **1073,** 600–608.

LIEBMANN, C., REISSMANN, S., ROBBERCHT, P. & AROLD, H. (1987). Bradykinin action in the rat duodenum: Receptor binding and influence on the cyclic AMP system. *Biomed. Biochim. Acta.*, **46,** 469–478.

MIASIRO, N., PAIVA, T.B., PEREIRA, C.C. & SHIMUTA, S.I. (1985). Reactivity to bradykinin and potassium of the isolated duodenum from rats with genetic and renal hypertension. *Br. J. Pharmacol.*, **85,** 639–646.

NABIKA, T., NARA, Y., YAMORI, Y., LOVENBERG, W. & ENDO, J. (1985). Angiotensin II and phorbol ester enhance isoproterenol- and vasoactive intestinal peptide (VIP)- induced cyclic AMP accumulation in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **131,** 30–36.

PAIVA, A.C.M., PAIVA, T.B., PEREIRA, C.C. & SHIMUTA, S.I. (1989). Selectivity of bradykinin analogues for receptors mediating contraction and relaxation of the rat duodenum. *Br. J. Pharmacol.*, **98,** 206–210.

PEREIRA, C.C. & PAIVA, T.B. (1989). Characterization of the receptors responsible for the diphasic effect of bradykinin in the rat duodenum. *Braz. J. Med. Biol. Res.*, **22,** 1137–1140.

RANSOM, R.W., GOODMAN, C.B. & YOUNG, G.S. (1992). Bradykinin stimulation of phosphoinositide hydrolysis in guinea-pig ileum longitudinal muscle. *Br. J. Pharmacol.*, **105,** 919–924.

REGOLI, D. & BARABE, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32,** 1–46.

REGOLI, D., DRAPEAU, G., ROVERO, P., DION, S., D'ORLEANS-JUSTE & BARABE, J. (1986). The actions of kinin antagonists on B_1 and B_2 receptor systems. *Eur. J. Pharmacol.*, **123,** 61–65.

SCHACHTER, M., USHIDA, Y., LONGRIDGE, D.J., LABEDZ, T., WHALLEY, E.T., VAVREK, R.J. & STEWART, J.M. (1987). New synthetic antagonists of bradykinin. *Br. J. Pharmacol.*, **92,** 851–855.

SHIMUTA, S.I., KANASHIRO, C.A., OSHIRO, M.E.M., PAIVA, T.B. & PAIVA, A.C.M. (1990). Angiotensin II desensitization and Ca^{++} and Na^+ fluxes in cultured intestinal smooth muscle cells. *J. Pharmacol. Exp. Ther.*, **253,** 1215–1221.

STEWART, J.M. & VAVREK, R.J. (1986). Bradykinin competitive antagonists for classical kinin systems. *Adv. Exp. Med. Biol.*, **198a,** 537–542.

TOVEY, K.C., OLDHAM, K.G. & WHELAN, J.A.M. (1974). A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. *Clin. Chim. Acta,* **56,** 221–234.

(Received January 23, 1992
Revised July 26, 1992
Accepted July 30, 1992)

Br. J. Pharmacol. (1992), **107**, 996–1000

Phosphoramidon blocks big-endothelin-1 but not endothelin-1 enhancement of vascular permeability in the rat

Stéphanie Lehoux, [1]Gérard E. Plante, Martin G. Sirois, Pierre Sirois & Pedro D'Orléans-Juste

Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, J1H 5N4, Canada

1 Changes in vascular permeability following intravenous injections of human big-endothelin-1 (big-ET-1) and endothelin-1 (ET-1) were measured by extravasation of Evans blue dye (EB, 20 mg kg^{-1}) in selected tissues.

2 A low dose of big-ET-1 (40 pmol kg^{-1}) failed to alter vascular permeability but a dose of 400 pmol kg^{-1} increased EB extravasation in the trachea, upper and lower bronchi, and lung parenchyma by 55 to 69% ($P<0.05$). Vascular permeability was also enhanced in the liver, spleen, kidney, heart, and diaphragm by 20, 14, 41, 25, and 67%, respectively ($P<0.05$).

3 Upon injection of ET-1 (400 pmol kg^{-1}), EB extravasation increased in the upper and lower bronchi, lung parenchyma, liver, pancreas, kidney, heart, and diaphragm.

4 Administration of ET-1 and big-ET-1 was not associated with significant systemic responses.

5 Pretreatment with phosphoramidon (PA) blocked the response to big-ET-1 in all tissues examined but this inhibitor failed to alter the response to ET-1.

6 We conclude from these results that the dose-dependent increase in vascular permeability induced by big-ET-1 in various tissues follows its conversion to ET-1 by the endothelin converting enzyme, a PA-sensitive process.

Keywords: Big-endothelin-1; endothelin-1; phosphoramidon; vascular permeability

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide which evokes long lasting pressor responses when injected intravenously (Yanagisawa *et al.*, 1988). These responses are dose-dependent (Brain *et al.*, 1988; Highsmith *et al.*, 1989) and have been observed in a spectrum of target organs including the heart, liver, spleen, kidney, pancreas, skeletal muscle, duodenum, and diaphragm (Clozel & Clozel, 1989). ET-1 acts through specific receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990) and may lead to the activation of calcium channels (Loutzenhiser *et al.*, 1990; Yanagisawa *et al.*, 1988). ET-1 also triggers the release of prostaglandins and thromboxanes (Antunes *et al.*, 1988; de Nucci *et al.*, 1988; Reynolds & Mok, 1989; Walder *et al.*, 1989).

ET-1 is formed through the conversion of its precursor big-endothelin (big-ET-1) via a metalloprotease named endothelin converting enzyme (ECE) (Yanagisawa *et al.*, 1988). Big-ET-1 and ET-1 elicit constriction of aortic rings yet the precursor (big-ET-1) is 100 times less potent than ET-1 (Kashiwabara *et al.*, 1989; McMahon *et al.*, 1991). However, both ET-1 and its precursor increase blood pressure equally in the anaesthetized rat and guinea-pig (Fukuroda *et al.*, 1990), and in the anaesthetized rabbit (D'Orléans-Juste *et al.*, 1990). In addition, big-ET-1 releases prostanoids and thromboxanes in the guinea-pig (D'Orléans-Juste *et al.*, 1991a). *In vivo* effects of the two peptides differ in the early stage of the blood pressure response, where ET-1 causes first a transient hypotension, which is not seen after administration of big-ET-1, before inducing a sustained hypertension. This is probably due to a gradual conversion of the precursor to the metabolically active peptide (McMahon *et al.*, 1991), which also explains a delayed release of prostaglandins after injection of big-ET-1 (D'Orléans-Juste *et al.*, 1991b).

Phosphoramidon (PA) inhibits ECE both *in vivo* (Fukuroda *et al.*, 1990; Matsumura *et al.*, 1990) and *in vitro* (McMahon *et al.*, 1991), blocking the effects of big-ET-1 but not ET-1. Studies show that PA inhibits big-ET-1 induced hypertension (Matsumura *et al.*, 1990; Hisaki *et al.*, 1991; McMahon *et al.*, 1991), aortic ring contractions (Fukuroda *et al.*, 1990; McMahon *et al.*, 1991), increased airway resistance (Fukuroda *et al.*, 1990), and eicosanoid release (D'Orléans-Juste *et al.*, 1990; 1991a). The results agree with the finding that there is a signficant increase in immunoreactive ET-1 following big-ET-1 injection in the rabbit (D'Orléans-Juste *et al.*, 1990), and generation of ET-1 correlates with the pressor effects of the precursor (Hisaki *et al.*, 1991).

Increased vascular permeability to dye-bound albumin in different tissues has recently been demonstrated in response to ET-3 (Valentin *et al.*, 1991) and ET-1 (Filep *et al.*, 1991; Sirois *et al.*, 1992). The present study was undertaken to verify the effects of human big-ET-1 on albumin extravasation across selected capillary beds in the rat, with or without PA pretreatment, and to compare these effects to those obtained with an equal dose of ET-1.

To verify that the results observed were due to the permeabilizing effects of ET-1 and big-ET-1, and not to other cardiovascular responses, we tested for changes in peripheral arterial pressure and haematocrit. Renal haemodynamics were also assayed to evaluate further possible systemic changes. Finally, we assessed vascular permeability responses to histamine dihydrochloride for complete validation of our experimental model.

Methods

Differences in vascular permeability to serum albumin were measured by extravasation of Evans blue dye (EB) by a modification of the method described by Jancar *et al.* (1988). Briefly, male Wistar rats (250–300 g) received a bolus injection of EB (20 mg kg^{-1}) in the caudal vein 10 min before being killed. Big-ET-1 and ET-1 were injected with the dye, whereas PA (2 mg kg^{-1}) was administered 5 min earlier. Two doses of big-ET-1 (40 and 400 pmol kg^{-1}) were tested, and because of its greater effects on the target organs selected, the higher dose was used in combination with PA and chosen for

[1] Author for correspondence.

comparative studies with ET-1. In an additional group of experiments (6 rats), histamine dihydrochloride (10 $\mu g\ kg^{-1}$) was injected in the caudal vein with EB. Experimental groups in which the effects of ET-1 and big-ET-1 were examined were as follows:

		Treatment		
Group	*n*	−5	0	10 min
1	5		EB	Killed
2	5		EB + big-ET-1 (40)[a]	Killed
3	6		EB + big-ET-1 (400)	Killed
4	5	PA[b]	EB + big-ET-1 (400)	Killed
5	5		EB + ET-1 (400)	Killed
6	5	PA	EB + ET-1 (400)	Killed
7	5	PA	EB	Killed

[a]Numbers in parentheses indicate doses administered (pmol kg^{-1}).
[b]PA was given in a single dose of 2 mg kg^{-1}.

The rats were killed and exsanguinated 10 min after the EB injection. The thorax was cut open and the lungs perfused with 15 ml of Krebs solution (10 ml min^{-1}) via a cannula inserted in the pulmonary artery to remove intravascular pulmonary dye. Specimens from the trachea, upper and lower bronchi, lung parenchyma, liver, spleen, pancreas, kidney, heart, and diaphragm were removed and weighed. Half of each tissue was dried at 60°C for 24 h to avoid underevaluation of changes due to oedema formation. The other half was placed in formamide (4 ml g^{-1} wet tissue) for dye extraction. The concentration of EB in the formamide was determined by spectrophotometry at 620 nm with a Titertek Multiskan (Flow Lab.) and 96 well microplates.

The results, calculated from a standard curve of EB (0.5–25 $\mu g\ ml^{-1}$), are expressed as EB $\mu g\ g^{-1}$ dry weight of tissue. Data are reported as mean ± s.e.mean, and analysis of variance was the statistical method used. Values of $P<0.05$ were considered as significant.

In a second set of experiments, 12 male Wistar rats (200–250 g) were anaesthetized with pentobarbitone (50 mg kg^{-1}, i.p.). The animals were tracheotomized and placed on a heated (37°C) table. The left jugular and femoral veins were catheterized for drug administration and continuous infusions, respectively. The left femoral artery was also catheterized for pressure monitoring and blood sampling. Isotonic sodium chloride, containing tritiated inulin (4 $\mu Ci\ ml^{-1}$), was administered throughout the experiment to measure glomerular filtration rate. After surgery, 60 min was allowed for equilibration. Animals were then injected with equal acute doses (400 pmol kg^{-1}) of big-ET-1 (6 rats) or ET-1 (6 rats). Blood pressure changes (detected with a Statham pressure transducer, model P-23A) were monitored and blood samples (15 µl) taken for haematocrit measurements before, and 10, 20, and 60 s after drug treatment, whereas inulin was measured before and 20 min after treatment. Manipulation of specimens and analytical methods have been described in previous papers (Jobin *et al.*, 1977; Plante *et al.*, 1981).

Human big-ET-1 and ET-1 were purchased from Hukabel (Longueuil, Canada), phosphoramidon was obtained from Peninsula Laboratories (Belmont, U.S.A.), Evans blue dye and histamine dihydrochloride were purchased from Sigma (St. Louis, U.S.A.), pentobarbitone came from Abbott (Toronto, Canada), and inulin was obtained from New England Nuclear (Dupont; Boston, U.S.A.).

Results

The first group of rats, injected with Evans blue dye only, served as controls. Subsequent observations were made for EB combined with big-ET-1 and ET-1, in the presence or absence of PA. Results in the figures represent the mean EB extravasation for each experimental group in each tissue. Values obtained after administration of PA alone (group seven) were not different from baseline in any tissues (data not shown).

In the second and third series of experiments, two doses of big-ET-1 (40 and 400 pmol kg^{-1}) were tested to evaluate vascular permeability of selected organs. The low dose of big-ET-1 failed to alter the tissue content of albumin, whereas the higher dose increased extravasation significantly from control in the trachea (195 ± 16 vs 126 ± 22 EB $\mu g\ g^{-1}$ dry tissue weight, $P<0.05$), upper (94 ± 13 vs 56 ± 5, $P<0.05$) and lower bronchi (84 ± 4 vs 54 ± 2, $P<0.01$), lung parenchyma (103 ± 7 vs 61 ± 9, $P<0.01$), liver (118 ± 3 vs 99 ± 3, $P<0.05$), kidney (175 ± 5 vs 123 ± 10, $P<0.01$), heart (147 ± 6 vs 118 ± 6, $P<0.01$), and diaphragm (103 ± 5 vs 61 ± 5, $P<0.01$) as shown in Figures 1, 2, and 3.

In experimental group 4, the high dose of big-ET-1 (400 pmol kg^{-1}) was injected 5 min after a PA pretreatment (2 mg kg^{-1}). This latter compound blocked the increased vascular permeability response observed with big-ET-1 in all tissues, bringing extravasation values back to baseline. Inhibition of ECE therefore abolished the rise in vascular permeability induced by big-ET-1 in the tissues examined.

The results obtained for group 5 (ET-1, 400 pmol kg^{-1}) were similar to those obtained for the same dose of the precursor big-ET-1. The rise in vascular permeability obtained with ET-1 was not statistically different from that obtained with big-ET-1 in the trachea, upper bronchi, lung parenchyma, liver, spleen, pancreas, and diaphragm, whereas a statistically significant difference ($P<0.05$) in permeability between equal doses of big-ET-1 and ET-1 was noted in the lower bronchi, kidney, and heart. Of these three organs, the effect of big-ET-1 was more pronounced than that of ET-1 in the kidney only, while the effect of ET-1 was greater than that of big-ET-1 in the other two tissues.

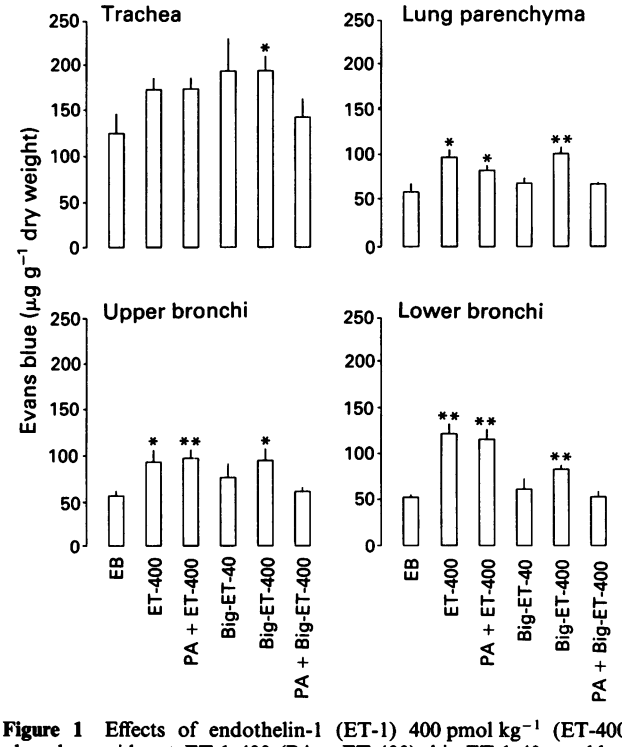

Figure 1 Effects of endothelin-1 (ET-1) 400 pmol kg^{-1} (ET-400), phosphoramidon + ET-1 400 (PA + ET-400), big-ET-1 40 pmol kg^{-1} (Big-ET-40), big-ET-1 400 pmol kg^{-1} (Big-ET-400), and phosphoramidon + big-ET-1 400 pmol kg^{-1} (PA + big-ET-400), on the vascular permeability of the trachea, upper and lower bronchi, and lung parenchyma. EB designates control values. Data represent mean ± s.e.mean (vertical lines) of 5–6 experiments. $*P<0.05$ and $**P<0.01$ compared to control values only.

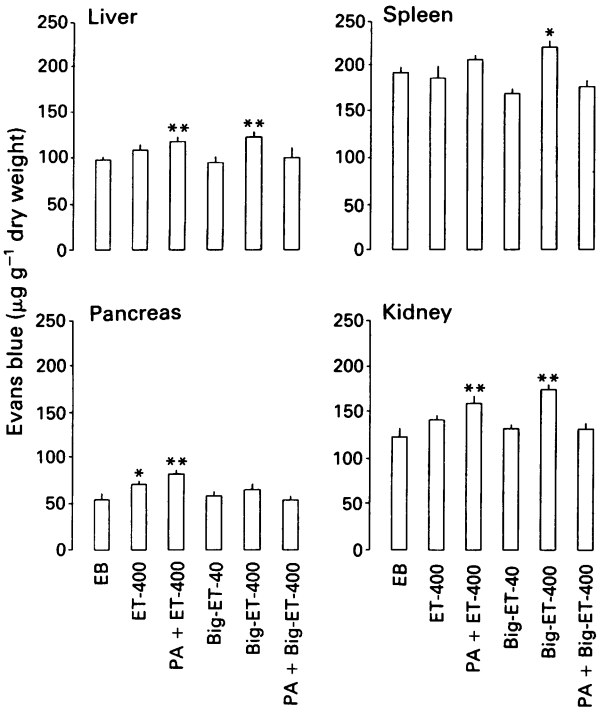

Figure 2 Effects of endothelin-1 (ET-1) 400 pmol kg^{-1} (ET-400), phosphoramidon + ET-1 400 (PA + ET-400), big-ET-1 40 pmol kg^{-1} (Big-ET-40), big-ET-1 400 pmol kg^{-1} (Big-ET-400), and phosphoramidon + big-ET-1 400 pmol kg^{-1} (PA + big-ET-400), on the vascular permeability of the liver, spleen, pancreas, and kidney. EB designates control values. Data represent mean ± s.e.mean (vertical lines) of 5–6 experiments. $*P<0.05$ and $**P<0.01$ compared to control values only.

Finally, in the sixth experimental group, ET-1 (400 pmol kg^{-1}) was injected after PA pretreatment. EB extravasation was significantly increased from control in the upper (97 ± 9 vs 56 ± 5 EB μg g^{-1} dry tissue weight) and lower bronchi (117 ± 11 vs 54 ± 2), lung parenchyma (84 ± 5 vs 61 ± 9), liver (119 ± 5 vs 99 ± 3), pancreas (83 ± 4 vs 55 ± 7), kidney (160 ± 8 vs 123 ± 10), heart (210 ± 12 vs 118 ± 6), and diaphragm (104 ± 13 vs 61 ± 5), shown in Figures 1, 2, and 3. Hence, inhibition of ECE with PA failed to alter vascular permeability induced by ET-1 in the tissues examined.

Further studies were performed to evaluate haemodynamics in big-ET-1- and ET-1-treated rats. ET-1 (400 pmol kg^{-1}) induced a transient reduction (from 169 ± 3 to 158 ± 6 mmHg; 10 s), then a rise (to 179 ± 5 mmHg; 20 s) in peripheral blood pressure. Within 60 s, however, blood pressure stabilized at control values (167 ± 2 mmHg). Peripheral arterial haematocrit averaged 46.5 ± 0.3, 47.0 ± 0.2, 47.0 ± 0.3, and 47.5 ± 0.3%, before, and 10, 20, and 60 s after ET-1 administration. Finally, inulin clearance studies indicated that glomerular filtration rate remained unchanged in the 20 min that followed ET-1 treatment (1.41 ± 0.11 to 1.52 ± 0.15 ml min^{-1}). Big-ET-1 (400 pmol kg^{-1}) failed to alter any of these peripheral and renal parameters (data not shown).

Experiments were done with histamine dihydrochloride (10 μg kg^{-1}) to validate our vascular permeability model. Histamine increased vascular permeability in the upper bronchi, liver and kidney, by 101, 25 and 40% respectively.

Discussion

The Evans blue dye method has been widely used by different groups (Dahlén *et al.*, 1981; Evans *et al.*, 1987) and further validated in our laboratory (Sirois *et al.*, 1988). The dye

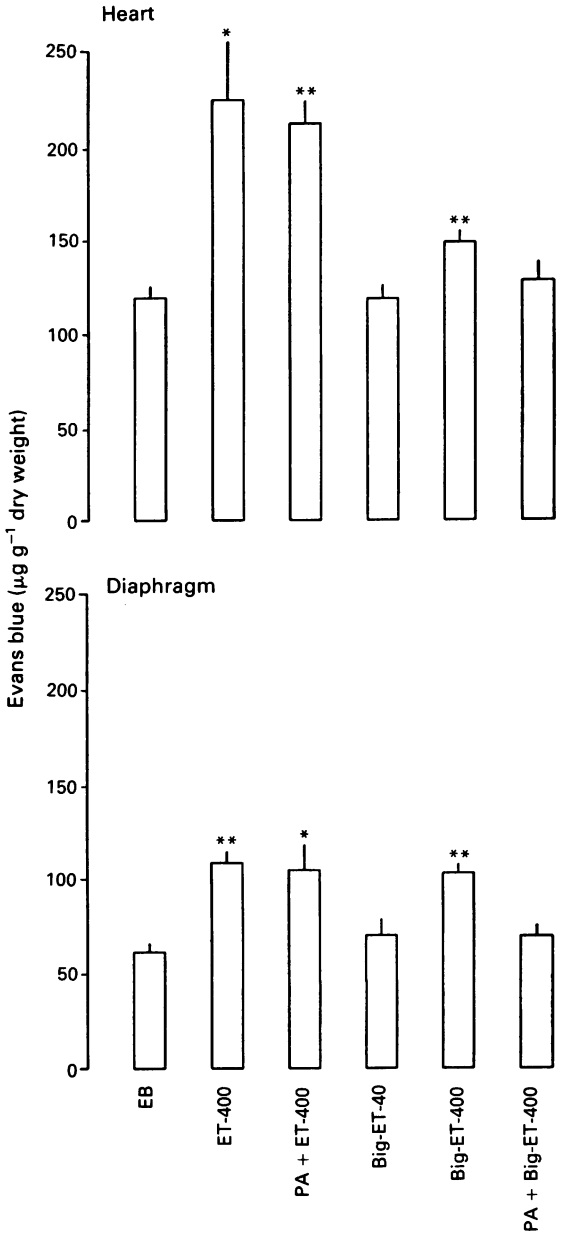

Figure 3 Effects of endothelin-1 (ET-1) 400 pmol kg^{-1} (ET-400), phosphoramidon + ET-1 400 (PA + ET-400), big-ET-1 40 pmol kg^{-1} (Big-ET-40), big-ET-1 400 pmol kg^{-1} (Big-ET-400), and phosphoramidon + big-ET-1 400 pmol kg^{-1} (PA + big-ET-400), on the vascular permeability of the heart and diaphragm. EB designates control values. Data represent mean ± s.e.mean (vertical lines) of 5–6 experiments. $*P<0.05$ and $**P<0.01$ compared to control values only.

which binds to plasma albumin (Rawson, 1943) is an indicator of its extravasation from the vascular to the interstitial compartment (LeVeen & Fishman, 1947; Rogers *et al.*, 1989).

To evaluate relationships between increased vascular permeability and possible systemic effects of the drugs, we studied peripheral pressure and haematocrit profiles after administration of big-ET-1 and ET-1. Changes in pressure were minimal and returned to baseline within 60 s of ET-1 injection, while a uniform haematocrit indicated constant vascular volume. Renal haemodynamics also remained unchanged. Likewise, big-ET-1 had little impact on peripheral or renal parameters. Since the drugs did not produce significant systemic effects at the doses used, they probably acted directly on the vessels or induced the release of

inflammatory mediators which could modify vascular permeability.

In an experiment carried out to validate further the vascular permeability model, histamine, a mediator known to increase vascular permeability (Gabbiani *et al.*, 1970), augmented EB extravasation in selected tissues. These results confirm that changes in vascular permeability could be detected by our experimental model. In an earlier study in our laboratory, we reported that noradrenaline, a potent vasoconstrictor (Miller & Vanhoutte, 1985), did not alter vascular permeability (Sirois *et al.*, 1990). Vasoconstriction tends to decrease vascular volume, so unchanged EB content after noradrenaline indicates that changes in vascular volume are not important enough to be detected with our method. By comparing concentrations needed to elicit a permeability response, relative potency was established as ET-1 (0.4 nmol kg^{-1}) > histamine (54.3 nmol kg^{-1}).

The administration of big-ET-1 has been associated with an increase in circulating immunoreactive ET-1 (D'Orléans-Juste *et al.*, 1991b), and the pressor response to the precursor has been correlated with the increase in immunoreactive ET-1 (D'Orléans-Juste *et al.*, 1990; Hisaki *et al.*, 1991). The similar increase in EB extravasation obtained with big-ET-1 and ET-1 in the present study therefore points to the conversion of the precursor to its active moiety on vascular permeability. Pretreatment with PA blocked the conversion of the precursor to the peptide and maintained extravasation at baseline values. In addition, the fact that PA alone failed to reduce vascular permeability below control values suggests that endogenous physiological levels of big-ET-1 are probably low, or at least below detectable changes in permeability.

A mechanism of action has been ascribed to the vascular effects of ET-1 involving the release of cyclo-oxygenase products. Studies have shown that ET-1 stimulates the release of certain prostaglandins (PGE_2 and PGI_2) and thromboxanes (TXA_2) in a variety of vascular preparations (Antunes *et al.*, 1988; de Nucci *et al.*, 1988; Reynolds & Mok, 1989; Walder *et al.*, 1989). It has recently been demonstrated in our laboratories that the increased EB extravasation induced by ET-1 is blocked by cyclo-oxygenase and thromboxane synthase inhibitors (Sirois *et al.*, 1992), indicating that these mediators are either directly involved in the vascular permeability responses of the peptide, or indirectly responsible for the effect on permeability through their actions on precapillary and/or postcapillary resistances (Reynolds & Mok, 1989). PGE_2, PGI_2 and TXA_2 were reported to be involved directly or indirectly in increased diapedesis and vascular permeability in selected tissue preparations (Williams, 1979; Doukas *et al.*, 1989; Okiji *et al.*, 1989).

The effect of PA reported above is further supported by the finding that both big-ET-1 and ET-1 stimulate the release of eicosanoids *in vitro* and *in vivo*. Since this response to big-ET-1 is inhibited by PA (D'Orléans-Juste *et al.*, 1991a,b), it would appear that the converting enzyme (ECE) is crucial in the vascular permeability response induced by big-ET-1, because the precursor alone cannot stimulate the release of vasoactive cyclo-oxygenase products.

Recent *in vitro* studies show that ET-1 induces more pronounced vascular reactivity responses than its precursor (Kashiwabara *et al.*, 1989; McMahon *et al.*, 1991), but experiments performed *in vivo* demonstrate, on the contrary, that the peptides are equipotent (Kashiwabara *et al.*, 1989; Fukuroda *et al.*, 1990; Hisaki *et al.*, 1991). Hence, it was of interest to find that EB extravasation induced by big-ET-1 was also comparable if not identical to the ET-1 induced enhancement of vascular permeability in most tissues. In the lower bronchi and heart, ET-1 induced more pronounced responses than big-ET-1, but this could be due to a delayed conversion time of the precursor to its active metabolite in those particular tissues (Matsumura *et al.*, 1990; McMahon *et al.*, 1991).

It is likely that under pathophysiological conditions, ET-1 plays a role in altering vascular permeability in specific capillary networks. The eventual possibility of modulating ECE by pharmacological manipulation of this critical enzyme may be of interest in controlling permeability-dependent vascular disorders.

We conclude from the results of this study that big-ET-1 leads to an increased vascular permeability, the magnitude of which is comparable to that obtained with ET-1 alone. These effects appear to be due to the conversion of big-ET-1 to ET-1 by a PA-sensitive enzyme (ECE). Because of their combined vasopressor effects (Yanagisawa *et al.*, 1988; Fukuroda *et al.*, 1990) and permeabilizing properties demonstrated in the present study, it is reasonable to propose that big-ET-1 and ET-1 probably play an important role in the selective distribution of body fluids across capillary beds under certain physiological conditions.

We thank Ms Marie Bergeron for her technical assistance in this work.

References

ANTUNES, E., DE NUCCI, G. & VANE, J.R. (1988). Endothelin releases eicosanoids from and is removed by perfused lungs of guinea pig. *J. Physiol.*, **407**, 40p.

ARAI, H., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, **348**, 730–732.

BRAIN, S.D., TIPPINS, J.R. & WILLIAMS, T.J. (1988). Endothelin induces potent microvascular constriction. *Br. J. Pharmacol.*, **95**, 1005–1007.

CLOZEL, M. & CLOZEL, J.-P. (1989). Effects of endothelin on regional blood flows in squirrel monkeys. *J. Pharmacol. Exp. Ther.*, **250**, 1125–1131.

DAHLÉN, S.E., BJÖRK, J., HEDQVIST, P., ARFORS, K.E., HAMMARSTRÖM, S., LINDGREN, J.A. & SAMUELSSON, B. (1981). Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: In vivo effects with relevance to the acute inflammatory response. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3887–3891.

DE NUCCI, G., THOMAS, R., D'ORLÉANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9797–9800.

D'ORLÉANS-JUSTE, P., TÉLÉMAQUE, S. & CLAING, A. (1991a). Different pharmacological profiles of big-endothelin-3 and big-endothelin-1 *in vivo* and *in vitro*. *Br. J. Pharmacol.*, **104**, 440–444.

D'ORLÉANS-JUSTE, P., LIDBURY, P.S., TÉLÉMAQUE, S., WARNER, T.D. & VANE, J.R. (1991b). Human big endothelin releases prostacyclin in vivo and in vitro through a phosphoramidon-sensitive conversion to endothelin-1. *J. Cardiovasc. Pharmacol.*, **17**, S251–S255.

D'ORLÉANS-JUSTE, P., LIDBURY, P.S., WARNER, T.D. & VANE, J.R. (1990). Intravascular big-endothelin increases circulating levels of endothelin-1 and prostanoids in the rabbit. *Biochem. Pharmacol.*, **39**, R21–R22.

DOUKAS, J., HECHTMAN, H.B. & SHEPRO, D. (1989). Vasoactive amines and eicosanoids interactively regulate each polymorphonuclear leukocyte diapedesis and albumin permeability in vitro. *Microvasc. Res.*, **37**, 125–137.

EVANS, T.W., CHUNG, K.F., ROGERS, D.F. & BARNES, P.J. (1987). Effect of platelet-activating factor on airway vascular permeability: possible mechanisms. *J. Appl. Physiol.*, **63**, 479–484.

FILEP, J.G., SIROIS, M.G., ROUSSEAU, A., FOURNIER, A. & SIROIS, P. (1991). Effects of endothelin-1 on vascular permeability in the conscious rat: interactions with platelet-activating factor. *Br. J. Pharmacol.*, **104**, 797–804.

FUKURODA, F., NOGUCHI, K., TSUCHIDA, S., NISHIKIBE, M., IKEMOTO, F., OKADA, K. & YANO, M. (1990). Inhibition of biological actions of big-endothelin-1 by phosphoramidon. *Biochem. Biophys. Res. Commun.*, **172**, 390–395.

GABBIANI, G., BADONNEL, M.C. & MAJNO, G. (1970). Intra-arterial injection of histamine, serotonin, or bradykinin: a topographic study of vascular leakage. *Proc. Soc. Exp. Biol. Med.*, **135**, 447–452.

HIGHSMITH, R.F., PANG, D.C. & RAPOPORT, R.M. (1989). Endothelial cell-derived vasoconstrictors: mechanisms of action in vascular smooth muscle. *J. Cardiovasc. Pharmacol.*, **13**, S36–S44.

HISAKI, K., MATSUMURA, Y., IKEGAWA, R., NISHIGUCHI, S., HAYASHI, K., TAKAOKA, M. & MORIMOTO, S. (1991). Evidence for phosphoramidon-sensitive conversion of big endothelin-1 to endothelin-1 in isolated rat mesenteric artery. *Biochem. Biophys. Res. Commun.*, **177**, 1127–1132.

JANCAR, S., DEGIACOBBI, G., MARIANO, M., SIROIS, P. & BRAQUET, P. (1988). Involvement of platelet-activating factor in acute immune-complex-induced pancreatitis. In *New Trends in Lipid Mediators Research*, Vol. 2. ed. Braquet, P., pp. 194–198. Basel: Karger.

JOBIN, J., NAWAR, T., CARON, C. & PLANTE, G.E. (1977). Effect of acetalozamide on renal bicarbonate excretion in volume expanded dogs. *Am. J. Physiol.*, **232**, F484–F489.

KASHIWABARA, T., INAGAKI, Y., OHTA, H., IWAMATSU, A., NOMIZU, M., MORITA, A. & NISHIKORI, K. (1989). Putative precursors of endothelin have less vasoconstrictor activity in vitro but a potent pressor effect in vivo. *FEBS Lett.*, **247**, 73–76.

LEVEEN, H.H. & FISHMAN, W.H. (1947). Combination of Evans blue with plasma protein: its significance in capillary permeability studies, blood dye disappearance curves, and its use as a protein tag. *Am. J. Physiol.*, **151**, 26–33.

LOUTZENHISER, R., EPSTEIN, M., HAYASHI, K. & HORTON, C. (1990). Direct visualization of effects of endothelin on the renal vasculature. *Am. J. Physiol.*, **258**, F61–F68.

MILLER, V.M. & VANHOUTTE, P.V. (1985). Endothelial a_2-adrenoreceptors in canine pulmonary and systemic blood vessels. *Eur. J. Pharmacol.*, **118**, 123–129.

MATSUMURA, Y., HISAKI, K., TAKAOKA, M. & MORIMOTO, S. (1990). Phosphoramidon, a metalloproteinase inhibitor, suppresses the hypertensive effect of big endothelin-1. *Eur. J. Pharmacol.*, **185**, 103–106.

MCMAHON, E.G., PALOMO, M.A., MOORE, W.M., MCDONALD, J.F. & STERN, M.K. (1991). Phosphoramidon blocks the pressor activity of porcine big endothelin-1-(1-39) in vivo and conversion of big endothelin-1-(1-39) to endothelin-1-(1-21) in vitro. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 703–707.

OKIJI, T., MORITA, I., SUNADA, I. & MUROTA, S. (1989). Involvement of arachidonic acid metabolites in increases in vascular permeability in experimental dental pulpal inflammation in the rat. *Arch. Oral Biol.*, **34**, 523–528.

PLANTE, G.E., ERIAN, R. & PETITCLERC, C. (1981). Renal excretion of levamisole. *J. Pharmacol. Exp. Ther.*, **216**, 617–623.

RAWSON, R.A. (1943). The binding of T-1824 and structurally related diazo dyes by the plasma proteins. *Am. J. Physiol.*, **138**, 708–717.

REYNOLDS, E.E. & MOK, L.L.S. (1989). Role of thromboxane A_2/prostaglandin H_2 receptor in the vasoconstrictor response of rat aorta to endothelin. *J. Pharmacol. Exp. Ther.*, **252**, 915–921.

ROGERS, D.F., BOSCHETTO, P. & BARNES, P.J. (1989). Correlation between Evans blue dye and radiolabeled albumin in guinea pig airways in vivo. *J. Pharmacol. Meth.*, **21**, 309–315.

SAKURAI, T., YANAGISAWA, M., TAUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide selective subtype of the endothelin receptor. *Nature*, **348**, 732–734.

SIROIS, M.G., FILEP, J.G., ROUSSEAU, A., FOURNIER, A., PLANTE, G.E. & SIROIS, P. (1992). Endothelin-1 increases protein extravasation in conscious rats: role of thromboxane A_2. *Eur. J. Pharmacol.*, **214**, 119–125.

SIROIS, M.G., PLANTE, G.E., BRAQUET, P. & SIROIS, P. (1990). Role of eicosanoids in PAF-induced increases of the vascular permeability in rat airways. *Br. J. Pharmacol.*, **101**, 896–900.

SIROIS, M.G., JANCAR, S., BRAQUET, P., PLANTE, G.E. & SIROIS, P. (1988). PAF increases vascular permeability in selected tissues: effect of BN-52021 and L-655,240. *Prostaglandins*, **36**, 631–644.

VALENTIN, J.-P., GARDNER, D.G., WIEDEMANN, E. & HUMPHREYS, M.H. (1991). Modulation of endothelin effects on blood pressure and hematocrit by atrial natriuretic peptide. *Hypertension*, **17**, 864–869.

WALDER, C.E., THOMAS, G.R., THIEMERMANN, C. & VANE, J.R. (1989). The hemodynamic effects of endothelin-1 in the pithed rat. *J. Cardiovasc. Pharmacol.*, **13**, S93–S97.

WILLIAMS, T.J. (1979). Prostaglandin E_2, prostaglandin I_2 and the vascular changes of inflammation. *Br. J. Pharmacol.*, **65**, 517–524.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

(Received July 7, 1992
Accepted July 30, 1992)

Br. J. Pharmacol. (1992), **107**, 1001–1007

Potentiation of the vasorelaxant activity of nitric oxide by hydroxyguanidine: implications for the nature of endothelium-derived relaxing factor

[1]Artur Zembowicz, Tomasz A. Swierkosz, Garry J. Southan, [2]Markus Hecker & John R. Vane

The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ

1 We recently demonstrated that N^G-hydroxy-L-arginine (L-HOArg) is a substrate for the constitutive nitric oxide (NO) synthase present in bovine aortic endothelial cells cultured on microcarrier beads (EC). Furthermore, L-HOArg reacts chemically with NO released from these cells to form a potent and more stable vasodilator. This is most likely through a reaction with the hydroxyguanidino group.

2 Here, we studied the interaction of a simpler molecule, hydroxyguanidine (HOG) with NO.

3 HOG (10 μM), like L-HOArg (10 μM) or N^G-hydroxy-D-arginine (D-HOArg, 10 μM), potentiated and stabilized the relaxant activity of authentic NO.

4 When NO was bubbled through the solution of HOG, a new compound was formed. It had similar physicochemical properties to those of the previously described L-HOArg/NO adduct. It was also a potent vasodilator and its action was inhibited by oxyhaemoglobin (10 μM), indicating formation of a NO-containing substance.

5 Moreover, HOG (10 μM) was not a substrate for the constitutive NO synthase present in the microsomal fraction of EC and did not affect the flow-induced or bradykinin-stimulated generation of prostacyclin, as measured by 6-ketoPGF$_{1\alpha}$.

6 We also studied the effect of HOG on the endothelium-derived relaxing factor (EDRF) released from the column of EC. HOG (10 μM) potentiated and stabilized the relaxations of rabbit aortic strips induced by EDRF released by bradykinin (5–20 pmol) or ADP (5–10 nmol). These relaxations were inhibited by N^G-nitro-L-arginine methyl ester (L-NAME, 10 μM) and L-arginine (L-Arg, 1 mM) reversed the inhibitory effects of L-NAME.

7 HOG (10 μM) augmented the basal (flow-induced) EC-dependent relaxations which were also inhibited by L-NAME (10 μM) and the effects of L-NOArg were reversed by L-Arg (1 mM).

8 Thus, the hydroxyguanidino moiety of L-HOArg is involved in the reaction with NO. Moreover, the comparable reaction of the hydroxyguanidino compounds with NO on the one hand and with flow-induced and agonist-triggered EDRF on the other, strongly supports their common identity.

Keywords: Endothelium; endothelium-derived relaxing factor; nitric oxide; arginine; hydroxyguanidine; N^G-hydroxy-L-arginine; bioassay; h.p.l.c.

Introduction

Nitric oxide (NO) is generated by mammalian cells from one of the N^G-guanidino nitrogens of L-arginine (L-Arg) and this reaction is catalysed by a NADPH-dependent dioxygenase (Kwon *et al.*, 1990). This enzyme, referred to as nitric oxide synthase exists in at least two distinct isoforms. One, a constitutive agonist-triggered and calcium-dependent NO synthase is mainly present in neuronal cells (Bredt & Snyder, 1990) and vascular endothelial cells (EC, Forstermann *et al.*, 1991). The other enzyme, a calcium-independent inducible NO synthase, is found in macrophages (Marletta *et al.*, 1988), hepatocytes (Billiar *et al.*, 1990), endothelial cells (Radomski *et al.*, 1990) and smooth muscle cells (Busse & Mulsch, 1990) after activation by bacterial lipopolysaccharide (LPS) or cytokines.

The formation from L-Arg of NO or a closely related substance by EC is involved in the maintenance of the tone and thromboresistance of the vascular wall (for review see, Vane *et al.*, 1990). In the central and peripheral nervous systems NO functions as a neurotransmitter (for review see, Snyder & Bredt, 1991). In the immune system NO made by the inducible NO synthase is responsible for the killing of tumour cells and microbial pathogens by activated macrophages (for review see Nathan & Hibbs, 1991).

The exact mechanisms of the conversion of L-arginine to NO by the isoforms of NO synthase are now known. N^G-hydroxylation of L-arginine (L-HOArg) has been proposed as a first step in the biosynthesis of NO (Marletta *et al.*, 1988). This notion was recently confirmed by Stuehr *et al.* (1991) who demonstrated that L-HOArg is, indeed, formed by the inducible NO synthase from macrophages. Moreover, L-HOArg is a substrate for this NO synthase and stable isotope studies revealed that this enzyme oxidizes exclusively the hydroxylated nitrogen of L-HOArg.

Recently we have demonstrated three mechanisms by which L-HOArg induces endothelium-dependent relaxations (Zembowicz *et al.*, 1991). It is a substrate for the constitutive NO synthase present in EC. L-HOArg induces a relatively stable, EC-dependent relaxation which is not affected by inhibitors of NO synthase. It also reacts with NO released from EC to form a potent and more stable vasorelaxant compound. The last of these effects is not specific for the L-isomer of hydroxyarginine. In contrast, L-Arg does not affect the EC-dependent relaxations nor does it react with NO. This suggested to us that the hydroxyguanidino moiety of L-HOArg is responsible for the EC-dependent relaxation or reaction with NO.

Here, we have investigated the effects of hydroxyguanidine (HOG) on the biosynthesis and vasorelaxant activity of

[1] Author for correspondence at present address: Department of Pharmacology, N. Copernicus Academy of Medicine, ul. Grzegorzecka 16, 31-531 Cracow, Poland.

[2] Present address: Institute of Applied Physiology, University of Freiburg, Hermann-Herder Strasse 7, D-W-7800 Freiburg i. Br. Germany.

EDRF released from cultured bovine aortic endothelial cells. We demonstrate that HOG potentiates both the agonist-triggered and flow-induced EC-dependent relaxations and that this is due to the reaction of HOG with NO released from endothelial cells and the formation of a new, more stable compound with similar physicochemical characteristics to those of the L-HOArg/NO adduct. Moreover, these results strongly support the notion that the EDRF released by endothelial cells which mediates the EC-dependent relaxations induced by flow or agonists is, indeed, NO.

Methods

Cell culture

Bovine aortic endothelial cells were harvested and grown on Cytodex-3 microcarrier beads (Pharmacia-LKB Ltd.) in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) supplemented with 4 mM L-glutamine and 10% (v/v) foetal calf serum (FCS; Gibco) as described previously (De Nucci *et al.*, 1988). LLC-PK_1 pig kidney epithelial cells (ATCC CL 101) were obtained from the European Collection of Animal Cell Cultures and grown on 96-well plates in medium 199 (Flow Laboratories) supplemented with 10% FCS.

Bioassay of endothelium-derived relaxing factor

Endothelium-derived relaxing factor (EDRF) was bioassayed as previously described (Gryglewski *et al.*, 1986). Briefly, male New Zealand White rabbits (2.2–2.8 kg) were anaesthetized with sodium pentobarbitone and their thoracic aorta were rapidly removed. After cleaning of adhering periadventitial fat and removal of the endothelium on a glass rod, the aortae were cut spirally into strips (3 × 30 mm). Approximately 9×10^7 endothelial cells cultured on beads (3 ml) were packed into a jacketed chromatography column and perfused at 5 ml min^{-1} with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution containing 5.6 μM indomethacin and in some experiments superoxide dismutase (SOD, 10–30 u ml^{-1}). The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 2.5, and glucose 5.6. The effluent from the column superfused a cascade of three or four spirally cut strips of rabbit aorta (detector tissues) which were preconstricted with 30 nM U46619. Relaxations of the detector tissues were recorded by auxotonic levels attached to Harvard isotonic transducers connected to a Graphtec WR3101 linearcorder. The amplifications of the recorders were adjusted so that standard doses of glyceryl trinitrate (22.5–90 pmol) induced similar recordings of the relaxations of all arterial strips. Bradykinin or ADP were given as bolus injections over the assay tissues (o.t.) or through the column (t.c.). Saturated solutions of NO (1–3 mM) were prepared by bubbling 2.5 ml of NO gas (Merck Ltd.) into 1 ml of helium-deoxygenated water kept in an air tight flask at 0–4°C. An aliquot of this solution (1 μl corresponding to 1–3 nmol) was injected t.c. as a bolus. All hydroxyguanidino-containing compounds were infused at 100 μl ml^{-1} either o.t. or t.c. In some experiments the column was packed with empty microcarrier beads (3 ml) containing no endothelial cells and experiments were performed as described above.

Preparation of subcellular fractions from cultured endothelial cells

All of the following procedures were carried out at 0–4°C. Approximately 10^9 bovine aortic endothelial cells cultured on beads were suspended in 50 mM Tris·HCl buffer, pH 7.4, containing 10 mM EDTA, 5 mM glucose, 1.15% (wt/v) KCl, 0.1 mM DL-dithiothreitol (DTT), 2 mg l^{-1} leupeptin, 2 mg l^{-1} pepstatin A, 10 mg l^{-1} trypsin inhibitor, and 44 mg l^{-1} phenylmethylsulphonyl fluoride (PMSF). The cell suspension was bubbled with helium for 15 min, sonicated, and the homogenate centrifuged at 1,000 *g* for 10 min to remove the empty beads. The 1,000 *g*-supernatant was centrifuged for 20 min at 10,000 *g*, the pellet discarded and the 10,000 *g*-supernatant centrifuged for 30 min at 200,000 *g*. The 200,000 *g*-supernatant (cytosol) which contains less than 10% of NO synthase activity (Zembowicz *et al.*, 1991) was discarded and the pellet (microsomes) was resuspended in 50 mM Tris·HCl buffer, pH 7.4 containing 0.1 mM EDTA, 0.1 mM DTT, 2 mg l^{-1} leupeptin, 2 mg l^{-1} pepstatin A, 10 mg l^{-1} trypsin inhibitor, 44 mg l^{-1} PMSF, and 10% (v/v) glycerol. Protein concentrations were determined by using a modified Lowry-method.

Determination of NO synthase activity

NO synthase activity was measured by monitoring NO-mediated increases in the levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in pig kidney epithelial cell line LLC-PK_1 with some modification of the previously described procedure (Zembowicz *et al.*, 1991). Aliquots of the microsomal fraction (50–100 μg of protein) were diluted in PBS buffer (with Ca^{2+} and Mg^{2+}), pH 7.4, containing SOD (100 u ml^{-1}), 100 μM NADPH, 100 μM DTT, 1 μM THB, and 0.1 μM calmodulin and incubated in the presence or absence of HOG, L-Arg, or L-HOArg (all at 100 μM) with the monolayers of LLC-PK_1 cells cultured in 96-well plates (5×10^5 cells/well) which were pretreated for 30 min with 0.5 mM isobutylmethylxanthine. The final volume of incubations was 100 μl. After 10 min, 100 μl of sodium acetate (pH 4.0) was added to each well and plates were rapidly frozen in liquid nitrogen in order to stop the reactions and break the cells. Contents of wells were diluted 50–100 fold with sodium acetate pH 5.6 (assay buffer) and cyclic GMP concentrations were measured by radioimmunoassay after prior acetylation of samples with acetic anhydride (Harper & Brooker, 1975) using a specific antiserum provided by Dr H. Strobach (Institute of Pharmacology, University of Dusseldorf, Germany) and [^{125}I]-cyclic GMP obtained from Amersham International (UK). The sensitivity of the assay was approximately 1–2 pmol of NO/ 10 min.

Measurement of 6-keto-$PGF_{1\alpha}$

The effluent from the column of endothelial cells was collected for 3 min under basal conditions or after a bolus injection of bradykinin (5–20 pmol, t.c.). The levels of 6-keto-$PGF_{1\alpha}$, a hydrolysis product of prostacyclin, were measured by a radioimmunoassay (Salmon, 1978) using antiserum purchased from Sigma (UK).

Isolation and h.p.l.c. analysis of hydroxyguanidine/nitric oxide adduct

Hydroxyguanidine/nitric oxide adduct was prepared by the reaction of hydroxyguanidine hemisulphate with sodium nitrite in water at pH 3. The precipitated product was dried and stored over calcium chloride at room temperature; 1–2 mg of this powder was reconstituted in distilled water and injected (10 μl) into isocractic high performance liquid chromatography (h.p.l.c.) system consisting of an aminopropyl stationary phase and a mobile phase of 60% acetonitrite and 40 phosphate buffer (10 mM KH_2PO_4, pH 4.3). The fraction corresponding to the HOG/NO adduct gave a single peak of u.v. absorbance at wavelengths of 205 or 320 nm and was collected for the determination of vasorelaxant activity.

Organ bath experiments

Rabbit thoracic aorta was obtained as described above taking care not to damage the endothelium. The aorta was cut into rings 5 mm wide which were suspended between stainless steel wires and mounted in 20 ml organ baths filled with

warmed (37°C) and oxygenated (95% O_2, 5% CO_2) Krebs buffer. Isometric force was measured with Biegestab K30 type 351 transducers (Hugo Sachs Electronic, Germany). A tension of 4 g was applied and rings were equilibrated (60–90 min) with changing the Krebs buffer and adjusting a preload to 4 g every 15 min. After a stable baseline was obtained, rings were contracted with KCl (15 mM) and relaxed with acetylcholine (ACh; 0.3 μM) which was added to the baths in order to verify the functional integrity of the endothelium. Only those rings which relaxed to ACh were used for further studies. After changing the buffer 4 times every 15 min the preparations were contracted with increasing concentrations of phenylephrine (0.01–10 μM) in the absence or presence of HOG (10 μM) which was added to the baths 15 min before addition of phenylephrine. Alternatively, rings were constricted with phenylephrine (0.1 μM) and cumulative concentration-response curves to ACh (0.01–1 μM) were obtained with or without prior preincubation of the tissues in the presence of HOG (10 μM). Contractions were calculated as % of the contraction induced by KCl (15 mM) and relaxations were expressed as % of the tone induced by phenylephrine.

Materials

Indomethacin, adenosine disphosphate (ADP), bradykinin, superoxide dismutase (from bovine erythrocytes; SOD), N^G-nitro-L-arginine methyl ester, were purchased from Sigma Chemical Co. (U.K.), (6**R**,**S**)-5,6,7,8-tetrahydro-L-biopterin (THB) from Dr B. Schricks Laboratories (Switzerland), and glyceryl trinitrate (Nitronal) from Lipha Pharmaceuticals Ltd (U.K.). 9,11-Dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$ (U46619) was a generous gift from the Upjohn Co. (U.S.A.). Hydroxyguanidine sulphate was synthesized by the method of Walker & Walker (1959). L-HOArg was synthesized by the Glaxo Group Research Ltd. (U.K.) and D-HOArg was synthesized by Dr Paul L. Feldman (Medicinal Chemistry, Glaxo Inc. Research Institute, Research Triangle Park, N.C., U.S.A.). Oxyhaemoglobin was prepared by reduction of bovine haemoglobin (obtained from Sigma) with sodium hydrosulphite followed by gel filtration with a prepacked disposable column PD-10 (Pharmacia) equilibrated with Tris·HCl (50 mM) at pH 7.4. All other reagents and solvents were of the highest commercially available quality from either Sigma Chemical Co. (U.K.) or Merck Ltd. (U.K.).

Statistical analysis

All values in the figures and text are expressed as mean ± s.e.mean of n observations. A one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni test or a two-tailed one sample t test was used, where appropriate, to assess the statistical significance of results. A P value less than 0.05 was considered statistically significant.

Results

Effects of hydroxyguanidine on the biological activity of endothelium-derived relaxing factor

HOG infused over the detector tissues (o.t.) at a concentration of 10 μM did not affect the tone of the detector tissues ($n = 12$; Figures 1, 2) and was without effect on the relaxations induced by GTN ($n = 12$; 45–90 pmol; o.t.; Figures 1, 2, 5). However, when HOG (10 μM) was infused through the column of EC (t.c.), in 6 out of 8 experiments there was a relaxation of the detector tissues which was the biggest on the first detector tissue and then smaller but relatively stable down the cascade. As seen in Figure 1 these relaxations were abolished in the presence of L-NAME (10 μM) and the effects of L-NAME were reversed by a concomitant infusion of L-Arg (1 mM).

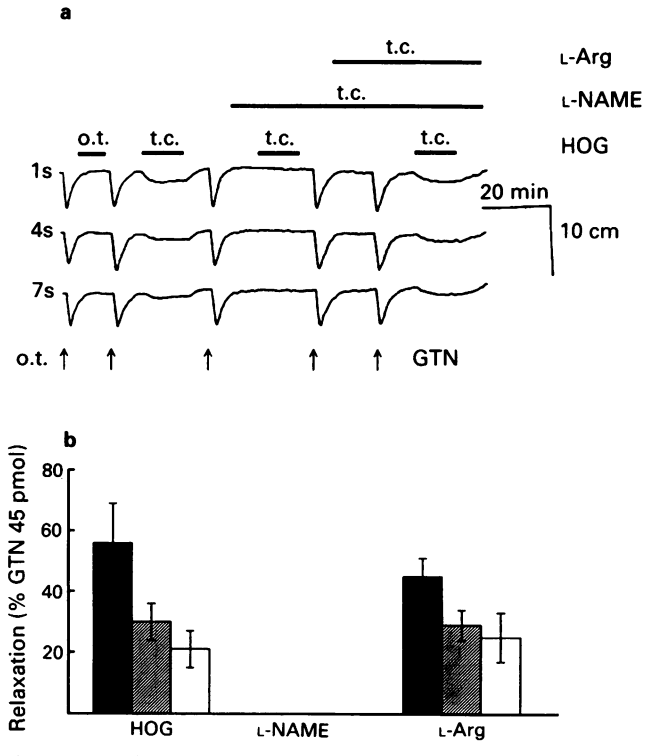

Figure 1 Hydroxyguanidine potentiates the vasorelaxant activity of flow-induced EDRF released from cultured endothelial cells (EC) as detected by the cascade bioassay. For experimental details refer to Methods. In (a) is shown an original trace of three rabbit aortic strips separated from the column of endothelial cells by 1, 4 and 7 s, respectively. Glyceryl trinitrate (GTN; 45 pmol) was given as a bolus injection over the tissues (o.t.) to calibrate their responses. Hydroxyguanidine (HOG; 10 μM) infused o.t. did not affect the tone of the detector tissues. When HOG (10 μM) was infused through the column of endothelial cells (t.c.) there was a relaxation of all three detector tissues. These relaxations were abolished in the presence of N^G-nitro-L-arginine methyl ester (L-NAME; 10 μM, t.c.) and this abolition was prevented by co-infusion of L-arginine (L-Arg; 1 mM). This trace is representative of a series of experiments ($n = 3$) summarized in graph (b). The relaxations (expressed as % relaxation relative to the response caused by GTN) of the first (solid columns), second (hatched columns) and third (open columns) detector tissues are shown.

HOG (10 μM) infused t.c., potentiated the relaxations induced by EDRF released from endothelial cells by bradykinin (BK, 5–20 pmol; t.c.; $n = 9$) or ADP (5–10 nmol; t.c.; $n = 6$). As depicted in Figure 2, these relaxations were more stable on the cascade than those induced in the absence of HOG. Moreover, they were abolished by L-NAME (10 μM; t.c.) in a manner that was reversible by L-Arg (1 mM; t.c.). Infusion of HOG (10 μM) o.t. did not affect the responses of the first two detector tissues to EDRF. However, on the third detector tissue, a slight potentiation was consistently observed (Figure 2). The effects of HOG were not due to the scavenging by HOG of superoxide anions for similar results were obtained in the presence of SOD (10–30 u ml^{-1}; t.c.; $n = 5$; data not shown).

Metabolism of hydroxyguanidino-containing compounds by nitric oxide synthase

We have previously reported (Zembowicz *et al.*, 1991) that the activity of the constitutive NO synthase is largely recovered in the membrane fraction from endothelial cells. The activity of NO synthase was assayed by monitoring NO-mediated increases in the level of cyclic GMP in LLC-PK_1 cells. Incubation of microsomal protein (50–100 μg) with L-Arg (100 μM) or L-HOArg (100 μM) increased the

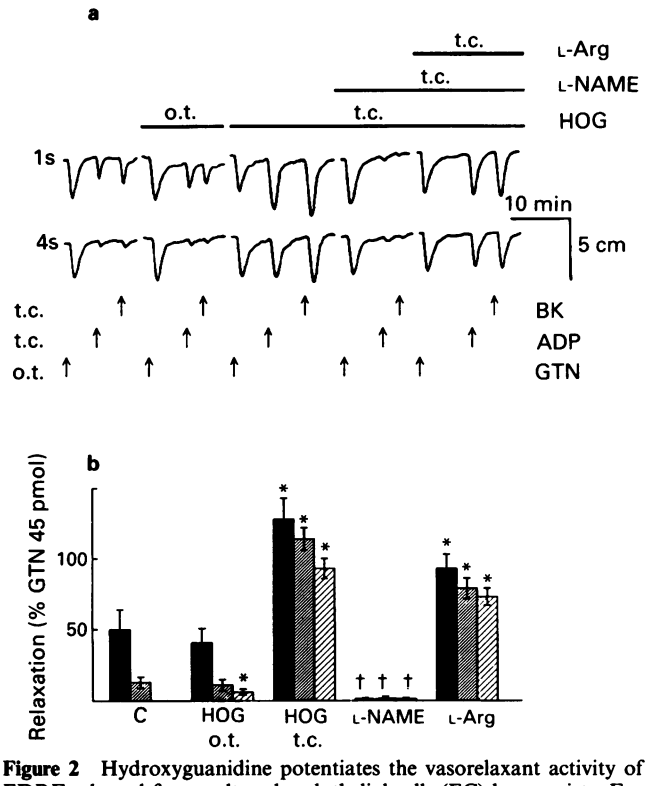

Figure 2 Hydroxyguanidine potentiates the vasorelaxant activity of EDRF released from cultured endothelial cells (EC) by agonists. For experimental details refer to Methods. An original trace is shown of two (out of three) rabbit aortic strips separated from the column of EC by 1 and 4 s, respectively. Glyceryl trinitrate (GTN; 45 pmol) was given as a bolus injection over the tissues (o.t.) to calibrate their responses. ADP (5 nmol) or bradykinin (BK, 5 pmol) injected through the column of endothelial cells (t.c.), caused the release of EDRF which was detected as the relaxations of the detector tissues which disappeared down the cascade. Hydroxyguanidine (HOG; 10 μM) infused o.t. had no effect on the relaxant responses to ADP or BK. When infused t.c., HOG (10 μM) potentiated and stabilized the ADP- or BK-induced relaxations. In the presence of N^G-nitro-L-arginine methyl ester (L-NAME; 10 μM, t.c.) these relaxations were blocked and the effect of L-NAME was prevented by co-infusion of L-arginine (L-Arg; 1 mM, t.c.). The results from 4 similar experiments are summarized in (b). The relaxations (expressed as % relaxation relative to the response caused by GTN) of the first (solid columns), second (hatched columns) and third (open columns) detector tissues induced by BK (5–10 pmol; t.c.) are shown. In the absence of HOG, BK induced the relaxations of only the first two tissues. However, in the presence of HOG (10 μM, o.t.) relaxations of the third tissue were also observed. C, control.
*$P<0.05$ when compared to control; †$P<0.05$ when compared to control.

intracellular concentrations of cyclic GMP in LLC-PK_1 cells by 24 ± 10 pmol mg^{-1} protein $10\ min^{-1}$ ($n = 4$ different batches of EC) in the case of L-Arg and by 37 ± 18 pmol mg^{-1} protein min^{-1} ($n = 4$) in the case of L-HOArg. HOG (100 μM) had no effect ($n = 4$), indicating that it is not a substrate for NO synthase.

Effect of hydroxyguanidine on the generation of prostacyclin by endothelial cells

The effect of HOG on the formation by endothelial cells of prostacyclin was measured by monitoring the concentrations of 6-ketoPGF$_{1\alpha}$, a product of its hydrolysis. The basal generation of 6-ketoPGF$_{1\alpha}$ was 3.3 ± 0.8 ng $3\ min^{-1}$ in the absence and 2.9 ± 0.5 ng $3\ min^{-1}$ in the presence of HOG (10 μM; $n = 5$). Similarly, HOG did not affect the generation of 6-ketoPGF$_{1\alpha}$ by EC stimulated with BK (20 pmol) which was 15 ± 5 in the absence and 11 ± 3 in the presence of HOG (10 μM; $n = 5$).

Potentiation of the biological activity of authentic nitric oxide by hydroxyguanidine

NO (1–3 nmol), injected through the column of empty beads, caused characteristic relaxations of the detector tissues which rapidly diminished down the cascade. HOG (10 μM; t.c.; $n = 6$), L-HOArg (10 μM; t.c.; $n = 7$) or D-HOArg (10 μM; t.c.; $n = 7$) significantly potentiated relaxations induced by NO which in the presence of hydroxyguanidino-containing compounds were more stable down the cascade (Figure 3). When HOG (10 μM), L-HOArg (10 μM) or D-HOArg (10 μM) were infused o.t. the relaxant responses to NO (1–3 nmol, t.c.) of only the third, but not the first two, detector tissue were potentiated ($n = 3$; data not shown). These results all strongly suggested that the potentiation of the biological activity of EDRF by HOG is due to a chemical reaction with NO (taking a few seconds) and formation of a novel and more stable vasorelaxant compound.

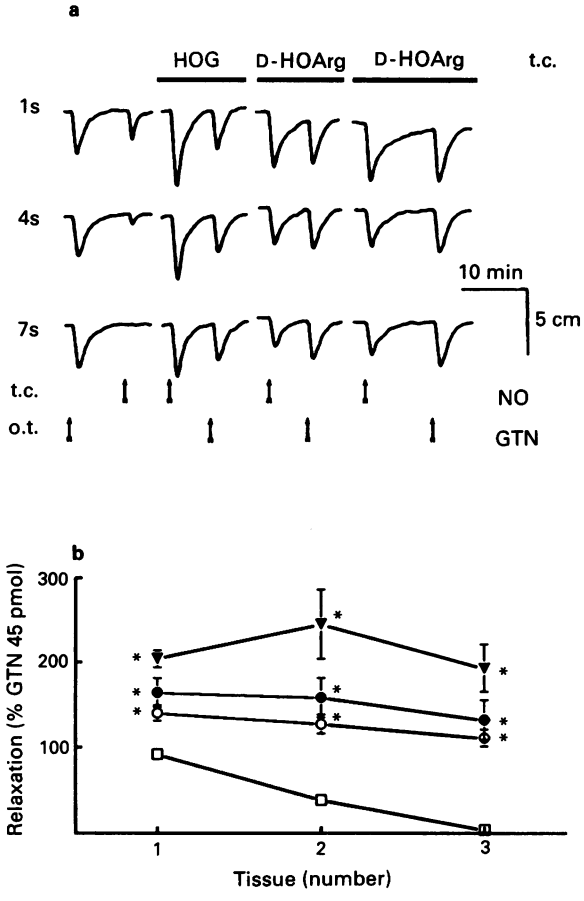

Figure 3 Hydroxyguanidine potentiates the vasorelaxant activity of nitric oxide. For experimental details refer to Methods. In (a) is shown an original trace of three rabbit aortic strips superfused by the effluent from the column containing empty beads and separated from the column by 1, 4 and 7 s, respectively. Glyceryl trinitrate (GTN; 45 pmol) was given as a bolus injection over the tissues (o.t.) to calibrate their responses. Nitric oxide (NO; 1–2 nmol) given through the column (t.c.) caused characteristic relaxations of the detector tissues which disappeared down the cascade. The vasorelaxant activity of NO was significantly increased and stabilized by hydroxyguanidine (HOG; 10 μM, t.c.), N^G-hydroxy-L-arginine (L-HOArg; 10 μM, t.c.) or N^G-hydroxy-D-arginine (D-HOArg; 10 μM, t.c.). The results of 4 similar experiments are summarized in the graph (b). The relaxations (expressed as % relaxation relative to the relaxation induced by GTN) of the first, second and third detector tissue are shown in the absence (□; $n = 18$) of hydroxyguanidino compounds or in the presence of HOG (10 μM; $n = 6$; ▲), L-HOArg (10 μM; $n = 7$; ●) or D-HOArg (10 μM; $n = 7$; ○). Values represent means ± s.e. mean (vertical bars) of n separate measurements. *$P<0.05$ when compared to control.

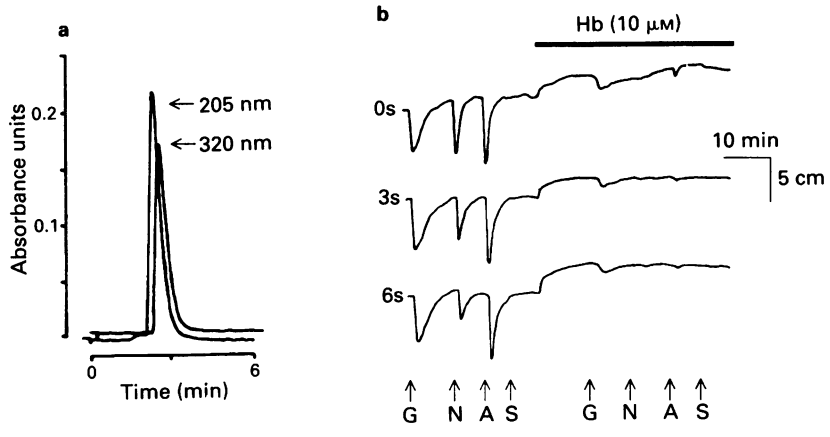

Figure 4 Hydroxyguanidine (HOG) reacts with nitric oxide (NO) to form a novel vasodilator. (a) Typical normal phase h.p.l.c. chromatogram of the HOG/NO adduct (for experimental details refer to Methods). The h.p.l.c. fraction, after injection of 10 μl of 1 mg ml^{-1} solution of the HOG/NO adduct, was collected and its vasorelaxant activity assayed using the cascade bioassay. In (b) an original trace of three rabbit aortic strips superfused with Krebs solution separated from each other by 3 s is shown. Glyceryl trinitrate (G; 90 pmol) was given as a bolus injection over the tissues (o.t.) to calibrate their responses. Nitric oxide (N; 1–2 nmol, o.t.) caused the relaxation of the detector tissues which disappeared down the cascade. In contrast, relaxations induced by the h.p.l.c. fraction containing HOG/NO adduct (A; corresponding to approximately 20 ng of compound) were stable. The h.p.l.c. solvent (S; 1 μl; o.t.) had no vasorelaxant activity. Oxyhaemoglobin (Hb; 10 μM; o.t.) significantly inhibited vasorelaxant responses to all tested compounds. This figure is representative of 4 experiments using different preparations of the HOG/ NO adduct.

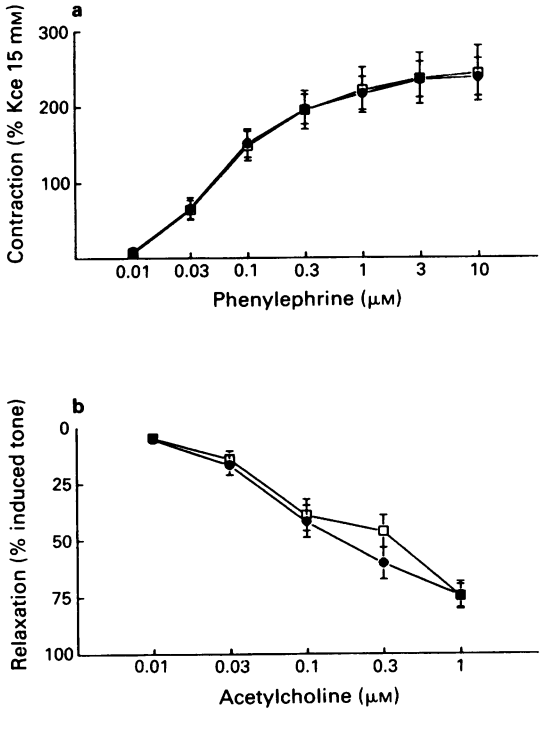

Figure 5 Hydroxyguanidine (HOG) does not affect the endothelium-dependent relaxations of rabbit aortic rings. Rings were contracted with KCl (15 mM) and the functional integrity of endothelium was confirmed by relaxations to acetylcholine (0.3 μM). After extensive rinsing, the cumulative concentration-response curves to phenylephrine (0.01–10 μM; a) were constructed in the presence (●) or in the absence (□) of HOG (10 μM). Alternatively (b), the rings were preconstricted with phenylephrine (0.1 μM) and cumulative concentration-response curves to acetylcholine (0.1–1 μM) were produced in the presence (●) or absence (□) of HOG (10 μM). In both series of experiments, HOG was added to organ baths 15 min before addition of phenylephrine. Contractions are expressed as % of the contraction induced by KCl and relaxations as % of the phenylephrine-induced tone. Values represent means ± s.e.mean (vertical bars) of $n = 6-12$ rings from 6 different animals.

Vasorelaxant properties of hydroxyguanidine/NO adduct

HOG (10 mM), when incubated with authentic NO or NO_2^- in acid, exhibited u.v. spectrum with a maximum of 320 nm not seen for HOG alone. This HOG/NO adduct was isolated by h.p.l.c. (Figure 4a) and, when injected o.t. in the cascade bioassay, caused a stable relaxation of the detector tissues which was blocked by oxyhaemoglobin (oxy-Hb; 10 μM; Figure 4b). The h.p.l.c. solvent did not affect the tone of the detector tissues.

Organ bath experiments

Incubation of rabbit aortic rings with HOG (10 μM) had no effect on the resting tone of the tissues (not shown) and it did not affect the concentration-dependent contractions to phenylephrine (Figure 5a). When rings were preconstricted with phenylephrine (0.1 μM), ACh produced a concentration-dependent relaxation which was not changed by pretreatment with HOG (10 μM; 10 min; Figure 5b).

Discussion

Our results demonstrate that HOG potentiates the vasorelaxant activity of EDRF released from bovine aortic endothelial cells by flow as well as by agonists. This effect is due to the chemical reaction of HOG with NO released from endothelial cells and formation of a novel, potent vasodilator.

Both the EC-dependent relaxation induced by HOG as well as relaxations triggered by BK or ADP in the presence or absence of HOG were totally dependent on the activity of the L-Arg/NO pathway, for they were abolished by L-NAME, an inhibitor of L-Arg-dependent NO synthase (Mulsh & Busse, 1990; Rees *et al.*, 1990) and the inhibitory effect of L-NAME was reversed by L-Arg, a substrate for this enzyme (Palmer *et al.*, 1988). The potentiation of the agonist-triggered EC-dependent relaxation cannot be explained by a direct activation by HOG of EC, for HOG had no effect on the generation of prostacyclin and, in our bioassay system, the receptor-mediated release of EDRF and prostacyclin are coupled (DeNucci *et al.*, 1988). Moreover, unlike L-Arg or L-HOArg, HOG is not a substrate for the constitutive NO

synthase present in EC showing that the effects of HOG are not due to an increased generation of NO by endothelial cells. The responses to GTN, which is metabolized to NO by vascular smooth muscle cells (Feelish & Kelm, 1991; Salvemini *et al.*, 1992), were not affected by HOG demonstrating that HOG does not release an EC-derived factor potentiating the action of NO. Clearly, both flow- and agonist-triggered relaxations were more stable down the cascade in the presence of HOG than in its absence.

The results summarized above exclude pharmacological activity of HOG on endothelial cells and all strongly suggest a chemical interaction between HOG and NO, either flow-induced or agonist-released from EC, leading to the formation of a novel potent vasodilator. This notion is further supported by the potentiation and stabilization by HOG of the vasorelaxant activity of authentic NO in the absence of EC and the formation of a more stable, chromatographically distinct vasodilator following reaction of HOG with NO in a test tube. The formation of this stable vasodilator in a test tube clearly shows that the reaction between HOG and NO is not an artefact of cultured cells or microcarrier beads.

HOG infused o.t. did not potentiate the relaxations of the first two detector tissues induced by EDRF or NO. However, a slight potentiation could be observed on the third detector tissue which was separated from the first one by 6 s. Infusion of HOG t.c., which markedly potentiated the vasorelaxant activity of EDRF or NO, further increased the time of interaction of HOG with EDRF or NO to 15–20 s, equivalent to the transit time of the perfusate through the column. Thus, the effects of HOG were proportional to the time of interaction between HOG and NO, indicating that the chemical reaction between HOG and NO is not instantaneous but requires some time (seconds) to occur.

Recently we have shown (Zembowicz *et al.*, 1991) that L-HOArg, an intermediate in the oxygenation of L-Arg to NO by the inducible NO synthase from macrophages (Stuehr *et al.*, 1991), is also a substrate for the constitutive endothelial NO synthase and that it is preferentially used by this enzyme despite saturating concentrations of L-Arg. Moreover, both L-HOArg and D-HOArg react with NO to form a more stable vasodilator with similar physicochemical properties to those of the HOG/NO adduct. Thus, the hydroxyguanidino moiety of L-HOArg is essential for its reactivity with NO and other hydroxyguanidino-derivatives should have similar properties. We have also shown (Zembowicz *et al.*, 1991) that, unlike L-Arg, L-HOArg causes EC-dependent relaxations which are resistant to the blockade by the inhibitors of NO synthase. In contrast, relaxant responses to HOG were completely abolished by L-NAME, demonstrating that they solely represent the potentiation of the vasorelaxant activity of EDRF released by flow (shear-stress), a process abrogated by inhibitors of NO synthase (Hutcheson & Griffith, 1991). In our previous study we have shown that the relaxant activity of flow-induced EDRF was only little affected by D-HOArg. This may be best explained by the differences in experimental conditions. In the present experiments, we used larger numbers (9×10^7 vs 6×10^7) and volumes (3 vs 2 ml) of EC which resulted in greater production of NO and increased its reaction time with HOG, hence allowing for more efficient formation of the HOG/NO adduct.

There has been much debate as to whether EDRF is NO or a closely related compound. Earlier evidence for the chemical identity of EDRF and NO (Hutchinson *et al.*, 1987; Radomski *et al.*, 1987; Palmer *et al.*, 1987) was questioned after the demonstration of poor correlation of the biological activity and chemiluminescence or EPR signal of authentic NO when compared with those of EDRF released from cultured bovine endothelial cells (Myers *et al.*, 1989; 1990; Rubanyi *et al.*, 1991). These authors suggested that EDRF more closely resembles a S-nitrosothiol than NO. This debate will probably continue until a direct chemical method for the selective detection of NO has been developed. The chemical reaction of the hydroxyguanidino moiety with NO suggests one possible approach towards the development of such a method, i.e. monitoring of the formation of the stable HOG/adduct by u.v. photometry. More importantly, our results are consistent with the chemical identity of EDRF with NO.

HOG was without effect on the phenylephrine-induced contractions and acetylcholine-induced relaxations of intact rabbit aortic rings, hence indicating that HOG does not potentiate the biological activity of EDRF released from an intact endothelium by flow or agonists. Moreover, HOG does not have a hypotensive effect in the anaesthetized rat (Dr C. Theimermann, unpublished observation). This indicates that the diffusion time for NO between the endothelial NO synthase and vascular smooth muscle guanylate cyclase is too short for the efficient reaction with HOG. This explanation is consistent with the lack of effect of HOG given o.t. in the bioassay cascade.

We conclude that the hydroxyguanidino moiety of L-HOArg is essential for its reaction with NO and that EDRF released from endothelial cells by flow or agonist-mediated cell activation has the chemical characteristics of nitric oxide.

We are indebted to Prof. Richard Gryglewski and Prof. Kenneth K. Wu for helpful discussions and reviewing this manuscript; Elizabeth G. Wood for the culture of endothelial and LLC-PK_1 cells; Carolyn Hynes and Graham Allcock for their help with some bioassay experiments and Ivana Vojnovic for expert technical assistance. This work was supported by a grant from Glaxo Group Research Ltd.

References

BILLIAR, T.R., CURRAN, R.D., STUEHR, D.J., STADLER, J., SIMMONS, R.L. & MURRAY, S.A. (1990). Inducible cytosolic enzyme activity for the production of nitrogen oxides from L-arginine in hepatocytes. *Biochem. Biophys. Res. Commun.*, **168**, 1034–1040.

BREDT, D.S. & SNYDER, S.H. (1990). Isolation of nitric oxide synthase, a calmodulin requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 682–685.

BUSSE, R. & MULSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.*, **275**, 87–90.

DENUCCI, G., GRYGLEWSKI, R.J., WARNER, T.D. & VANE, J.R. (1988). Receptor mediated release of endothelium-derived relaxing factor and prostacyclin from bovine endothelial cells are coupled. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 2334–2337.

FEELISH, M. & KELM, M. (1991). Biotransformation of organic nitrates to nitric oxide by vascular smooth muscle and endothelial cells. *Biochem. Biophys. Res. Commun.*, **180**, 286–293.

FORSTERMANN, U., GORSKY, L.D., POLLOCK, J.S., SCHMIDT, H.H.H.W., HELLER, M. & MURAD, F. (1991). Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 1788–1792.

GRYGLEWSKI, R.J., MONCADA, S. & PALMER, R.M.J. (1986). Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine endothelial cells. *Br. J. Pharmacol.*, **87**, 685–694.

HARPER, J.F. & BROOKER, G. (1975). Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'O acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.*, **1**, 207–218.

HUTCHESON, I.R. & GRIFFITH, T.M. (1991). Release of endothelium-derived relaxing factor is modulated both by frequency and amplitude of pulsatile flow. *Am. J. Physiol.*, **261** (*Heart. Circ. Physiol.*, **30**), H257–H262.

HUTCHINSON, P.J.A., PALMER, R.M.J. & MONCADA, S. (1987). Comparative pharmacology of EDRF and nitric oxide on vascular strips. *Eur. J. Pharmacol.*, **141**, 445–451.

KWON, N.S., NATHAN, C.F., GILKER, C., GRIFFITH, O.W., MATTHEWS, D.E. & STUEHR, D.J. (1990). L-Citrullin production from L-arginine by macrophage nitric oxide synthase: the ureido oxygen derives from dioxygen. *J. Biol. Chem.*, **265**, 13442–13445.

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.

MULSH, A. & BUSSE, R. (1990). N^G-nitro-L-arginine (N^G-[immino (nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 143–147.

MYERS, P.R., GUERRA, R. & HARRISON, D.G. (1989). Release of NO and EDRF from cultured bovine endothelial cells. *Am. J. Physiol.*, **256** (*Heart Circ. Physiol.*, **25**), H1030–H1037.

MYERS, P.R., MINOR, R.L., GUERRA, R., BATES, J.N. & HARRISON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature*, **345**, 161–163.

NATHAN, C.F. & HIBBS, J.B. (1991). Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opinion Immunol.*, **3**, 65–70.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M.J., FERRIDGE, A.G. & MONCADA, S. (1987). Nitric oxide release from vascular endothelial cells accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br. J. Pharmacol.*, **92**, 181–187.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 10043–10047.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br. J. Pharmacol.*, **101**, 746–752.

RUBANYI, G.M., JOHNS, A., WILCOX, D., BATES, F.N. & HARRISON, D.G. (1991). Evidence that s-nitrosothiol, but not nitric oxide, may be identical with endothelium-derived relaxing factor. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 3), S41–S45.

SALMON, J.A. (1978). A radioimmunoassay for 6-keto-prostagladin $F_{2\alpha}$. *Prostaglandins*, **15**, 383–397.

SALVEMINI, D., MOLLACE, V., PISTELLI, A., ANGGARD, E.E. & VANE, J.R. (1992). Metabolism of glyceryl trinitrate to nitric oxide by endothelial cells and smooth muscle cells and its induction by Eschericha coli lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 982–986.

SNYDER, S.H. & BREDT, D.S. (1991). Nitric oxide as a neuronal messenger. *Trends Pharmacol. Sci.*, **12**, 125–128.

STUEHR, D.J., KWON, N.S., NATHAN, C.F. & GRIFFITH, O.W. (1991). N^G-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J. Biol. Chem.*, **266**, 6259–6263.

VANE, J.R., ANGGARD, E.E. & BOTTING, R.M. (1990). Regulatory functions of the vascular endothelium. *N. Engl. J. Med.*, **323**, 27–36.

WALKER, J.B. & WALKER, M.S. (1959). The enzymatic reduction of hydroxyguanidine. *J. Biol. Chem.*, **234**, 1481–1484.

ZEMBOWICZ, A., HECKER, M., MACARTHUR, H., SESSA, W. & VANE, J.R. (1991). Nitric oxide and another potent vasodilator are formed from N^G-hydroxy-L-arginine by cultured endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11172–11176.

(Received April 5, 1992
Revised July 25, 1992
Accepted July 31, 1992)

Br. J. Pharmacol. (1992), **107**, 1008–1012

Analysis of the agonist activity of fenoldopam (SKF 82526) at the vascular 5-HT_2 receptor

[1]Mark I. Christie, Diane Harper & George W. Smith

Fisons plc, Pharmaceutical Division, Department of Pharmacology, Bakewell Road, Loughborough, Leicestershire LE11 0RH

1 The 5-HT_2 receptor agonist activity of fenoldopam (SKF 82526) was characterized in the rabbit isolated aorta preparation.

2 Fenoldopam was an agonist at the vascular 5-HT_2 receptor with lower affinity and efficacy than the naturally occurring agonist 5-hydroxytryptamine (5-HT). Fenoldopam had an affinity (pK_A) of 5.84 ± 0.04 and efficacy (τ) of 0.57 ± 0.04, whereas 5-HT had a pK_A of 6.65 ± 0.12 and τ of 2.66 ± 0.41.

3 The constrictor effects of fenoldopam and 5-HT were competitively antagonized by the 5-HT_2 antagonist, ketanserin, with pK_B values of 8.81 ± 0.11 and 8.83 ± 0.10 respectively.

4 Prior incubation with fenoldopam produced a concentration-related rightward shift of a subsequent 5-HT concentration-response curve. This inhibition was specific for 5-HT since constrictor responses to angiotensin II were unaffected.

5 This study indicates that the D_1 receptor agonist, fenoldopam, acts as an agonist at the vascular 5-HT_2 receptor, but with an affinity and efficacy less than that of the naturally occurring agonist, 5-HT.

Keywords: Rabbit aorta; 5-HT_2 receptor; fenoldopam

Introduction

Fenoldopam (SKF 82526) is a 3-benzazepine D_1 receptor agonist currently in clinical trials as a novel vasodilator for the treatment of hypertension, congestive heart failure and renal failure. Whilst it is relatively selective as an agonist at the D_1 receptor (Hahn *et al.*, 1982), fenoldopam also acts as an antagonist at α_1- and α_2-adrenoceptors (Ohlstein *et al.*, 1985). More recently, fenoldopam has also been reported to act as an agonist at the 5-HT_2 receptor expressed in canine tracheal smooth muscle (Gretler *et al.*, 1992). In order to analyse fully the activity of fenoldopam at the vascular 5-HT_2 receptor, we examined its properties in the endothelium-denuded rabbit aortic ring preparation. This preparation was chosen because its response to 5-hydroxytryptamine (5-HT) is consistent and well characterized and, in contrast to other preparations such as rat caudal artery, the 5-HT_2 antagonist, ketanserin, behaves as a simple competitive antagonist (Leff & Martin, 1986).

A preliminary report of these results has been presented to the British Pharmacological Society (Christie & Smith, 1991).

Methods

Tissue preparation

New-Zealand White/Half-lop rabbits (1.9–3.5 kg) were killed by an overdose of pentobarbitone (100 mg kg^{-1}) injected into a marginal ear vein. The thoracic aorta was removed, cleaned of excess fat and connective tissue, everted and the intimal surface rubbed with a moistened cotton bud to remove the endothelium. After re-eversion the aorta was cut into 2–3 mm wide rings and mounted on parallel wires in 30 ml tissue baths containing ascorbic Krebs solution of the following composition (mM); NaCl 117.56, NaH_2PO_4 0.89, $NaHCO_3$ 25.0, $MgSO_4$ 1.18, D-glucose 11.1, KCl 5.36, $CaCl_2$ 2.55 and L-ascorbic acid 1.1, maintained at 37°C and gassed with 95% O_2: 5% CO_2. Isometric tension changes were detected with a force transducer attached to the top wire and recorded on a chart recorder.

[1] Author for correspondence.

Experimental protocols

Aortic rings were equilibrated for approximately 60 min with frequent washing and re-adjustment to a resting tension of 2 g until a steady tension was maintained. They were then challenged with a maximally effective concentration of 5-HT (10 μM) or angiotensin II (0.1 μM) followed by acetylcholine (1 μM) to confirm the absence of endothelium. Any rings that relaxed in response to acetylcholine were discarded. This initial constrictor response to 5-HT or angiotensin II was used as a reference to correct for differences in tension developed by different tissues. After washout and return to resting tension, rings were exposed to the irreversible α_1-adrenoceptor antagonist, benextramine (10 μM) and the cyclo-oxygenase inhibitor, indomethacin (3 μM) for 30 min. The tissues were then washed four or five times before one of the following protocols was employed.

Fenoldopam affinity estimation These studies were carried out using a paired curve protocol. After construction of a control cumulative concentration-effect (E/[A], where E is the pharmacological effect and [A] is the concentration of the agonist) curve to 5-HT (0.01–10 μM) followed by wash-out and return of tension to baseline, tissues were exposed to either incubation with the irreversible antagonist phenoxybenzamine (0.1 μM for 30 min) followed by a second E/[A] curve to 5-HT (0.03–30 μM), incubation with vehicle for 30 min followed by a second E/[A] curve to 5-HT (0.01–10 μM) or a cumulative E/[A] curve to fenoldopam (0.1–100 μM).

Antagonism of constrictor responses by ketanserin A single curve protocol was used in this series of experiments, as pilot experiments indicated that constrictor responses to fenoldopam were not readily reversed after washout. Cumulative E/[A] curves to 5-HT (0.01–300 μM) or fenoldopam (0.1–300 μM) were constructed 45 min after the addition of either vehicle or ketanserin (3–100 nM).

Interaction between fenoldopam and 5-HT These studies were also carried out with a single curve protocol. Cumulative E/[A] curves to 5-HT (0.01–100 μM) or angiotensin II (0.1–30 nM) were constructed 45 min after the addition

of vehicle or as soon as the initial constriction to fenoldopam (10–100 μM) had plateaued.

Drugs used

Acetylcholine chloride, angiotensin II, benextramine hydrochloride, 5-hydroxytryptamine (creatinine sulphate complex) and indomethacin were purchased from Sigma Chemical Co. Ltd., Poole, Dorset. Ketanserin tartrate and phenoxybenzamine hydrochloride were purchased from Semat technical (UK) Ltd., St. Albans, Herts. Fenoldopam methane sulphonate was a gift from SmithKline Beecham Ltd. Ketanserin, phenoxybenzamine and indomethacin were dissolved in ethanol, benextramine and 5-HT dissolved in Krebs solution and fenoldopam dissolved in acidified distilled water to give stock solutions of 30 mM. Angiotensin II was dissolved in distilled water to give a stock solution of 10 mM. Bath concentrations of ethanol did not exceed 0.43% and addition of the maximum vehicle volumes to the tissue bath produced no constrictor effects.

Analysis of data

Fenoldopam affinity estimation A combination of analytical methods was employed–the receptor inactivation method (Furchgott, 1966) to obtain the system parameters and a modification of the comparative method (Barlow *et al.*, 1967) to establish the affinity and efficacy of fenoldopam at the 5-HT$_2$ receptor:

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (1)$$

in which E is the pharmacological effect, [A] is the concentration of the agonist, E_m is the maximum possible effect in the system, K_A is the agonist dissociation constant (estimated as the negative logarithm, that is, pK_A), τ is the efficacy of the agonist (estimated as a logarithm) and n is the slope of the occupancy-effect relation.

The comparative method for K_A estimation depends on the comparison of a partial agonist with a full agonist. In operation model terms a full agonist is need to provide estimates of E_m and n in equation (1) so that K_A and τ for a partial agonist can be estimated (Leff *et al.*, 1989). 5-HT is not a full agonist in this tissue (Black *et al.*, 1985) and thus could not be used directly to analyse fenoldopam. However, it has earlier been shown that E_m and n estimates can be obtained in these circumstances by using the receptor inactivation method allowing the comparative analysis to proceed (Black *et al.*, 1985). Four curves, the E/[A] data for 5-HT, in the absence and presence of receptor inactivation, together with the E/[A] data for 5-HT and fenoldopam, were fitted simultaneously to the above equation providing a common estimate of E_m and n along with a value of τ for each curve in the set. The K_A for 5-HT was assumed to be the same for the three 5-HT curves: a different K_A was allowed for fenoldopam.

The data from the receptor inactivation method enabled E_m and n to be estimated whereas the data from the fenoldopam curve gave the additional information needed to produce the K_A of fenoldopam.

All data fitting procedures were carried out with the BMDP statistical package on a Vax 11/780 mainframe computer. Constrictor responses to 5-HT and fenoldopam were expressed as a percentage of the control 5-HT concentration-effect curve maximum. Results are expressed as mean ± s.e.mean.

Antagonism of constrictor responses by ketanserin, and full/partial interaction Tissue constrictor responses were expressed as a percentage of the initial constrictor response to 5-HT (10 μM) or angiotensin II (0.1 μM), and the experimental data fitted to the following logistic equation:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \quad (2)$$

where [A] is the concentration of the agonist, m is the slope parameter, α represents the asymptote and the $[A_{50}]$ is estimated as a logarithm.

The pK_B values for ketanserin and (in the full/partial interaction study) pA_2 values for fenoldopam were determined by fitting the computed log $[A_{50}]$ values to a linear form of the Schild equation as previously described (Leff & Martin, 1986). One way analysis of variance was used to test for treatment effects on E/[A] curve $[A_{50}]$'s, asymptotes and slopes, and statistical significance assigned at the $P<0.05$ level. Results are expressed as mean ± s.e.mean.

Results

Fenoldopam affinity estimation

Both 5-HT and fenoldopam produced smooth monophasic E/[A] curves, with fenoldopam acting as a partial agonist in this system, approximately 30 times weaker than 5-HT (Figure 1). The constrictor responses to fenoldopam developed more slowly than those to 5-HT and were less readily reversed by wash-out.

Incubation with phenoxybenzamine produced a rightward shift and depression of the subsequent E/[A] curve to 5-HT, an interaction typical of an irreversible receptor antagonist (Figure 1). However, incubation with vehicle for 45 min did not significantly alter the agonist parameters of the second 5-HT E/[A] curve: the first 5-HT curve had a $p[A_{50}]$ of 6.88 ± 0.05 and a maximum of 100% and the second 5-HT curve a $p[A_{50}]$ of 6.80 ± 0.04 and a maximum of 106.4 ± 2.3% (data from 6 experiments).

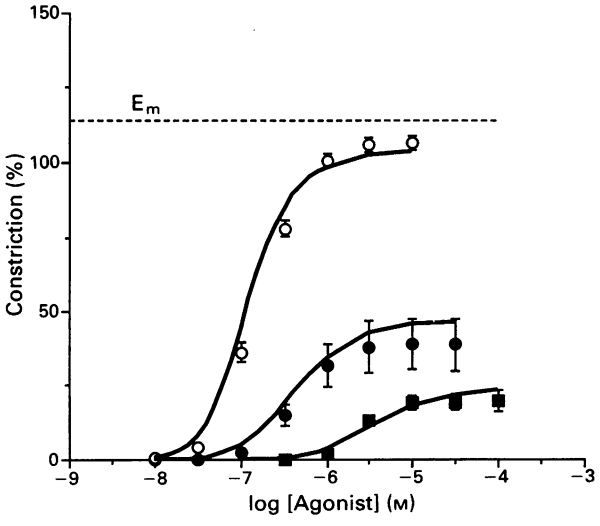

Figure 1 Constrictor responses of rabbit aortic rings expressed as a percentage of the initial 5-hydroxytryptamide (5-HT) concentration-response curve maximum. Each point represents the mean of six experiments; vertical lines show s.e.mean. 5-HT control (○); 5-HT after incubation with phenoxybenzamine 0.1 μM for 30 min (●); fenoldopam (■). The lines fitted through the data are calculated according to equation (1) in the text, E_m indicating the calculated maximum possible response in the system. The maximum constrictor response to 5-HT for all tissues was 5.56 ± 0.42 g.

Table 1 Parameter estimates for 5-hydroxytryptamine (5-HT) and fenoldopam at the rabbit aorta 5-HT_2 receptor

Parameter	*Fenoldopam*	*5-HT*
τ	0.57 ± 0.04	2.66 ± 0.41
pK_A	5.84 ± 0.04	6.65 ± 0.12

Curves were fitted to equation (1) expressing responses as a percentage of the control 5-HT concentration response curve maximum. The system had an E_m (system maximum) of $114.1 \pm 3.4\%$ and n (slope) of 2.37 ± 0.15. Agonist dissociation constants are given as the negative logarithm of the K_A and efficacies as the logarithm of τ. The results are the mean ± s.e.mean of 6 experiments.

The system and agonist parameters, calculated according to equation (1) in the methods section, are summarized in Table 1. Note that in the case of 5-HT, its asymptote (100%) was less than the calculated E_m (indicating that it generated only 88% of the maximal possible response), confirming that 5-HT acts as a partial agonist in this system.

Antagonism of constrictor responses by ketanserin

Prior incubation with the 5-HT_2 receptor antagonist, ketanserin, produced a concentration-related rightward shift of the subsequent E/[A] curve to 5-HT and fenoldopam. The Schild slopes were not significantly different from unity (0.89 ± 0.13 and 0.97 ± 0.32 for 5-HT and fenoldopam respectively), indicating that the antagonism was competitive in nature with pK_B values against 5-HT and fenoldopam of 8.83 ± 0.05 and 8.81 ± 0.06 respectively (pooled data from 3–4 experiments). The initial constrictor responses to 5-HT for all tissues used in 5-HT and fenoldopam experiments were 3.40 ± 0.38 g and 3.65 ± 0.18 g respectively.

Interaction between fenoldopam and 5-HT or angiotensin II

Prior incubation with fenoldopam (10–100 μM) produced a concentration-related rightward shift of the subsequent E/[A] curve to 5-HT (Figure 2). However, the interaction between fenoldopam and 5-HT indicated that fenoldopam was acting non-competitively at the 5-HT_2 receptor, as all concentrations of fenoldopam used produced a statistically significant reduction ($P<0.05$) in the maximum response to 5-HT. This non-competitive behaviour precluded the quantitative estimate of a pK_B for fenoldopam: empirically Schild analysis gave an estimated pA_2 value for fenoldopam of 5.23 ± 0.23 and a slope of 1.20 ± 0.05, significantly greater than unity ($P<0.05$) (data from 6 experiments).

Prior incubation with fenoldopam (100 μM) did not alter the $[A_{50}]$ or the maximum response of a subsequent angiotensin II E/[A] curve, thus showing that the inhibition of the 5-HT response (Figure 2) was specific: the control angiotensin II E/[A] curve had a $p[A_{50}]$ of 8.59 ± 0.10 and a maximum of $78.4 \pm 8.3\%$, and the post fenoldopam angiotensin E/[A] curve a $p[A_{50}]$ of 8.74 ± 0.11 and a maximum of $97.0 \pm 14.6\%$ (data from 5 experiments). The results are shown in Figure 3.

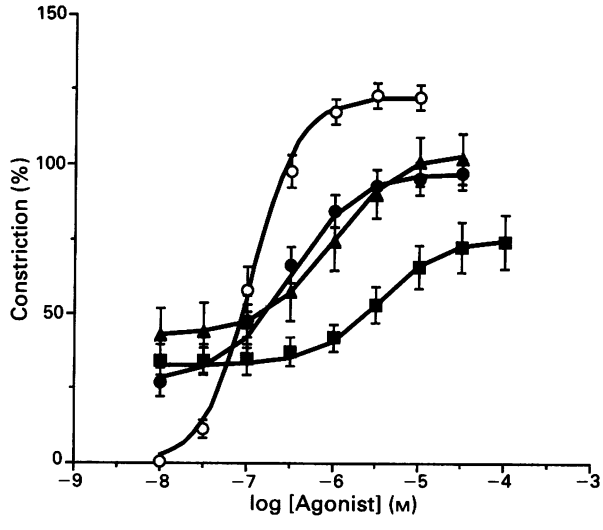

Figure 2 Constrictor responses of rabbit aortic rings expressed as a percentage of the initial constrictor response to 5-hydroxytryptamine (5-HT). Cumulative concentration-response curves to 5-HT after incubation with either vehicle (○) or fenoldopam 10 μM (●), 30 μM (▲) or 100 μM (■). Each point represents the mean of six experiments; vertical lines show s.e.mean. The lines fitted through the data are calculated according to equation (2) in the text. The initial constrictor response to 5-HT for all tissues was 4.20 ± 0.21 g.

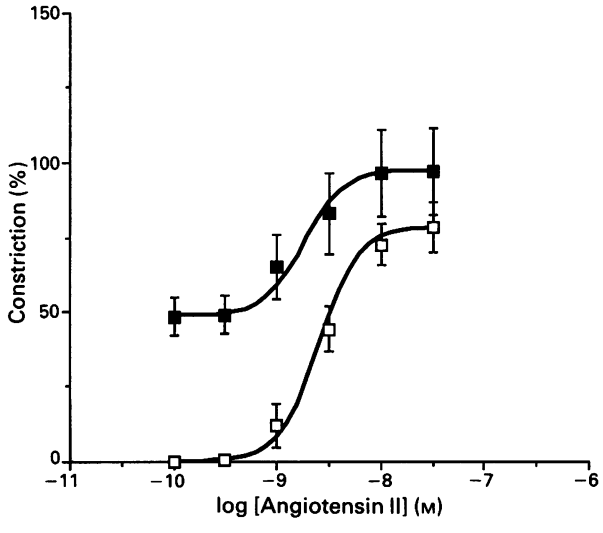

Figure 3 Constrictor responses of rabbit aortic rings expressed as a percentage of the initial constrictor response to angiotensin II. Cumulative concentration-response curves to angiotensin II after incubation with either vehicle (□) or fenoldopam 10 μM (■). Each point represents the mean of five experiments; vertical bars show s.e.mean. The lines fitted through the data are calculated according to equation (2) in the text. The initial constrictor response to angiotensin II for all tissues was 5.26 ± 1.45 g.

Discussion

In addition to acting as an antagonist at the peripheral D_1-receptor (Hahn *et al.*, 1982), fenoldopam (SKF 82526) shows significant antagonism at vascular α_2-adrenoceptors (dog saphenous vein pA_2 of 7.78, Ohlstein *et al.*, 1985) with a lower affinity for vascular α_1-adrenoceptors (rabbit aorta pA_2 of 5.41, Ohlstein *et al.*, 1985). Following a recent report indicating that fenoldopam acts at the canine tracheal smooth muscle 5-HT receptor (Gretler *et al.*, 1992), we have shown that fenoldopam is also an agonist at the well-characterized rabbit aorta vascular 5-HT_2-receptor. The constrictor effects of fenoldopam are unlikely to be mediated by

D_1 agonism as dopamine was inactive in this preparation at up to 100 μM (data not shown). This study extends the structure-activity relations reported for other benzazepines structurally related to fenoldopam such as SCH 23390 and SKF 83566 which are active as antagonists at the vascular 5-HT$_2$-receptor (Ohlstein & Berkowitz, 1985) and our observations with another benzazepine, SKF 38393 (Christie M.I. & Hollick L.J., unpublished), which acts as a weak, competitive 5-HT$_2$ antagonist with a pK_B of 4.76 ± 0.16 (data from 4 experiments). Furthermore this study confirms the finding with SKF 87516 (Le Monnier de Gouville *et al.*, 1991) that compounds in the benzazepine series may also act as agonists at the vascular 5-HT$_2$ receptor.

Our results with fenoldopam are qualitatively similar to those obtained in canine tracheal smooth muscle (Gretler *et al.*, 1992), in that constrictor responses are slow in onset and not readily reversed by washout. In both studies fenoldopam has a lower affinity and efficacy than the naturally occurring agonist 5-HT, such that any 5-HT$_2$ receptor agonism produced by fenoldopam will be dependent on the tissue receptor reserve as well as the concentration of fenoldopam. The results reported by Gretler *et al.* (1992) suggest that the canine tracheal smooth muscle has a higher 5-HT receptor reserve than the rabbit aorta as 5-HT appears more potent, with a p[A_{50}] of 7.08. This may also explain the higher intrinsic activity of fenoldopam (ca. 84% of the maximum response to 5-HT) in the canine trachea. However, both studies are consistent with fenoldopam acting as a low efficacy agonist at the 5-HT$_2$ receptor.

We obtained two estimates of the affinity of fenoldopam for the 5-HT$_2$ receptor, one from the comparative analysis (pK_A of 5.84 ± 0.04) and one from Schild analysis of the full/partial interaction between 5-HT and fenoldopam (apparent pA_2 of 5.23 ± 0.23). The Schild result is likely to be an unreliable estimate of the affinity of fenoldopam for the 5-HT$_2$ receptor for three reasons: firstly fenoldopam caused depression as well as rightward shift of subsequent 5-HT curves and therefore did not satisfy the criteria for competitive antagonism, secondly the agonism caused by fenoldopam introduces a slight error in the pA_2 value (Kenakin, 1987) and thirdly, the steep transducer slope of this system introduces another source of error in full/partial interaction studies (Leff P., unpublished). We therefore believe that the pK_A value for fenoldopam obtained by the comparative method is the most accurate estimate of its affinity for the 5-HT$_2$ receptor.

The 5-HT$_2$ agonist activity of fenoldopam is confirmed by the blockade of the effects of fenoldopam by the 5-HT$_2$-receptor antagonist, ketanserin, with a pK_B value against fenoldopam of 8.81, similar to that obtained against 5-HT in this study (8.83), by Leff & Martin (1986) against 5-HT in the rabbit aorta (8.56 ± 0.09) and by Gretler *et al.* (1992) against fenoldopam in the canine trachea (8.37). Ketanserin also has affinity for α_1-adrenoceptors, but the antagonism of fenoldopam by ketanserin is unlikely to be due to either direct or indirect (as occurs with 5-HT, due to a tyramine-like effect) α_1-adrenoceptor antagonism, as the benextramine pretreatment used throughout this study effectively abolished the α_1-adrenoceptor-mediated constrictor response to phenylephrine (> 20,000 fold rightward shift, data not shown).

Further confirmation that 5-HT and fenoldopam act at the same receptor was provided by the observation that fenoldopam produced a concentration-related rightward shift of subsequent 5-HT E/[A] curves. The reduction in the maximum response to 5-HT indicates that a component of the action of fenoldopam is non-competitive in nature, but whether this is due to desensitization of the 5-HT-mediated contractile response in this tissue (Ben-Harari *et al.*, 1991) or irreversible binding to the receptor is unclear. However, the lack of effect of fenoldopam on constrictor responses to angiotensin II indicates that the effects of fenoldopam are specific for the 5-HT$_2$ receptor.

In conclusion, these data confirm that fenoldopam is a low efficacy 5-HT$_2$-receptor agonist that displays characteristics of partial agonism in the rabbit aorta. Thus whilst fenoldopam acts primarily as a vasodilator *in vivo* (Hahn *et al.*, 1982), our evidence suggests that in states where the 5-HT$_2$ receptor-mediated constrictor response is functionally up-regulated, such as following endothelial loss (Cocks & Angus, 1983) or vascular smooth muscle damage (De Meyer *et al.*, 1990; Sobey *et al.*, 1991) fenoldopam may be capable of producing localized vasoconstriction *via* 5-HT$_2$ receptor agonism. Conversely in tissues where the 5-HT$_2$ receptor reserve is low, fenoldopam would be expected to behave as a 5-HT$_2$ receptor antagonist.

The authors thank Mrs A. Verity and Miss L.J. Hollick for technical assistance, and Dr P. Leff for his constructive criticism of the manuscript.

References

BARLOW, R.B., SCOTT, N.C. & STEPHENSON, R.P. (1967). The affinity and efficacy of onium salts on the frog rectus abdominis. *Br. J.Pharmacol. Chemother.*, **31**, 188–196.

BEN-HARAR, R.R., DALTON, B.A., OSMAN, R. & MAAYANI, S. (1991). Kinetic characterisation of 5-hydroxytryptamine receptor desensitisation in isolated guinea-pig trachea and rabbit aorta. *J. Pharmacol Exp. Ther.*, **257**, 416–424.

BLACK, J.W., LEFF, P., SHANKLEY, N.P. & WOOD, J. (1985). An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. *Br. J. Pharmacol.*, **84**, 561–571.

CHRISTIE, M.I. & SMITH, G.W. (1991). Fenoldopam (SKF 82526) is a low efficacy agonist at the 5-HT$_2$ receptor. *Br. J. Pharmacol.*, **104**, 108P.

COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305**, 627–630.

DE MEYER, G.R.Y., BULT, H., MARTIN, J.F., VAN HOYDONCK, A. & HERMAN, A.G. (1990). The effect of a developing neo-intima on serotonergic and adrenergic contractions. *Eur. J. Pharmacol.*, **187**, 519–524.

FURCHGOTT, R.F. (1966). The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor agonist complexes. *Adv. Drug Res.*, **3**, 21–55.

GRETLER, D.D., JONES, K.C. & MURPHY, M.B. (1992). 5-hydroxytryptamine receptor activity of the dopamine receptor agonist fenoldopam in canine tracheal smooth muscle. *J. Pharmacol. Exp. Ther.*, **260**, 491–498.

HAHN, R.A., WARDELL, J.R., SARAU, H.M. & RIDLEY, P.T. (1982). Characterisation of the peripheral and central effects of SK&F 82526, a novel dopamine receptor agonist. *J. Pharmacol. Exp. Ther.*, **223**, 305–313.

KENAKIN, T.P. (1987) In *Pharmacologic Analysis of Drug-Receptor Interaction*. New York: Raven press.

LE MONNIER DE GOUVILLE, A., LAWSON, K., THIRY, C. & CAVERO, I. (1990). SK&F 87516, a close analogue of fenoldopam, is a partial agonist at dopamine-1 and alpha-2 receptors and produces stimulation of 5-hydroxytryptamine-2 receptors in the cardiovascular system of the rat. *J. Pharmacol. Exp. Ther.*, **256**, 1049–1056.

LEFF, P. & MARTIN, G.R. (1986). Peripheral 5-HT$_2$ receptors. Can they be classified with the available antagonists? *Br. J. Pharmacol.*, **88**, 585–593.

LEFF, P., PRENTICE, D.J., GILES, H., MARTIN, G.R. & WOOD, J. (1989). Estimation of agonist affinity and efficacy by direct, operational model fitting. *J. Pharmacol. Methods*, **23**, 225–237.

OHLSTEIN, E.H. & BERKOWITZ, B.A. (1985). SCH 23390 and SK&F 83566 are antagonists at vascular dopamine and serotonin receptors. *Eur. J. Pharmacol.*, **108**, 205–208.

OHLSTEIN, E.H., ZABKO-POTAPOVICH, B. & BERKOWITZ, B.A. (1985). The DA_1 receptor agonist fenoldopam (SK&F 82526) is also an α2-adrenoreceptor antagonist. *Eur. J. Pharmacol.*, **118**, 321–329.

SOBEY, C.G., DUSTING, G.J. & WOODMAN, O.W. (1991). Enhanced vasoconstriction by serotonin in rabbit carotid arteries with atheroma-like lesions *in vivo*. *Clin. Exp. Pharmacol. Physiol.*, **18**, 367–370.

(Received May 12, 1992
Revised July 30, 1992
Accepted July 31, 1992)

Br. J. Pharmacol. (1992), **107**, 1013–1019

Differential sensitivities of the prostacyclin and nitric oxide biosynthetic pathways to cytosolic calcium in bovine aortic endothelial cells

Heydar Parsaee, Jean R. McEwan, Sunil Joseph & [1]John MacDermot

Department of Clinical Pharmacology, Royal Postgraduate Medical School, Ducane Road, London W12 0NN

1 Bovine aortic endothelial cells were cultured *in vitro*, and shown to release both prostacyclin (PGI_2; K_{act} = 24.1 nM) and endothelium-derived relaxing factor (EDRF, NO; K_{act} = 0.7 nM) in a concentration-dependent manner when exposed to bradykinin.

2 The bradykinin-dependent release of PGI_2 (but not EDRF) was inhibited by 1 μM isoprenaline or 5 μM forskolin, and the inhibitory effect of isoprenaline could be reversed by the β_2-adrenoceptor antagonist, ICI 118551. In contrast, isoprenaline had no capacity to inhibit PGI_2 release stimulated by exogenous arachidonic acid.

3 Exposure of cells to bradykinin increased the cytosolic concentration of Ca^{2+} ions ($[Ca^{2+}]_i$; K_{act} = 4.8 nM), and the effect was inhibited by both 1 μM isoprenaline and 5 μM forskolin.

4 In similar experiments, exposure of cells to ionomycin also increased $[Ca^{2+}]_i$ and the values of $[Ca^{2+}]_i$ were calibrated in terms of the ionomycin concentration. In subsequent experiments involving exposure of endothelial cells to selected concentrations of ionomycin, it was possible to show that the biosynthesis of NO was triggered at ionomycin concentrations about one tenth of that required for PGI_2 biosynthesis and that these corresponded to a $[Ca^{2+}]_i$ threshold of 350 nM for PGI_2 release while that for EDRF release was less than 200 nM.

5 These differences in Ca^{2+} ion sensitivity explain the selective inhibition of bradykinin-stimulated PGI_2 biosynthesis (to the exclusion of NO biosynthesis) by isoprenaline or forskolin, both of which attenuate bradykinin-dependent increases in $[Ca^{2+}]_i$.

Keywords: Prostacyclin; endothelium-derived relaxing factor; endothelium; calcium; β_2-adrenoceptors; NO synthase; phospholipase A_2

Introduction

Endothelial cells are located on the luminal surface of blood vessels and play an important role in the regulation of vascular tone. At least two major dilator substances are released, namely prostacyclin (epoprostenol, PGI_2) (Moncada *et al.*, 1976), and endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), which has now been identified as nitric oxide (NO) (Palmer *et al.*, 1987). PGI_2 and EDRF are both released from endothelial cells following activation of phospholipase C by agonists such as bradykinin, ADP or thrombin (de Nucci *et al.*, 1988). The generation of inositol 1,4,5-*tris*phosphate (IP_3) triggers a transient release of calcium ions from internal stores, leading to a rise in the concentration of calcium ions in the cytosol ($[Ca^{2+}]_i$) (Berridge, 1987). This is then followed by a more sustained increase in $[Ca^{2+}]_i$, which is mediated by influx of Ca^{2+} from outside the cell (Hallam *et al.*, 1988). The other metabolite of phospholipase C activation is diacylglycerol, which mediates activation of protein kinase C (Berridge, 1987).

PGI_2 is an oxidative metabolite of arachidonic acid that is produced by an enzymatic cascade involving cyclo-oxygenase (Moncada *et al.*, 1976). In most tissues the rate limiting step in PGI_2 biosynthesis is the release of arachidonic acid from phospholipid by the activity of phospholipase A_2 (Chang *et al.*, 1987). This enzyme activity is itself triggered by the rise in $[Ca^{2+}]_i$ (Hallam *et al.*, 1988). EDRF is formed from L-arginine by NADH-dependent nitric oxide synthase, which is also a calcium-sensitive enzyme, and is located in the endothelial cytosol (Forstermann *et al.*, 1991). Clearly, both biosynthetic pathways may be activated in parallel by rises in $[Ca^{2+}]_i$ following agonist-dependent stimulation of phospholipase C activity. In addition to the phospholipase C-linked receptors, endothelial cells have receptors linked to activation of adenylate cyclase. These include the β_2-adrenoceptor (Steinberg *et al.*, 1984), a receptor for calcitonin gene-related peptide (CGRP) (Crossman *et al.*, 1987; McEwan *et al.*, 1989) and the adenosine A_2 receptor (Luty *et al.*, 1989). The physiological role of these receptors in the regulation of endothelial cell function and vascular tone is uncertain, but is reported to include regulation of angiotensin converting enzyme activity (Iwai *et al.*, 1987) and inhibition of secretion of tissue-type plasminogen activator (Francis & Neely, 1989).

In the present study we have examined the possibility that adenylate cyclase activation might modify the release of PGI_2 or EDRF. Measurements were made of the release of PGI_2 and EDRF from cultured bovine aortic endothelial cells. We have examined the effects of direct or receptor-mediated activation of adenylate cyclase on the Ca^{2+} transient produced by a phospholipase C-linked agonist, bradykinin (BK). The response was then related to the release of the two vasodilators. Our results demonstrate differential sensitivities of the biosynthetic pathways of PGI_2 and NO to increases in intracellular Ca^{2+} ion concentration. Furthermore, the findings explain how adenylate cyclase activation attenuates PGI_2 but not NO release through a reduction in the magnitude of the calcium transient.

Methods

The Krebs-Henseleit buffer pH 7.4 (components from BDH) contained (mM): $NaHCO_3$ 25, glucose 11, NaCl 118, KCl

[1] Author for correspondence.

4.5, NaH_2PO_4 1.2, $MgSO_4$ $7H_2O$ 1.2 and $CaCl_2$ 1.25. The salts (except $CaCl_2$) were dissolved in distilled water, and thereafter $CaCl_2$ (1 M solution) was added gradually (to avoid precipitation of $Ca_3(PO_4)_2$). The buffer was gassed with 5% CO_2 and 95% air for 30 min before each perfusion experiment. [^{3}H]-6-oxo-$PGF_{1\alpha}$ was obtained from Amersham International, UK; ionomycin and fura 2-AM were obtained from Calbiochem, UK; bradykinin, phenylephrine, N^{ω}-mono-methyl-L-arginine (L-NMMA), isoprenaline and forskolin were obtained from Sigma, UK.

Culture of AG6780 bovine aortic endothelial cells

Bovine aortic endothelial cells (AG7680) at passage 8 were obtained from the National Institute of Aging cell repository (U.S.A.). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 15% foetal calf serum (Gibco), in 75 cm^2 flasks as described previously for AG4762 cells (McEwan *et al.*, 1989). Experiments were performed on cells between passages 13 and 20. The endothelial cells were subcultured in multi-well culture plates, on glass coverslips, or on microcarrier beads as described below.

Subculture of endothelial cells on microcarrier beads

The confluent cells from a 75 cm^2 flask were removed with 0.05% trypsin and 0.02% EDTA, and transferred to a siliconized 125 ml stirring flask (Techne) containing 25 ml of culture medium and 2.5 ml of superbead microcarrier inert beads (Flow Laboratories). The cells were stirred constantly at 37°C, in 95% air and 5% CO_2 until the cells were confluent over the surface of the beads (3–4 days).

Determination of EDRF in a bioassay system

EDRF was measured by a modification of the method described previously (Furchgott & Zawadzki, 1980). A sealed column for cell superfusion was prepared from the barrel of a 2 ml syringe. The rubber bung of the syringe was removed and a small hole made in the centre. Microbore plastic tubing no. 800/100/200, (Portex, UK) was then threaded through, with a 1 ml disposable pipette tip used as a guide. The bottom of the syringe was plugged with a circle of Whatman no.4 filter paper (Whatman Paper Ltd). Under sterile conditions cell-covered beads were transferred from the stirring culture flask to the column until the final packed volume of beads was 0.5 ml, which provided approximately 10^7 cells (60–80 cells per confluent bead). The modified bung with tubing was inserted into the barrel, and pushed down until it rested on the top of a second circle of filter paper resting on top of the beads. The column was then placed in a warm jacket (37°C), and perfused before any measurements for at least 30 min. The direction of flow of Krebs-Henseleit buffer (which had previously been warmed and gassed with 5% CO_2 and 95% air) was from the bottom upwards, at a rate of 2 ml min^{-1}.

Ring segments of rat thoracic aorta (4 mm thickness) were cut, dissected free of fat and connective tissues, and the endothelium removed by gentle rotation of the ring around a closed pair of fine forceps. The rings were mounted on hooks attached to a force transducer (Grass model FT 03C), connected to a polygraph (Grass model 79D) and a recorder (Grass Instrument Co.), which had been calibrated previously with a 2 g weight. The rings were superfused at 2 ml min^{-1} with Krebs-Henseleit buffer, pH 7.4, gassed with 5% CO_2 and 95% air, at 37°C.

Initially, the ring was set to a resting tension of 1.5 g, and allowed to relax for 2 h, after which the resting tension fell to approximately 1 g. The contractility of the rings was confirmed by superfusion for 30 s with Krebs-Henseleit buffer containing a high potassium concentration (140 mM) and low sodium concentration (26 mM). Thereafter, the rings were again superfused with authentic Krebs-Henseleit buffer, and then contracted by the addition of 1 μM phenylephrine.

The lack of relaxation of the precontracted rings on direct stimulation with bradykinin confirmed that the endothelium had been removed. The eluate from columns of cell-covered beads (2 ml min^{-1}) was then dripped on to the aortic ring, and 1 μM phenylephrine in the buffer originally bathing the ring was replaced with 2 μM phenylephrine, so that the final phenylephrine concentration on the rings was maintained at 1 μM. The columns of cell-covered beads were exposed to bradykinin or ionomycin for periods of 45 s, and the superfusate dripped on to the rings for measurement of EDRF release. Relaxation was expressed as mm movement of the pen recorder needle towards the ground state (i.e. before addition of phenylephrine to the endothelium-free rat aortic ring), or as the % inhibition of the constriction mediated by phenylephrine.

Two separate protocols were employed (each in triplicate), and one of a pair of rings was used as a control in each experiment. In the first protocol, the endothelial cells on the columns were stimulated three times with bradykinin (10–100 nM). Thereafter, the column perfusates were isolated from the aortic rings, and the endothelial cells superfused with plain buffer (control) or buffer containing forskolin (5 μM) or isoprenaline (1 μM) for selected times. After a brief washout, the column eluates were then re-directed to the rings, and the endothelial cells exposed again to the same concentrations of bradykinin. In the second protocol (see below), the endothelial cells on the columns were stimulated with selected concentrations of ionomycin (10–500 nM), and the release of EDRF was measured. Small amounts of superfusate were diverted from the columns (before reaching the aortic rings) for the simultaneous measurement of PGI_2 and EDRF release.

Prior perfusion of the column with N^{ω}-mono-methyl-L-arginine (L-NMMA), or simultaneous superfusion of the aortic ring with haemoglobin, abolished the relaxation of the phenylephrine-precontracted ring mediated by the eluate from the column of endothelial cells which had been exposed to bradykinin or ionomycin. The findings confirmed that relaxation of the ring was mediated by EDRF.

Prostacyclin determination

Bovine aortic endothelial cells were subcultured in 12 well culture plates (23 mm diam. Flow Laboratories, U.K.), and used at confluence (3–4 days in culture, 4.5×10^5 cells per well). The culture medium was replaced 24 h before any experiment. The experiments were performed in a warm box at 37°C and in an atmosphere of 5% CO_2 in air, using methods described by Carter *et al.* (1988). The culture medium was replaced with serum-free 25 mM HEPES-buffered DMEM, pH 7.4. The medium was replaced carefully with 1 ml of the same medium after an interval of 5 min. Five minutes later, 0.2 ml of supernatant was removed (sample A) and another 0.2 ml of plain medium or medium containing 'pretreatment' reagents was added to each well. After 30 min, 0.2 ml of the supernatant was removed (sample B), and another 0.2 ml of medium with or without bradykinin or ionomycin was added. Finally 0.2 ml of supernatant was removed after another 5 min (sample C). The release of PGI_2 was measured as the difference in concentration between samples B and C. There was little or no difference in the levels measured in samples A and B. This relatively complex protocol was adopted to confirm that the measured release of PGI_2 was not due simply to excitation of the cells during sampling of the medium.

In other experiments, PGI_2 release from endothelial cells was determined using the cell-coated beads on columns (prepared as described above). In experiments where only PGI_2 release was examined, the flow rate of buffer was 0.5 ml min^{-1}, and at this perfusion rate the volume of the system was such that it took 2.5 min to perfuse end to end.

In all experiments the column was perfused initially for 30 min to allow the system to return to a stable state. Fractions of the column eluate were collected for periods of 1 min, and these were stored for the later measurement of 6-oxo-$PGF_{1\alpha}$. A three-way tap permitted switching of the column perfusate between plain buffer and buffer containing the particular pharmacological agents.

All supernatant and eluate samples were stored at − 20°C until required for measurement of 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$), the stable hydrolysis product of prostacyclin. The measurement involved specific radioimmunoassay, and the antibody to 6-oxo-$PGF_{1\alpha}$ was a generous gift from Dr Susan Barrow (UMDS, University of London).

Measurement of free intracellular calcium $[Ca^{2+}]_i$

AG7680 endothelial cells were cultured on 10 mm glass coverslips (Chance Proper) contained in 17 mm wells. The cells were grown to confluence, and the medium changed 24 h before the cells were studied by a modification of the method described by Hallam *et al.* (1988). Cell monolayers were incubated in the dark with the fluorescent calcium-sensitive dye, fura 2-acetoxy-methyl ester (4 μM fura 2-AM) at 37°C for 30 min (10 mM stock of fura 2-AM in dimethylsulphoxide was diluted in culture medium, with a final dimethylsulphoxide concentration of 0.05% (v/v)).

The coverslips were washed briefly 3 times with Krebs-Henseleit buffer, and left at room temperature until ready for use. The coverslip was mounted in a slide holder and placed diagonally (~40°) in a quartz cuvette (Helma), containing 1.2 ml Krebs-Henseleit buffer. The cuvette was placed in a 37°C thermostatted holder in a Shimadzu RF 5000 spectrophotofluorimeter and the buffer stirred continuously with a magnetic stir-bar. The fluorescence intensity of fura-2 was recorded with dual-wavelength excitation (340 nm and 380 nm) alternating at 2 s intervals, and emission measured at 500 nm. The ratio of the two fluorescent signals was used to calculate $[Ca^{2+}]_i$ from the equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_{f2}}{S_{b2}}$$

R_{min} and R_{max} are the minimum and maximum ratio of fluorescence intensity of fura-2 in a Ca^{2+}-free solution with 25 mM EGTA (Sigma), or in a Ca^{2+}-saturated solution with 1.25 mM Ca^{2+} respectively. S_{f2} and S_{b2} are the fluorescence values of fura-2 at 380 nm in a Ca^{2+}-free solution with 25 mM EGTA, and a Ca^{2+}-saturated solution with 1.25 mM Ca^{2+} respectively. The K_d value for the fura-2/Ca^{2+} complex is 224 nM at 37°C. To calibrate the fluorescence signal in each experiment, R_{max} was obtained by applying the calcium ionophore, ionomycin (4 μM) in the presence of external calcium (1.25 mM), and R_{min} was obtained by removal of Ca^{2+} with 25 mM EGTA.

Statistical analysis

Data are expressed as mean ± standard error of the mean (s.e.mean) and compared by non-paired Student's *t* tests. Values of $P \leq 0.05$ are accepted as significant.

Results

Release of PGI_2 from cells cultured on multiwell plates

The release of PGI_2 from endothelial cells grown in multiwell plates (3 or 4 replicates) was determined in the absence or presence of bradykinin at selected concentrations (1–100 nM). The accumulation of 6-oxo-$PGF_{1\alpha}$ was plotted as a function of bradykinin concentration, which revealed a concentration-dependent increase in 6-oxo-$PGF_{1\alpha}$ production by bradykinin (Figure 1). Basal production of 6-oxo-$PGF_{1\alpha}$ was 369 ± 164 pg min^{-1} per well, and the maximum stimulated release was 5120 ± 361 pg min^{-1} per well. The concentration of bradykinin for half-maximum stimulation (K_{act}) was 24.1 ± 1.2 nM.

Effect of forskolin or isoprenaline on the release of PGI_2 from endothelial cells

The effect of activation of adenylate cyclase on bradykinin-stimulated release of PGI_2 was determined in further experiments. Incubation of endothelial cells with 5 μM forskolin (which serves to activate adenylate cyclase directly) for 30 min reduced the production of 6-oxo-$PGF_{1\alpha}$ in the presence of 100 nM bradykinin from 2026 ± 251 to 367 ± 198 pg min^{-1} per well ($P<0.001$). Incubation of endothelial cells with 1 μM isoprenaline for 30 min also reduced the bradykinin-stimulated generation of 6-oxo-$PGF_{1\alpha}$ from a mean value of 2026 ± 251 to 1219 ± 149 pg min^{-1} per well ($P<0.05$). In other experiments, 1 μM isoprenaline was shown to have no effect on the basal production of 6-oxo-$PGF_{1\alpha}$.

The effect was also measured of isoprenaline or forskolin-pretreatment on the bradykinin-stimulated release of PGI_2 from endothelial cells on a column. Parallel columns of endothelial cells were exposed to 100 nM bradykinin for 3 min and the concentration of 6-oxo-$PGF_{1\alpha}$ quantified in the eluate. The results were compared with the release of 6-oxo-$PGF_{1\alpha}$ from those columns superfused previously for 30 min with buffer containing 1 μM isoprenaline or 5 μM forskolin. Figure 2 is typical of 3 similar experiments, and shows the inhibition of the bradykinin-stimulated release of 6-oxo-$PGF_{1\alpha}$ by pretreatment of AG7680 endothelial cells with 1 μM isoprenaline for 30 min. Similar inhibition was seen after treatment of the cells (at a higher passage, on a separate occasion) with 5 μM forskolin for 30 min (result not shown).

To confirm that the isoprenaline-induced inhibition of 6-oxo-$PGF_{1\alpha}$ release was mediated by β-adrenoceptors, the effect of ICI 118551 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol; a β_2-selective adrenoceptor antagonist) (Stadel *et al.*, 1987) was examined on cells cultured in wells. The inhibitory effect of isoprenaline on the release of PGI_2 was reversed with increasing concentrations of ICI 118551 (Figure 3). The addition of 1 μM ICI 118551 alone to unstimulated cells did not change significantly the production of 6-oxo-$PGF_{1\alpha}$.

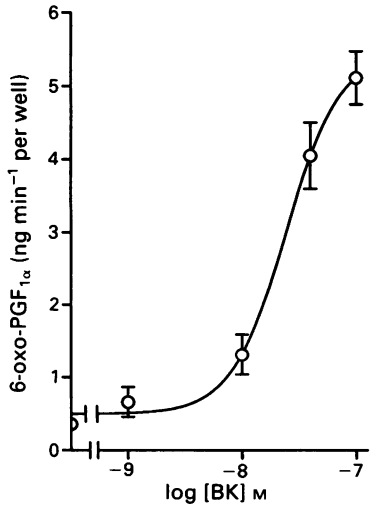

Figure 1 Concentration-response curve of the bradykinin (BK)-dependent release of 6-oxo-$PGF_{1\alpha}$ from AG7680 bovine aortic endothelial cells. Cells were cultured in multi-well plates, and exposed to selected concentrations of bradykinin, and the release of 6-oxo-$PGF_{1\alpha}$ measured during an interval of 5 min as described in Methods ($n = 3–4$, mean with s.e.mean shown by vertical bars).

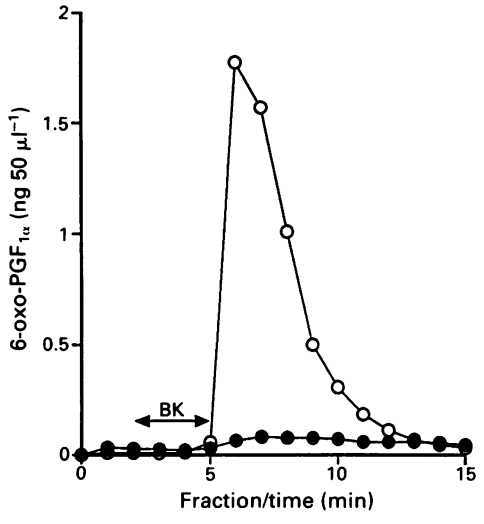

Figure 2 Effect of preincubation of AG7680 bovine aortic endothelial cells with 1 μM isoprenaline on the bradykinin (BK)-dependent release of 6-oxo-$PGF_{1\alpha}$. The cells were cultured on microcarrier beads, and loaded on to a column as described in Methods. The results show basal and bradykinin (100 nM)-dependent release of 6-oxo-$PGF_{1\alpha}$ from control cells (○) or cells exposed to 1 μM isoprenaline for 30 min (●). The results are typical of 3 similar experiments.

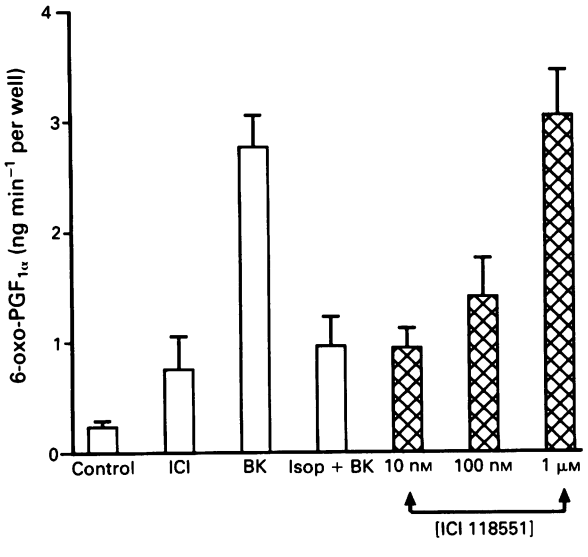

Figure 3 The inhibitory effect of 1 μM isoprenaline on the bradykinin (BK)-stimulated release of 6-oxo-$PGF_{1\alpha}$ is mediated by β_2-adrenoceptors. Results show the capacity of isoprenaline (Isop) to reduce BK-dependent 6-oxo-$PGF_{1\alpha}$ release from AG7680 endothelial cells. Results are also shown of the effect of ICI 118551 (10 nM to 1 μM, cross hatched columns) on the reduction by isoprenaline (1 μM) of the 6-oxo-$PGF_{1\alpha}$ release stimulated by bradykinin 100 nM ($n = 6$, mean with s.e.mean shown by vertical bars).

In further experiments, the effect was measured of isoprenaline pretreatment of endothelial cells stimulated with 100 nM bradykinin or 30 μM arachidonic acid for 5 min. Basal, bradykinin or arachidonic acid-stimulated levels of 6-oxo-$PGF_{1\alpha}$ in wells of control cells were 350 ± 70, 5820 ± 340 and 6120 ± 110 pg min^{-1} per well respectively ($n = 4-6$). Isoprenaline-pretreatment (1 μM) reduced the bradykinin-stimulated release of 6-oxo-$PGF_{1\alpha}$ to 3030 ± 360 pg min^{-1} per well ($P < 0.0001$) as before, but had no significant effect on arachidonic acid-stimulated release of 6-oxo-$PGF_{1\alpha}$ (6400 ± 1370 pg min^{-1} per well; Figure 4).

EDRF release from bovine aortic endothelial cells

EDRF was released from bovine aortic endothelial cells, and quantified by relaxation of endothelium-free and preconstricted rat aortic rings. The endothelial cells on the column were superfused with selected concentrations of bradykinin (0.1–100 nM) for periods of 45 s. The relaxation produced by EDRF (%) was plotted against bradykinin concentration. Figure 5 shows one of 3 similar experiments which revealed concentration-dependent stimulation of EDRF release by bradykinin. The maximum relaxation produced by bradykinin-stimulated release of EDRF was $71.0 \pm 4.3\%$ and the concentration of bradykinin for half-maximum release of EDRF (K_{act}) was 0.70 ± 0.14 nM.

Effect of forskolin or isoprenaline on EDRF release from endothelial cells

The capacity of bradykinin (10 nM) to stimulate release of EDRF from endothelial cells on a column was measured in the bioassay system (Figure 6). The superfusates from the columns were then isolated from the aortic rings, and the endothelial cells superfused for 30 min with Krebs-Henseleit buffer alone, or with buffer containing 1 μM isoprenaline or 5 μM forskolin. Thereafter, the capacity of 10 nM bradykinin to stimulate release of EDRF from the endothelial cells was re-examined. As shown in Figure 6, bradykinin-stimualted release of EDRF produced similar relaxation of aortic rings before and after perfusion of the columns with either forskolin or isoprenaline. These results are typical of 3 similar experiments, and show that neither receptor-mediated nor direct activation of adenylate cyclase affected release of EDRF from endothelial cells under these conditions.

Relationship between $[Ca^{2+}]_i$ elevation and prostacyclin or EDRF release from endothelial cells

The resting $[Ca^{2+}]_i$ level in unstimulated endothelial cells was 141 ± 12 nM ($n = 11$). Bradykinin (500 nM) caused a transient

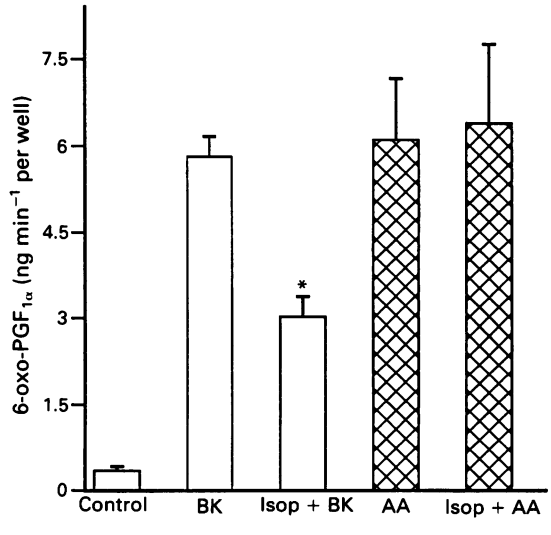

Figure 4 The effect of isoprenaline (Isop) on the release of 6-oxo-$PGF_{1\alpha}$ from endothelial cells exposed to bradykinin (BK) or arachidonic acid (AA). Results show the release of 6-oxo-$PGF_{1\alpha}$ from control cells or after exposure for 30 min to 1 μM isoprenaline. The release of 6-oxo-$PGF_{1\alpha}$ was triggered by exposure of the cells for 5 min to either 100 nM bradykinin (open columns) or 30 μM arachidonic acid (cross hatched columns). *$P < 0.001$ in comparison with cells exposed to bradykinin alone ($n = 4-6$, mean ± s.e.mean).

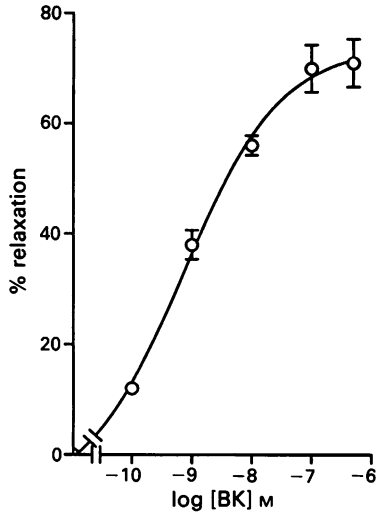

Figure 5 Concentration-response curve of bradykinin (BK)-dependent release of endothelium-derived relaxing factor from AG7680 bovine aortic endothelial cells. Endothelial cells were superfused on columns as described in the legend to Figure 2 and in Methods, and exposed to bradykinin for 45 s at selected concentrations. Results show mean values with s.e.mean (vertical bars) $n = 3$.

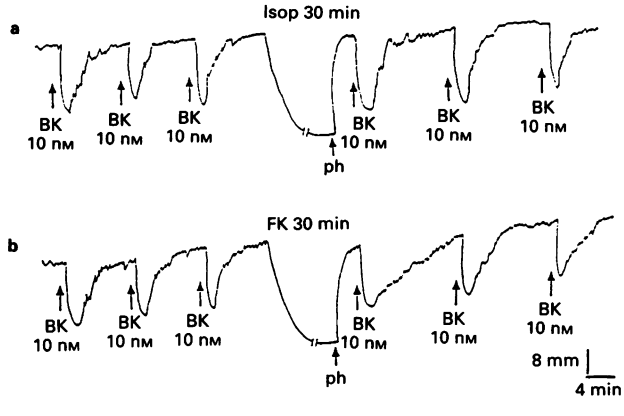

Figure 6 Bradykinin (BK)-stimulated release of endothelium-derived relaxing factor (EDRF) from AG7680 bovine aortic endothelial cells. The aortic rings were pre-constricted with 1 μM phenylephrine before measurement of EDRF release, and as shown by the arrow (ph). The results show the release of EDRF from endothelial cells following repeated (× 3) exposure for 45 s to 10 nM bradykinin. Thereafter, the cells were exposed to either (a) 1 μM isoprenaline (Isop) or (b) 5 μM forskolin (FK), and the bradykinin-dependent release of EDRF measured another 3 times.

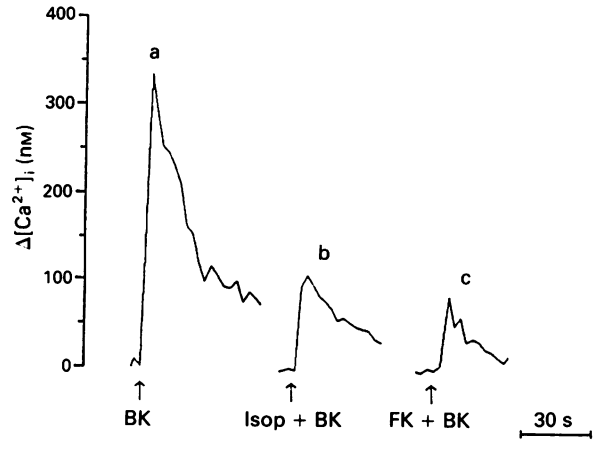

Figure 7 Isoprenaline (Isop) or forskolin (FK)-pretreatment of AG7680 bovine aortic endothelial cells decreases the bradykinin (BK)-stimulated increase in $[Ca^{2+}]_i$. Measurements were made of $[Ca^{2+}]_i$ in Fura 2-AM loaded AG7680 cells adherent to glass coverslips. Results show the bradykinin-dependent calcium transients in control cells (a), or cells exposed for 30 min to 1 μM isoprenaline (b) or 5 μM forskolin (c). The results are representative of 3–5 similar experiments.

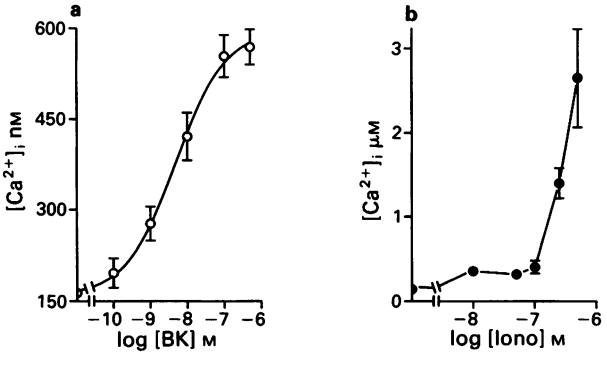

Figure 8 Concentration-response curves for changes in $[Ca^{2+}]_i$ mediated by selected concentrations of bradykinin (BK) (a) or ionomycin (Iono) (b) in bovine aortic endothelial cells. Endothelial cells adherent to glass coverslips were exposed to bradykinin or ionomycin at the concentrations shown, and $[Ca^{2+}]_i$ measured as described in Methods. The results are means with s.e.mean (vertical bars) of replicate determinations, $n = 3–6$.

rise in $[Ca^{2+}]_i$ as illustrated in Figure 7. Incubation of the endothelial cells with 1 μM isoprenaline or 5 μM forskolin had no significant effect on the resting level of $[Ca^{2+}]_i$, but the peak level of the transient rise in $[Ca^{2+}]_i$ stimulated by bradykinin was significantly reduced by pre-incubation of the cells for 30 min with isoprenaline or forskolin. These results show that direct or receptor-mediated activation of adenylate cyclase decreases the bradykinin-stimulated rise in $[Ca^{2+}]_i$.

Experiments were performed to investigate the relationship between the stimulated level of $[Ca^{2+}]_i$ and the rates of prostacyclin or EDRF synthesis. Concentration-response curves were generated relating the extracellular concentration of bradykinin or ionomycin to the level of $[Ca^{2+}]_i$. The endothelial cells were exposed to selected concentrations of bradykinin (0.1–500 nM) or ionomycin (10–500 nM) and the increase in $[Ca^{2+}]_i$ measured (Figure 8). The concentration of bradykinin that produced half-maximum increase in $[Ca^{2+}]_i$ was 4.8 nM (K_{act}).

Thereafter, to compare the $[Ca^{2+}]_i$ required for EDRF and prostacyclin release from AG7680 endothelial cells, microcarrier beads coated with endothelial cells were superfused with selected concentrations of ionomycin (1–500 nM). EDRF was quantified by measurement of the relaxation of rat aortic rings with the endothelium removed and small volumes of column eluate were diverted from the column for the simultaneous measurement of prostacyclin release. Both EDRF and prostacyclin were released by ionomycin. As shown in Figure 9 the minimum concentration of ionomycin required to trigger EDRF release was approximately 10 fold less than that required for prostacyclin release. It is possible to correlate indirectly the relationship between $[Ca^{2+}]_i$ and the release of the two vasodilators by extrapolating from the level of $[Ca^{2+}]_i$ raised by the selected concentrations of ionomycin (Figure 9). These results suggest that the $[Ca^{2+}]_i$ threshold for release of prostacyclin is approximately 350 nM, while that for release of EDRF is less than 200 nM.

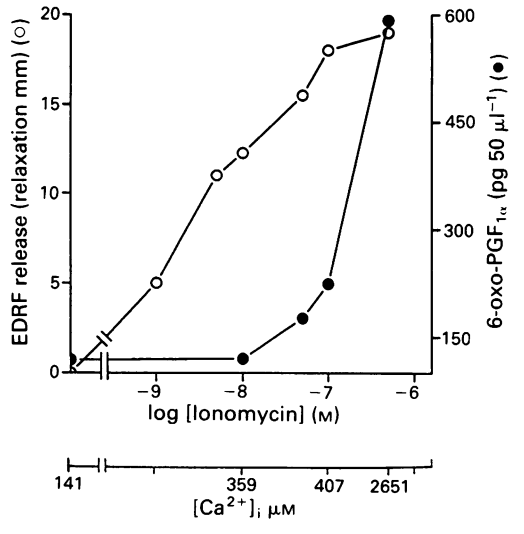

Figure 9 Simultaneous measurement of the release of endothelium-derived relaxing factor (EDRF) and 6-oxo-$PGF_{1\alpha}$ from ionomycin-stimulated AG7680 bovine aortic endothelial cells. The results show the release of EDRF (○) and 6-oxo-$PGF_{1\alpha}$ (●) following exposure of AG7680 endothelial cells to ionomycin (1–500 nM). The scale for the $[Ca^{2+}]_i$ was taken from the titration of ionomycin against $[Ca^{2+}]_i$ shown in Figure 8.

Discussion

Bradykinin and numerous other vasodilator substances mediate their effect on vascular smooth muscle by triggering release of EDRF from endothelial cells. Endothelial cells exposed to bradykinin also show simultaneous release of PGI_2 (de Nucci *et al.*, 1988), which contributes little to the vasodilator effects of bradykinin *in vitro*, but which (in some circumstances) may serve *in vivo* to inhibit platelet aggregation on the luminal surface of a vessel (Higgs *et al.*, 1977). There is compelling evidence that the release of both EDRF and PGI_2 are triggered by hormone-dependent rises in $[Ca^{2+}]_i$ (Hallam & Pearson, 1986; Luckhoff *et al.*, 1988), but in the present paper evidence is presented that the pathway for PGI_2 biosynthesis may be inhibited selectively by isoprenaline, with little or no effect on EDRF biosynthesis.

In preliminary experiments, the capacity of bradykinin to trigger release of PGI_2 was confirmed. Thereafter, experiments were performed which showed that pre-incubation of endothelial cells with the β-adrenoceptor agonist, isoprenaline (1 μM) or the diterpine, forskolin (5 μM) reduced substantially the release of PGI_2. At these concentrations, both agents increase adenylate cyclase activity (via a β-adrenoceptor or by direct action on the adenylate cyclase molecule respectively). Further experiments with the inhibitor ICI 118551 revealed that inhibition of PGI_2 release by isoprenaline was mediated by the β_2-subclass of adrenoceptors.

Inhibition of the pathway of PGI_2 biosynthesis by isoprenaline occurs before the increased availability of arachidonic acid from phospholipid, since isoprenaline had no capacity to inhibit PGI_2 synthesis when the response was triggered by the addition of exogenous arachidonic acid. The implication of these results was that both isoprenaline and forskolin most probably inhibit PGI_2 release by reduction of the magnitude of the bradykinin-dependent Ca^{2+} transient. Adenosine 3':5'-cyclic monophosphate (cyclic AMP)-mediated reduction in stimulated $[Ca^{2+}]_i$ has been reported in platelets (MacIntyre *et al.*, 1985; Yamanashi *et al.*, 1983; Zavoico *et al.*, 1984). The possibility of attenuated Ca^{2+} transients in these endothelial cells was later addressed experimentally, and shown indeed to be the case (Figure 7).

The present results confirm a previous report that activation of adenylate cyclase reduces agonist-stimulated rises in intracellular calcium in endothelial cells (Luckhoff *et al.*, 1990), but contrasts with a negative report from Ryan *et al.* (1986). The latter group examined endothelium derived from the pulmonary artery, which may account for the observed differences in response to isoprenaline, although measurements were also made of direct as well as receptor-mediated activation of adenylate cyclase.

The results presented here also confirm earlier work which reports the capacity of bradykinin to trigger release of EDRF from bovine aortic endothelial cells in culture (de Nucci *et al.*, 1988). A clear concentration-response relationship was established, but paradoxically, neither isoprenaline nor forskolin had any capacity to inhibit EDRF release, at concentrations shown previously to reduce PGI_2 biosynthesis by >90% (Figure 2). Similar inhibition of ATP-induced PGI_2 release has been reported previously (Luckhoff *et al.*, 1990), without inhibition of the release of NO when measured in a bioassay. However, that study suggested the initial rate of EDRF release might be attentuated by an increase in intracellular cyclic AMP. The results presented in this paper show no evidence of this.

Additional experiments were performed to address further the possibility that the biosynthetic pathways of PGI_2 and EDRF might be activated at different concentrations of $[Ca^{2+}]_i$. Furthermore, we examined the possibility that the capacity of adenylate cyclase-linked receptors to inhibit the PGI_2 biosynthetic pathway selectively might reflect reduction in $[Ca^{2+}]_i$ to a point below the threshold for activation of phospholipase A_2. Several pieces of experimental evidence emerged in support of this notion. Inspection of Figures 1, 5 and 8 reveals that the concentration-response relationships for the release of EDRF (measured as relaxation of the aortic ring) and the release of PGI_2 by bradykinin could not be superimposed, and had K_{act} values of 0.7 nM and 24.1 nM respectively. Thus, the biosynthetic pathway for EDRF was activated at lower occupancy of bradykinin receptors than was required for activation of phospholipase A_2. Furthermore, the concentration-response relationship for the rise in $[Ca^{2+}]_i$ with increasing bradykinin concentration (K_{act} = 4.8 nM) more closely approximated the K_{act} value for EDRF release than for PGI_2 release.

An effort was made to quantify the absolute values of $[Ca^{2+}]_i$ and then demonstrate unequivocally the differences in $[Ca^{2+}]_i$ required to activate the two biosynthetic pathways. The experiment was performed in two parts. First, $[Ca^{2+}]_i$ was increased by exposure of endothelial cells to ionomycin, and a calibration curve established relating $[Ca^{2+}]_i$ to the concentration of ionomycin. In the second half of the experiment, endothelial cells on a column were exposed to selected concentrations of ionomycin, and concentration-response relationships established for either (i) ionomycin concentration or (ii) $[Ca^{2+}]_i$ and the activities of the two biosynthetic pathways. The results are shown in Figure 9, and these confirm once again the different sensitivities of the two pathways to changes in $[Ca^{2+}]_i$. The level of $[Ca^{2+}]_i$ associated with release of PGI_2 was >350 nM, while that for EDRF release was less than 200 nM. Carter *et al.* (1988) reported that the threshold of $[Ca^{2+}]_i$ for detectable release of PGI_2 from human umbilical vein endothelial cells was 800–1000 nM. In other experiments, we have shown similar separation of agonist concentration-response curves for EDRF and PGI_2 release when endothelial cells are exposed to bradykinin.

$[Ca^{2+}]_i$ has been proposed as the major signalling pathway for release of both PGI_2 and EDRF (Carter *et al.*, 1988; Luckhoff *et al.*, 1988). However, the magnitude of either response may depend on the particular agonist, since ADP produces a transient release of PGI_2, but a more prolonged release of EDRF (Mitchell *et al.*, 1992). In contrast bradykinin triggers a transient release only of both vasodilators. This may reflect the involvement of different distal signalling pathways. There is evidence that the release

of PGI_2 may be increased by activation of protein kinase C when endothelial cells are exposed to phorbol ester (Demolle & Boeynaems, 1988). Similarly, the release of PGI_2 following exposure of AG4762 bovine aortic endothelial cells to U46619, a thromboxane receptor agonist, is inhibited by the putative protein kinase C antagonist, staurosporine (Clesham *et al.*, 1992). Intriguingly, the activation of protein kinase C by phorbol esters appears to reduce the size of $[Ca^{2+}]_i$ transients, while simultaneously serving to increase the sensitivity of phospholipase A_2 to $[Ca^{2+}]_i$ (Carter *et al.*, 1989). Cyclic GMP analogues inhibit the release of EDRF stimulated by substance P or acetylcholine, but not ATP (Evans *et al.*, 1988), which again suggests multiple distal signalling pathways, and differences in their sensitivities to specific kinases.

We conclude that the change in $[Ca^{2+}]_i$ in bovine aortic endothelial cells following exposure to bradykinin is responsible for activation of phospholipase A_2 (leading to PGI_2 release) and NO synthase (leading to NO release). However, the thresholds for activation of these two biosynthetic pathways are dissimilar, and higher concentrations of Ca^{2+} ions are required for activation of phospholipase A_2 than for NO synthase, which is more Ca^{2+}-sensitive. These differences in Ca^{2+} sensitivity may be exploited experimentally to reveal selective inhibition of PGI_2 synthesis by reduction in the magnitude of bradykinin-dependent Ca^{2+} transients by drugs that elevate intracellular cyclic AMP.

This work was supported in part by a Programme Grant from the Wellcome Trust. H.P. was in receipt of a scholarship from the Ministry of Health and Medical Education, Iran.

References

BERRIDGE, M.J. (1987). Inositol trisphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.*, **56**, 159–193.

CARTER, T.D., HALLAM, T.J., CUSACK, N.J. & PEARSON, J.D. (1988). Regulation of P_{2y}-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br. J. Pharmacol.*, **95**, 1181–1190.

CARTER, T.D., HALLAM, T.J. & PEARSON, J.D. (1989). Protein kinase C activation alters the sensitivity of agonist-stimulated endothelial-cell prostacyclin production to intracellular Ca^{2+}. *Biochem. J.*, **262**, 431–437.

CHANG, J., MUSSER, J.H. & MCGREGOR, H. (1987). Phospholipase A_2: function and pharmacological regulation. *Biochem. Pharmacol.*, **36**, 2429–2436.

CLESHAM, G., PARSAEE, H., JOSEPH, S., MCEWAN, J.R. & MACDERMOT, J. (1992). Activation of bovine endothelial thromboxane receptors triggers release of prostacyclin but not EDRF. *Cardiovasc. Res.*, **26**, 513–517.

CROSSMAN, D., MCEWAN, J., MACDERMOT, J., MACINTYRE, I. & DOLLERY, C.T. (1987). Human calcitonin gene-related peptide activates adenylate cyclase and releases prostacyclin from human umbilical vein endothelial cells. *Br. J. Pharmacol.*, **92**, 695–701.

DEMOLLE, D. & BOEYNAEMS, J.M. (1988). Role of protein kinase C in the control of vascular prostacyclin: study of phorbol esters' effect in bovine aortic endothelium and smooth muscle. *Prostaglandins*, **35**, 243–257.

DE NUCCI, G., RICHARD, J., GRYGLEWSKI, R.J., WARNER, T.D. & VANE, J.R. (1988). Receptor-mediated release of endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 2334–2338.

EVANS, H.G., SMITH, J.A. & LEWIS, M.J. (1988). Release of endothelium-derived relaxing factor is inhibited by 8-bromocyclic guanosine monophosphate. *J. Cardiovasc. Pharmacol.*, **12**, 672–677.

FORSTERMANN, U., POLLOCK, J.S., SCHMIDT, H.H.H.W., HELLER, M. & MURAD, F. (1991). Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 1788–1792.

FRANCIS, R.B. & NEELY, S. (1989). Inhibition of endothelial secretion of tissue-type plasminogen activator and its rapid inhibition by agents which increase intracellular cyclic AMP. *Biochem. Biophys. Acta*, **1012**, 207–213.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HALLAM, T.J. & PEARSON, J.D. (1986). Exogenous ATP raises cytoplasmic free calcium in fura-2 loaded piglet aortic endothelial cells. *FEBS Lett.*, **207**, 95–99.

HALLAM, T.J., PEARSON, J.D. & NEEDHAM, L.A. (1988). Thrombin-stimulated elevation of human endothelial-cell cytoplasmic free calcium concentration causes prostacyclin production. *Biochem. J.*, **251**, 243–249.

HIGGS, G.A., MONCADA, S. & VANE, J.R. (1977). Prostacyclin (PGI_2) inhibits the formation of platelet thrombi induced by adenosine diphosphate (ADP) *in vivo*. *Br. J. Pharmacol.*, **61**, 137P.

IWAI, N., MATSUNAGA, M., KITA, T., TEI, M. & KAWAI, C. (1987). Regulation of angiotensin converting enzyme activity in cultured human vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **149**, 1179–1185.

LUCKHOFF, A., POHL, U., MÜLSCH, A. & BUSSE, R. (1988). Differential role of extra- and intra-cellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. *Br. J. Pharmacol.*, **95**, 189–196.

LUCKHOFF, A., MULSCH, A. & BUSSE, R. (1990). cAMP attenuates autacoid release from endothelial cells: relation to internal calcium. *Am. J. Physiol.*, **258**, H960–H966.

LUTY, J., HUNT, J.A., NOBBS, P.K., KELLY, E., KEEN, M. & MACDERMOT, J. (1989). Expression and desensitisation of A_2 purinoceptors in cultured bovine aortic endothelial cells. *Cardiovasc. Res.*, **23**, 303–307.

MACINTYRE, D.E., BUSHFIELD, M. & SHAW, A.M. (1985). Regulation of platelet cytosolic free calcium by cyclic nucleotides and protein kinase C. *FEBS Lett.*, **188**, 383–388.

MCEWAN, J.R., RITTER, J.M. & MACDERMOT, J. (1989). Calcitonin gene related peptide (CGRP) activates adenylate cyclase of bovine aortic endothelial cells: guanosine 5′ triphosphate dependence and partial agonist activity of tyrosynated analogue. *Cardiovasc. Res.*, **23**, 921–927.

MITCHELL, J.A., DE NUCCI, G., WARNER, T.D. & VANE, J.R. (1992). Different patterns of release of endothelium-derived relaxing factor and prostacyclin. *Br. J. Pharmacol.*, **105**, 485–489.

MONCADA, S., GRYGLEWSKI, R., BUNTING, S. & VANE, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263**, 663–665.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RYAN, U.S., AVDONIN, P.V., POSIN, E.Y.A., POPOV, E.G., DANILOV, S.M. & TKACHUK, V.A. (1988). Influence of vasoactive agents on cytoplasmic free calcium in vascular endothelial cells. *J. Appl. Physiol.*, **65**, 2221–2227.

STADEL, J.M., POKSAY, K.S., NAKADA, M.T. & CROOKE, S.T. (1987). Regulation of β-adrenoceptor number and subtype in 3T3-L1 preadipocytes by sodium butyrate. *Eur. J. Pharmacol.*, **143**, 35–44.

STEINBERG, S.F., JAFEE, E.A. & BILEZIKIAN, J.P. (1984). Endothelial cells contain beta adrenoceptors. *Naunyn Schmiedebergs Arch. Pharmacol.*, **325**, 310–313.

YAMANASHI, J., KAWAHARA, Y. & FUKOZAKI, H. (1983). Effect of cyclic AMP on cytoplasmic free calcium in human platelets stimulated by thrombin: direct measurement with Quin 2. *Thromb. Res.*, **32**, 183–188.

ZAVOICO, G.B. & FEINSTEIN, M.B. (1984). Cytoplasmic Ca^{2+} in platelets is controlled by cyclic AMP: antagonism between stimulators and inhibitors of adenylate cyclase. *Biochem. Biophys. Res. Commun.*, **120**, 579–585.

(Received July 13, 1992
Accepted August 3, 1992)

Br. J. Pharmacol. (1992), **107**, 1020–1028

Central administration of 5-HT activates 5-HT_{1A} receptors to cause sympathoexcitation and 5-HT_2/5-HT_{1C} receptors to release vasopressin in anaesthetized rats

[1]Ian K. Anderson, *Graeme R. Martin & Andrew G. Ramage

Academic Department of Pharmacology, Royal Free Hospital School of Medicine, Rowland Hill St, Hampstead, London NW3 2PF and *Analytical Pharmacology Group, Biochemical Sciences, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The effects of intracerebroventricular injections to the right lateral ventricle (i.c.v.) of 5-hydroxytryptamine (5-HT, 40 and 120 nmol kg^{-1}), N,N-di-n-propyl-5-carboxamidotryptamine (DP-5-CT; 3 nmol kg^{-1}), 5-carboxamidotryptamine (5-CT; 3 nmol kg^{-1}), 8-hydroxy-2-(di-N-propylamino) tetralin (8-OH-DPAT; 3, 40 and 120 nmol kg^{-1}) and 1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane (DOI; 40 and 120 nmol kg^{-1}) on renal sympathetic nerve activity, blood pressure, heart rate and phrenic nerve activity were investigated in normotensive rats anaesthetized with α-chloralose.

2 5-HT caused a long lasting pressor response which was associated with an initial bradycardia and renal sympathoinhibition followed by a tachycardia and renal sympathoexcitation. Pretreatment with the 5-HT_2/5-HT_{1C} receptor antagonists, cinanserin (300 nmol kg^{-1}, i.c.v.) or LY 53857 (300 nmol kg^{-1}, i.c.v.) reversed the initial bradycardia and sympathoinhibition to tachycardia and sympathoexcitation. Combined pretreatment with LY 53857 (300 nmol kg^{-1}, i.c.v.) and the 5-HT_{1A} antagonist, spiroxatrine (300 nmol kg^{-1}, i.c.v.), blocked the effects of 5-HT on all the above variables.

3 Pretreatment with the vasopressin V_1-receptor antagonist, β-mercapto-β,β-cyclopentamethylene-propionyl[1], O-Me-Tyr[2], Arg^8-vasopressin [$(d(CH_2)_5Tyr(Me)AVP$, 10 μg kg^{-1}, i.v.] did not affect the magnitude but reduced the duration of the pressor response produced by i.c.v. 5-HT and reversed the initial bradycardia and renal sympathoinhibition to tachycardia and sympathoexcitation.

4 1-(2,5-Di-methoxy-4-iodophenyl)-2-aminopropane (DOI) caused a pressor effect which was associated with a bradycardia and sympathoinhibition. These effects were blocked by pretreatment with BW501C67 (0.1 mg kg^{-1}, i.v.), a peripherally acting 5-HT_2/5-HT_{1C} receptor antagonist. However, BW501C67 (0.1 mg kg^{-1}, i.v.) failed to block the effects of i.c.v. 5-HT.

5 DP-5-CT, 5-CT and 8-OH-DPAT (3 nmol kg^{-1}, i.c.v.) caused sympathoexcitation, tachycardia and a rise in blood pressure. Pretreatment with methiothepin (1 mg kg^{-1}, i.v.) or spiroxatrine (300 nmol kg^{-1}, i.c.v.) attenuated the response to i.c.v. DP-5-CT.

6 It is concluded that i.c.v. administration of 5-HT activates 5-HT_{1A} receptors to cause sympathoexcitation and 5-HT_2 or 5-HT_{1C} receptors to cause the release of vasopressin.

Keywords: 5-HT_{1A} receptors; 5-HT_2 or 5-HT_{1C} receptors; vasopressin V_1-receptors; 8-OH-DPAT; DP-5-CT; 5-HT; DOI; blood pressure; sympathetic nerve activity; anaesthetized rat

Introduction

Intracerebroventricular (i.c.v.) injections of 5-hydroxytryptamine (5-HT) in anaesthetized rats cause a rise in blood pressure and variable effects on heart rate (Lambert *et al.*, 1975; 1978; Kristic & Djurkovic, 1976; 1980). In conscious rats i.c.v. administration of 5-HT also causes a pressor effect but in these animals consistently produces bradycardia (Sukamato *et al.*, 1984; Dalton, 1986). More recently, Inoue & Buñag (1989) were able to demonstrate that i.c.v. 5-HT induced a pressor response which was associated with a consistent bradycardia and sympathoinhibition in the anaesthetized rat. These authors also demonstrated that the pressor effect was attenuated by pretreatment with a vasopressin V_1-receptor antagonist. The precise nature of the 5-HT receptor involved in this central release of vasopressin was not determined, although (as in previous studies) the pressor effect of i.c.v. 5-HT could be antagonized by the non-selective 5-HT receptor antagonists, methysergide or bromolysergic acid diethylamide (see Ramage, 1985; Hoyer, 1991). In addition, it has also been reported that the rise in blood pressure produced by i.c.v. administration of 5-HT is attenuated by cervical transection of the spinal cord, adrenalectomy, adrenergic neurone blocking agents and α-adrenoceptor antagonists (Kristic & Djurkovic, 1980), suggesting that there is an additional sympathoexcitatory component to the response of 5-HT. However, Inoue & Buñag (1989) were unable to demonstrate such an action when recording from the splanchnic nerve. We therefore decided to investigate further the mechanism and nature of the receptors involved in effects of i.c.v. administration of 5-HT on blood pressure, heart rate and renal sympathetic nerve activity in rats anaesthetized with α-chloralose using more selective agonists and antagonists for the different 5-HT receptor subtypes. In addition respiratory variables were monitored. A preliminary account of these observations has been presented to the British Pharmacological Society (Anderson, 1991; Anderson *et al.*, 1992b).

Methods

Experiments were performed on male Sprague-Dawley normotensive rats (250–350 g). Anaesthesia was induced with halothane (2.5% in oxygen) and maintained with α-chloralose (80 mg kg^{-1}, i.v.). Supplementary doses of α-

[1] Author for correspondence.

chloralose (10–20 mg kg^{-1}, i.v.) were given as required. Depth of anaesthesia was assessed by the stability of cardiovascular and respiratory variables being recorded. The left carotid artery was cannulated for the measurement of blood pressure and for sampling arterial blood for analysis of pH and blood gases. Blood pressure was measured with a pressure transducer (Gould Statham P23XL) and the heart rate was derived electronically from the blood pressure signal (Gould Biotach Amplifier). The left jugular vein was cannulated for drug administration and a tracheal cannula was implanted. Body temperature was monitored by a rectal probe and maintained at 36–38°C with a homeothermic blanket system (Harvard). The animals were artificially ventilated (rate 50 min^{-1}, stroke volume 8 ml kg^{-1}) with oxygen-enriched room air by use of a positive pressure pump (Harvard Rodent Ventilator 683) and neuromuscular blockade was produced with decamethonium (3 mg kg^{-1}, i.v.). Blood samples were taken from a T-piece on the carotid arterial cannula and blood gases and pH were monitored with a Corning pH/blood gas analyser. Blood gases were maintained between 90–130 mmHg P_{O_2}, 40–50 mmHg P_{CO_2} and pH 7.3–7.4. Adjustments of the respiratory pump volume were made as necessary to maintain blood gas and pH balance. Once ventilated, the animals were infused (6 ml kg^{-1} h^{-1}) via the jugular vein with a solution comprising 10 ml plasma substitute (gelofusine), 10 ml distilled water, 0.04 g glucose, 0.168 g sodium bicarbonate and 30 mg decamethonium. This was to prevent the development of non-respiratory acidosis and to maintain blood volume and neuromuscular blockade.

Cannulation of the lateral cerebral ventricle

The rats were placed in a sterotaxic head holder and a stainless steel guide cannula (22 gauge) was implanted into the right lateral cerebral ventricle. The co-ordinates used from bregma were 4 mm ventral, 1.5 mm lateral and 1 mm posterior. Drug and vehicle solutions were administered through an i.c.v. injection cannula (28 gauge) attached by a length of polythene tubing to a 100 µl syringe (Hamilton). At the end of the experiment, the cannula placement was confirmed by the administration of 5 µl of 2% pontamine sky blue dye.

Recording of phrenic nerve and renal nerve activity

The right phrenic nerve was exposed by deflecting the scapula forwards and dissecting the nerve clear of overlying muscle and connective tissue. The nerve was cut peripherally and placed on a bipolar silver hook electrode as described previously (Dreteler *et al.*, 1991). Phrenic nerve activity was quantified by counting the number of action potentials above the noise level over 5 s with a spike processor (Digitimer D130). To maintain phrenic nerve activity, a measure of central inspiratory drive, the blood P_{CO_2} values in these animals were maintained at a slightly higher (40–50 mmHg) level than the physiological norm (35–49 mmHg). This usually locked the rate of phrenic nerve firing to the rate of the animals chest movements caused by the respiration pump and changes in phrenic nerve activity were the result of changes in the size of each inspiratory burst. The right kidney was exposed by a retroperitoneal approach and was deflected laterally to reveal the renal artery and nerve. Renal nerve activity was recorded as previously described (Ramage & Wilkinson, 1989). Renal nerve activity was quantified by integrating the signal above background noise over 5 s with a solid state integrator (Medical Electronics workshop, Royal Free Hospital School of Medicine). The noise levels were verified at the end of the experiment after the administration of pentobarbitone sodium (20 mg per animal).

At the beginning of each experiment the baroreceptor reflex response was tested by observing whether renal nerve activity and heart rate were reduced by a rise in blood pressure caused by noradrenaline (25 ng per animal, i.v.) and were raised by a reduction in blood pressure caused by sodium nitroprusside (0.6 µg per animal, i.v.). Only preparations with an intact baroreceptor reflex were used.

Experimental protocols

The preparation was allowed to stabilize for 30 min before the administration of saline (5 µl i.c.v.). After a 5 min control period a single dose of test compound or saline control was given i.c.v. and the response was followed for at least 30 min. In antagonist studies, antagonists administered i.c.v. were given 10 min before the injection of test drug. However, both LY 53857 and spiroxatrine were administered in two doses of 150 nmol kg^{-1} alone or combined, 5 min apart and the test drug administered 5 min after the last dose of these antagonists. Vehicle for spiroxatrine (0.01 N HCl) was administered in 2 volumes of 5 µl 5 min apart and the test agonist was given 5 min after the last dose of vehicle. When antagonists were given i.v. the test drug was administered 5 min later. However, for methiothepin the test drug was administered following a stabilization period of 20 min. These pretreatment times were chosen to allow stabilization of any changes in the variables being recorded caused by administration of the antagonists. In each rat the cardiovascular response of a single dose of the test drug was recorded.

Analysis of results

Baseline values were taken 1 min before the addition of drug or vehicle. All results are expressed as changes from baseline values. Nerve activity was measured as the average of the integrated values over 1 min in arbitrary units and was expressed as the percentage change from baseline. Changes in mean blood pressure, heart rate, renal and phrenic nerve activity caused by the test drug were compared with time-matched vehicle controls by two-way analysis of variance and were subsequently analysed by the least significant difference test (Sokal & Rohlf, 1969). Biphasic responses in some variables were observed following i.c.v. administration of 5-HT and these phases were analysed separately. Thus, the maximum change for each phase of the 5-HT response was measured and compared to the maximum change in vehicle controls during the same period by Student's t test for unpaired data. Changes in variables caused by antagonist or vehicle pretreatments were compared to the pre-dose baseline by Student's t test for paired data. All values are expressed as the mean ± s.e.mean, differences in the mean were taken as significant when $P < 0.05$.

Drugs and solutions

The drugs used were BW501C67, (2-anilino-N-(2-(3-chlorophenoxy)propyl) acetamide HCl), 5-carboxamidotryptamine maleate (5-CT), N,N-di-n-propyl-5-carboxamidotryptamine maleate (DP-5-CT; these were gifts from Wellcome Laboratories, Beckenham, Kent), α-chloralose (Sigma Chemical Co., Poole, Dorset); DOI, 1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane (Research Biochemicals Inc., Semat, St. Albans); decamethonium iodide (Koch-Light, Haverhill, Suffolk); Gelofusine (Consolidated Chem., Wrexham, Clwyd); 8-hydroxy-2-(di-N-propylamino)tetralin HBr (8-OH-DPAT; Research Biochemicals Inc., Semat, St. Albans); 5-hydroxytryptamine creatinine sulphate, 5-HT (BDH, Poole, Dorset); halothane (ICI Pharmaceuticals Ltd, Macclesfield); hexamethonium bromide (Koch-Light, Haverhill, Suffolk); [β-mercapto-β,β-cyclopentamethylenepropionyl^1,O-Me-Tyr^2, Arg^8]-vasopressin, (d$(CH_2)_5$Tyr(Me)AVP; Sigma Chemical Co., Poole, Dorset); methiothepin mesylate (Research Biochemicals Inc., Semat, St. Albans); noradrenaline acid tartrate (Winthrop, Guildford, Surrey); sodium nitroprusside (Sigma Chemical Co., Poole, Dorset) and spiroxatrine (a gift from Janssen, Wantage, Oxon). Drugs given i.c.v. were dis-

solved in 0.9% w/v saline except for spiroxatrine and the combination of spiroxatrine and LY 53857 which were dissolved in 0.01 N hydrochloric acid (HCl). Solutions were administered in a dose volume of 5 μl over a 20 s period. All drugs given i.v. were dissolved in saline.

Results

Effect of i.c.v. administration of saline

Saline i.c.v. (5 μl; $n = 6$) had little effect on blood pressure, heart rate, renal or phrenic nerve activity and these variables remained stable for the duration of the experiment (see Figure 1). Baseline values for blood pressure and heart rate for this group of experiments were 122 ± 6 mmHg and 417 ± 13 beats min^{-1} (mean ± s.e.mean).

Effect of i.c.v. administration of 5-HT

5-HT [40 ($n = 6$); 120 ($n = 8$) nmol kg^{-1}] caused immediate, dose-related increases in arterial blood pressure (Figures 1 and 2) which reached maxima, between 1 and 5 min after injection, of 6 ± 1 and 19 ± 2 mmHg respectively. The rise in blood pressure remained elevated for 5 min after the low dose and 20 min after the high dose. In addition, 5-HT (40 and 120 nmol kg^{-1}) caused initial significant ($P<0.05$) falls in heart rate of 17 ± 5 and 16 ± 2 beats min^{-1} and in renal nerve activity of 23 ± 8% and 41 ± 8% respectively. The initial decrease in these variables (first phase) was followed by an increase (second phase). Maximum increases in heart rate and renal nerve activity produced by 5-HT (40 and 120 nmol kg^{-1}) were 19 ± 5 and 63 ± 14 beats min^{-1} and 47 ± 15 and 137 ± 32% respectively and occurred between 10–20 min. These biphasic changes in heart rate and renal nerve activity were temporally matched. 5-HT (120 nmol kg^{-1}) caused an initial (1–5 min) significant reduction in phrenic nerve activity of 22 ± 5%. In 3 animals this was followed by a secondary rise in phrenic nerve activity (227 ± 84%, 10–20 min). However, in the remaining animals phrenic nerve activity returned to baseline levels after 5 min. Baseline values for blood pressure and heart rate in the low and high dose groups were 112 ± 6 mmHg and 417 ± 31 beats min^{-1} and 112 ± 4 mmHg and 417 ± 18 beats min^{-1} respectively.

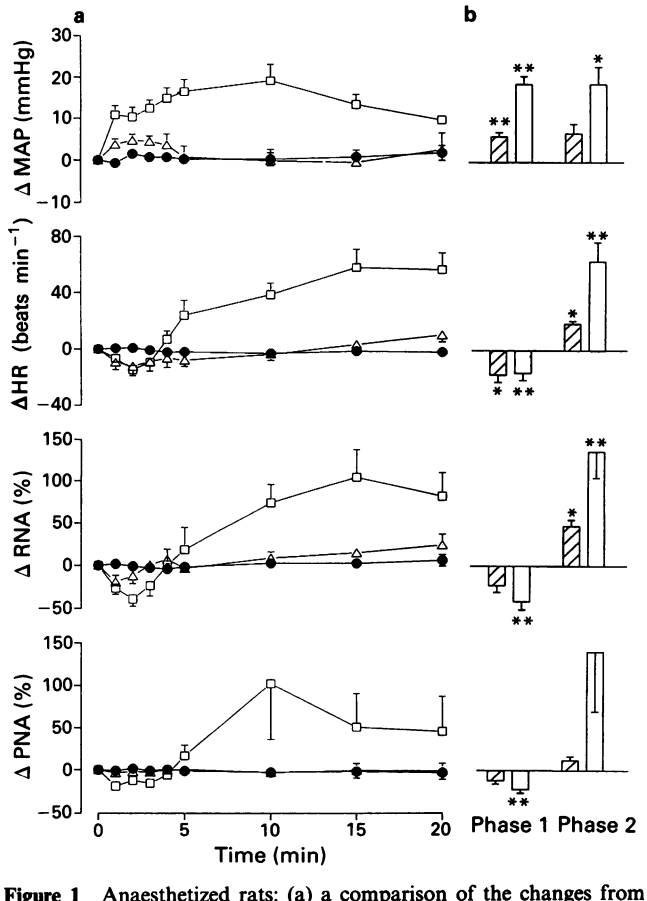

Figure 1 Anaesthetized rats: (a) a comparison of the changes from baseline values over time (min) caused by i.c.v. saline (●; $n = 6$), 5-HT 40 nmol kg^{-1} (△; $n = 6$) and 5-HT 120 nmol kg^{-1} (□; $n = 8$) in mean arterial blood pressure (MAP), heart rate (HR), renal nerve activity (RNA) and phrenic nerve activity (PNA). Each point represents the mean value and the vertical bars show s.e.mean. (b) Histograms showing the maximal changes in mean arterial blood pressure (MAP), heart rate (HR), renal nerve activity (RNA) and phrenic nerve activity (PNA) during the first phase (1–5 min) and the second phase (10–30 min) of the response to i.c.v. 5-HT (40 nmol kg^{-1}, hatched columns; 120 nmol kg^{-1}, open columns). Each point represents the mean value and the vertical bars show s.e.mean. *$P<0.05$ and **$P<0.01$ compared to i.c.v. saline over the same periods.

The effect of pretreatment (i.c.v.) with either cinanserin or LY 53857 on the response to 5-HT

Cinanserin (300 nmol kg^{-1}; $n = 6$) or LY 53857 (300 nmol kg^{-1}; $n = 6$) had no significant effect on blood pressure, heart rate, renal nerve activity or phrenic nerve activity. Baseline values for blood pressure and heart rate were 102 ± 4 mmHg and 398 ± 10 beats min^{-1} and 102 ± 7 mmHg and 417 ± 21 beats min^{-1} respectively. Neither drug prevented the rise in blood pressure caused by 5-HT (120 nmol kg^{-1}) but the initial bradycardia and renal sympathoinhibition were reversed to tachycardia and sympathoexcitation (Figures 2 and 3). The 5-HT induced changes in phrenic nerve activity were not significantly altered (Figure 3).

Effect of i.v. pretreatment with BW501C67 on the response to i.c.v. 5-HT

5-HT (120 nmol kg^{-1}) administered i.c.v. in animals pretreated with BW501C67 (0.1 mg kg^{-1}, i.v.; $n = 4$) caused similar effects to those observed in non-pretreated animals; there was an immediate increase in blood pressure and biphasic changes in heart rate and renal nerve activity (Figure 3). However, the duration of the bradycardia and the renal sympathoinhibition was significantly prolonged. Baseline values for blood pressure and heart rate were 115 ± 11 mmHg and 420 ± 22 beats min^{-1}.

The effect of combined pretreatment (i.c.v.) with LY 53857 and spiroxatrine on the response to 5-HT

Combined pretreatment with LY 53857 (300 nmol kg^{-1}) and spiroxatrine (300 nmol kg^{-1}; $n = 4$) did not significantly change baseline values (baseline values for blood pressure and heart rate were 98 ± 7 mmHg and 380 ± 7 beats min^{-1}). However, the effect of 5-HT (120 nmol kg^{-1}, i.c.v.) on all variables was significantly reduced in animals pretreated with this combination compared to animals pretreated with LY 53857 (300 nmol kg^{-1}, i.c.v.) alone (Figure 4). The combination of LY 53857 and spiroxatrine was dissolved in 0.01 N HCl (vehicle for spiroxatrine) whereas previously LY 53857 had been dissolved in saline. Therefore, in 2 separate experiments, animals were pretreated with LY 53857 dissolved in 0.01 N HCl. In these experiments the response to 5-HT (120 nmol kg^{-1}; i.c.v.; data not shown) was similar to that observed previously in animals pretreated with LY 53857 alone dissolved in saline.

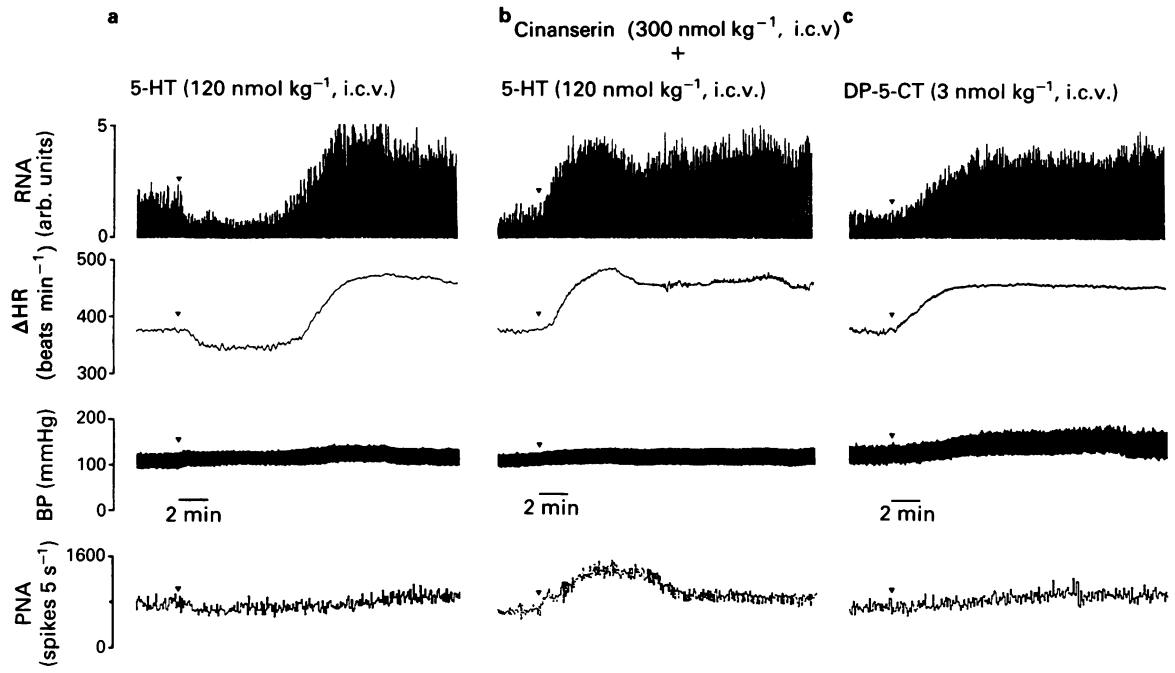

Figure 2 Traces showing the effects of i.c.v. 5-HT (120 nmol kg^{-1}) in absence (a) and presence (b) of cinanserin (300 nmol kg^{-1}, i.c.v.) and (c) i.c.v. N,N-di-n-propyl-5-carboxamidotryptamine (DP-5-CT, 3 nmol kg^{-1}) on arterial blood pressure (BP), heart rate (HR), renal nerve activity (RNA) and phrenic nerve activity (PNA) in anaesthetized rats.

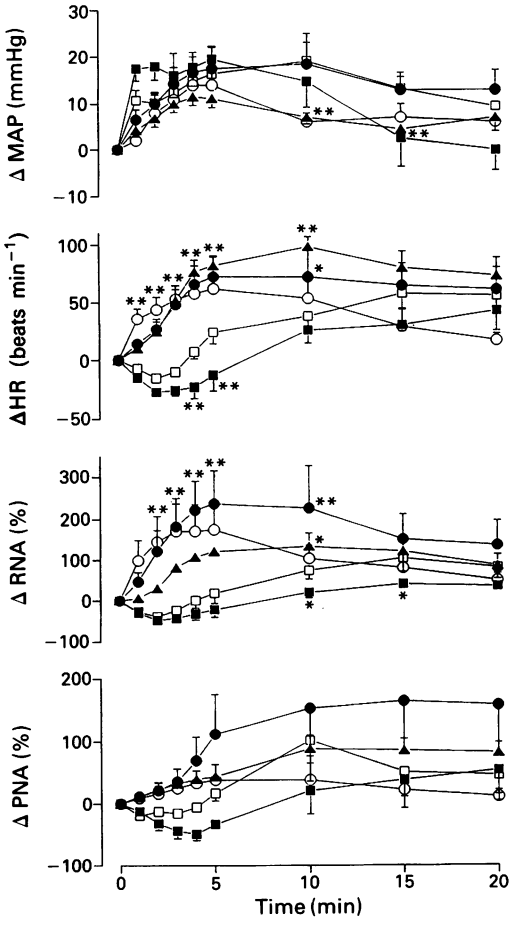

Figure 3 Anaesthetized rats: a comparison of the changes from baseline or post-pretreatment values over time (min) caused by 5-HT (120 nmol kg^{-1}, i.c.v.) in the presence of saline (□; 5 μl; $n = 8$), cinanserin (●; 300 nmol kg^{-1}, i.c.v.; $n = 6$), LY 53857 (○; 300 nmol kg^{-1}, i.c.v.; $n = 6$), BW501C67 (■; 0.1 mg kg^{-1}, i.v.; $n = 4$) and $d(CH_2)_5Tyr(Me)AVP$ (▲; 10 μg kg^{-1}, i.v.; $n = 6$) in mean arterial blood pressure (MAP), heart rate (HR), renal nerve activity (RNA) and phrenic nerve activity (PNA). Each point represents the mean value and the vertical bars show s.e.mean $*P < 0.05$ and $**P < 0.01$ compared to 5-HT saline pretreatment. Statistical significance for the effects of 5-HT on HR and RNA in animals pretreated with cinanserin, LY 53857 and $d(CH_2)_5Tyr(Me)AVP$ between 1–5 min are the same and are illustrated by a single symbol for the sake of clarity.

The effect of pretreatment (i.v.) with $d(CH_2)_5Tyr(Me)$-AVP on the response to 5-HT

$d(CH_2)_5Tyr(Me)AVP$ (10 μg kg^{-1} i.v.; $n = 6$) had no significant effect on blood pressure, heart rate, renal or phrenic nerve activity. Baseline values for blood pressure and heart rate were 104 ± 4 mmHg and 388 ± 8 beats min^{-1}. Pretreatment with $d(CH_2)_5Tyr(Me)AVP$ did not prevent the rise in blood pressure caused by 5-HT (120 nmol kg^{-1}, i.c.v.) but the duration of the pressor rise was significantly attenuated, see Figure 3. The 5-HT-induced bradycardia and renal sympathoinhibition were reversed to an immediate tachycardia and sympathoexcitation in the presence of $d(CH_2)_5Tyr(Me)$-AVP (Figure 3). Changes in phrenic nerve activity caused by 5-HT were unaffected.

Effect of i.c.v. and i.v. administration of 1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane (DOI) in the absence and presence of BW501C67

DOI 12 nmol kg^{-1} (i.c.v.) had no effect on blood pressure, heart rate, renal and phrenic nerve activity (data not shown). DOI (40 nmol kg^{-1}, $n = 4$; 120 nmol kg^{-1}, n = 6; i.c.v.) produced maximum increases in blood pressure of 7 ± 1 and 10 ± 2 mmHg respectively and decreases in heart rate and

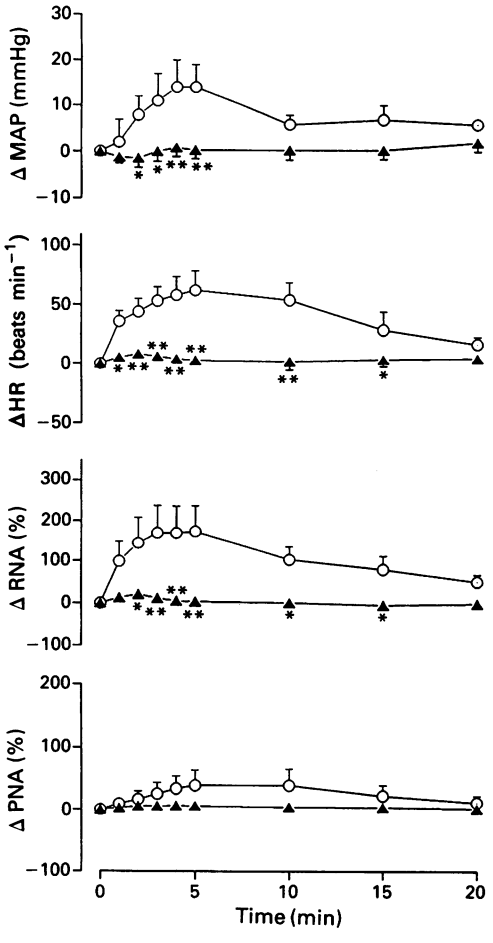

Figure 4 Anaesthetized rats: a comparison of the changes from post-pretreatment values over time (min) caused by 5-HT (120 nmol kg^{-1}, i.c.v.) in the presence of LY 53857 (300 nmol kg^{-1}, i.c.v.; O; $n = 6$) with the combined pretreatment of LY 53857 (300 nmol kg^{-1}, i.c.v.) and spiroxatrine (300 nmol kg^{-1}, i.c.v.; ▲; $n = 4$) in mean arterial blood pressure (MAP), heart rate (HR), renal nerve activity (RNA) and phrenic nerve activity (PNA). Each point represents the mean value and the vertical bars show s.e.mean. *$P<0.05$ and **$P<0.01$ compared to 5-HT in the presence of LY 53857

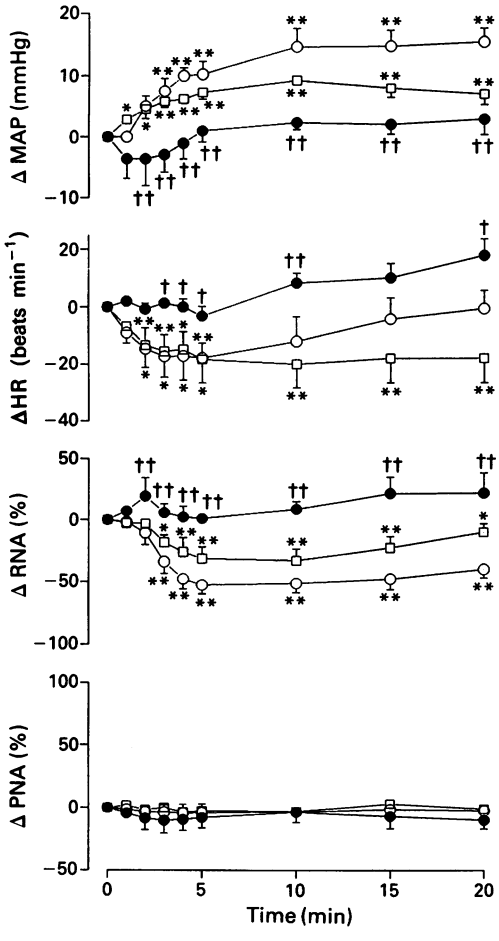

Figure 5 Anaesthetized rats: a comparison of the changes with over time (min) from baseline or post-pretreatment values caused by i.c.v. 1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane (DOI) 40 nmol kg^{-1} (□; $n = 6$) and i.c.v. DOI 120 nmol kg^{-1} in the absence (O; $n = 6$) and presence of BW501C67 (0.1 mg kg^{-1}, i.v.; ●; $n = 4$) in mean arterial blood pressure (MAP), heart rate (HR), renal nerve activity (RNA) and phrenic nerve activity (PNA). Each point represents the mean value and the vertical bars show s.e.mean. *$P<0.05$ and **$P<0.01$ compared to i.c.v. saline (not illustrated for the sake of clarity). †$P<0.05$ and ††$P<0.01$ comparing the effects of DOI (120 nmol kg^{-1}, i.c.v.) in the absence and presence of BW501C67.

renal nerve activity of 18 ± 6 and 19 ± 9 beats min^{-1} and 31 ± 9 and 53 ± 7% respectively, 5 min after injection. There was no change in phrenic nerve activity (Figure 5). Baseline values for blood pressure and heart rate were 126 ± 5 mmHg and 424 ± 21 beats min^{-1} and 122 ± 2 mmHg and 406 ± 20 beats min^{-1} respectively. Pretreatment with BW501C67 (0.1 mg kg^{-1}, i.v.), which had no effect *per se*, significantly attenuated the response to i.c.v. DOI (120 nmol kg^{-1}; $n = 4$; Figure 5) on these variables. Baseline values for blood pressure and heart rate in animals pretreated with BW501C67 were 112 ± 2 mmHg and 406 ± 20 beats min^{-1}.

DOI, 120 nmol kg^{-1} ($n = 3$), given i.v. produced a rise in blood pressure of 22 ± 2 mmHg which was maintained over 20 min. Again bradycardia and renal sympathoinhibition were observed and reached maxima of 21 ± 3 beats min^{-1} and 54 ± 6% respectively 1 min following injection. The bradycardia was not maintained and had returned to baseline by 10 min. However, the renal sympathoinhibition was maintained for 20 min. Phrenic nerve activity was not measured in these animals. Baseline values for blood pressure and heart rate were 117 ± 4 mmHg and 429 ± 22 beats min^{-1}. Pretreatment with BW501C67 (0.1 mg kg^{-1}, i.v.; $n = 2$) abolished the rise in blood pressure caused by i.v. DOI (data not illustrated).

Effect of i.c.v. administration of 5-CT, DP-5-CT and 8-OH-DPAT

DP-5-CT (3 nmol kg^{-1}; $n = 8$) caused an immediate and significant rise in blood pressure, heart rate and renal nerve activity reaching a maximum by 5 min of 9 ± 3 mmHg, 39 ± 5 beats min^{-1} and 83 ± 15%, respectively (Figures 2 and 6). These changes were maintained for at least 30 min. There was no change in phrenic nerve activity (Figure 6). Baseline values for blood pressure and heart rate were 114 ± 4 mmHg and 391 ± 11 beats min^{-1}.

5-CT (3 nmol kg^{-1}; $n = 5$) also caused an immediate significant rise in blood pressure of 17 ± 5 mmHg after 2 min. This rise in blood pressure was associated with significant increases in heart rate of 48 ± 7 beats min^{-1} and in renal nerve activity of 77 ± 35% (Figure 6). Both the rise in heart rate and in renal nerve activity were well maintained returning to near baseline values by 30 min. However, blood pressure remained elevated for only 2 min (Figure 6) and in three experiments, between 3 and 5 min after injection, the rise in blood pressure was followed by a substantial fall of 21 ± 3 mmHg below baseline. 5-CT caused no significant changes in phrenic nerve activity. Baseline values for blood

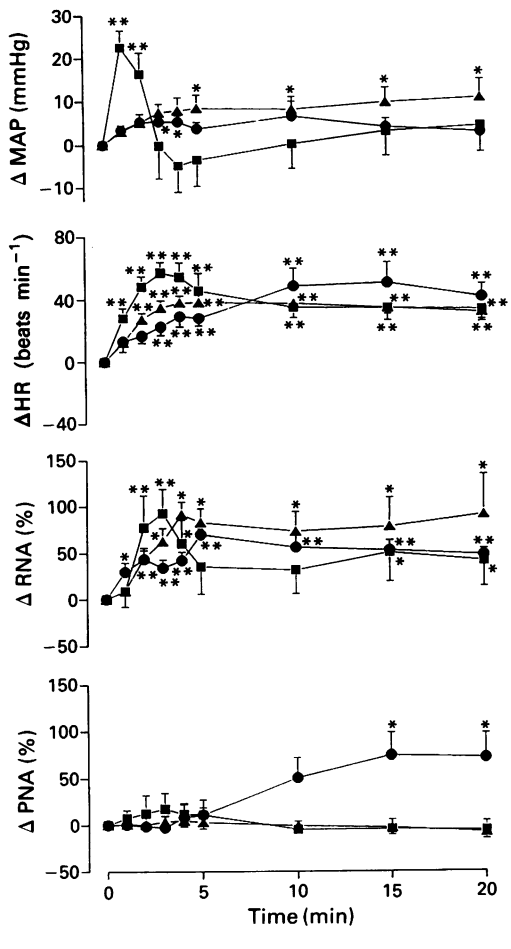

Figure 6 Anaesthetized rats: a comparison of the changes with time (min) from baseline values induced by i.c.v. N,N-di-n-propyl-5-carboxamidotryptamine (DP-5-CT, ▲; 3 nmol kg^{-1}; $n = 8$), 5-carboxamidotryptamine (5-CT, ■; 3 nmol kg^{-1}; $n = 5$) and 8-hydroxy-2-(di-N-propylamino)tetralin (8-OH-DPAT, ●; 3 nmol kg^{-1}; $n = 6$) in mean blood pressure (MAP), heart rate (HR), renal nerve activity (RNA) and phrenic nerve activity (PNA). Each point represents the mean value and the vertical bars show s.e.mean. $*P<0.05$ and $**P<0.01$ compared to i.c.v. saline (not illustrated for the sake of clarity).

pressure and heart rate were 114 ± 4 mmHg and 442 ± 2 beats min^{-1}.

8-OH-DPAT (3 nmol kg^{-1}; $n = 6$) caused significant increases in blood pressure, heart rate and renal nerve activity (Figure 6). The onset of these changes was immediate and was maximal at 10 min, reaching 7 ± 3 mmHg, 49 ± 11 beats min^{-1} and 57 ± 17% respectively. Phrenic nerve activity was also significantly increased (75 ± 24%, 15 min), however the onset of this response was delayed, see Figure 6. Baseline values for blood pressure and heart rate were 96 ± 4 mmHg and 391 ± 11 beats min^{-1}. Higher doses, 40 and 120 nmol kg^{-1}, of 8-OH-DPAT produced small reductions in blood pressure and these were associated with dose-related tachycardia of 21 ± 5 and 38 ± 14 beats min^{-1}, respectively, after 10 min. These higher doses of 8-OH-DPAT did not significantly alter renal or phrenic nerve activity (data not illustrated). Baseline values were for blood pressure 122 ± 5 and 108 ± 8 mmHg and for heart rate 428 ± 10 and 411 ± 16 beats min^{-1} respectively.

The effect of pretreatment with either methiothepin or spiroxatrine on the response to DP-5-CT

Methiothepin (1 mg kg^{-1} i.v.; $n = 4$) caused a significant reduction in blood pressure of 32 ± 2 mmHg 5 min following injection. This was associated with an initial increase in renal nerve activity, reaching a maximum between 1 and 5 min of 64 ± 14% and then returning to near baseline levels after 20 min. Methiothepin did not cause any significant changes in heart rate or phrenic nerve activity. Baseline values for blood pressure and heart rate were 114 ± 5 mmHg and 424 ± 10 beats min^{-1}. The response produced by DP-5-CT (3 nmol kg^{-1}) administered i.c.v. 20 min after methiothepin was significantly attenuated (Figure 7).

The effect of DP-5-CT (3 nmol kg^{-1}, i.c.v.) on all variables was significantly attenuated in spiroxatrine (300 nmol kg^{-1}, i.c.v.; $n = 6$) pretreated animals compared to vehicle (0.01 N HCl i.c.v.; $n = 6$) pretreated animals, see Figure 7. Spiroxatrine pretreatment did not alter baseline values *per se* (blood pressure 109 ± 4 mmHg and heart rate 404 ± 8 beats min^{-1}). Vehicle pretreatment caused a significant rise in heart rate of 19 ± 4 beats min^{-1} and in renal nerve activity of 28 ± 10%. Baseline values for blood pressure and heart rate were 109 ± 4 mmHg and 404 ± 8 beats min^{-1} respectively.

Discussion

In the present experiments in α-chloralose anaesthetized rats, treated with a neuromuscular blocking agent, i.c.v. injection of DP-5-CT, 5-CT, 8-OH-DPAT (low dose) and 5-HT [in

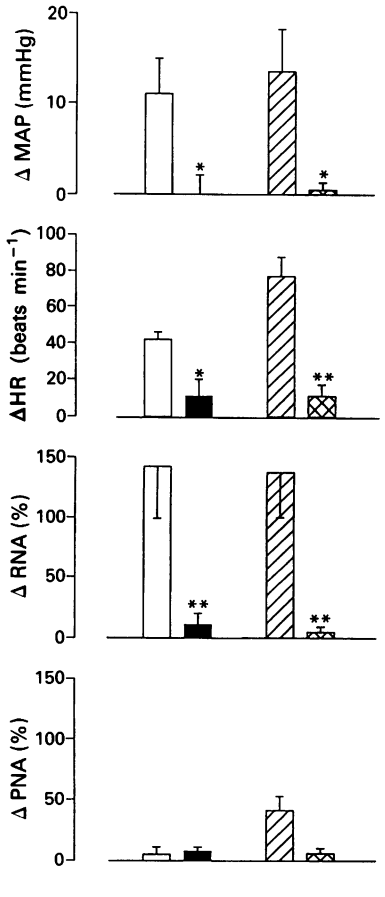

Figure 7 Anaesthetized rats: histogram comparing changes from post-treatment values 5 min after i.c.v. administration of N,N-di-n-propyl-5-carboxamidotryptamine (DP-5-CT, 3 nmol kg^{-1}) in the presence of saline (open columns; 5 μl i.c.v.; $n = 8$), methiothepin (solid columns; 1 mg kg^{-1}, i.v.; $n = 4$), 0.01 N HCl (vehicle control for spiroxatrine; hatched columns; 10 μl i.c.v.; $n = 6$) and spiroxatrine (cross hatched columns; 300 nmol kg^{-1}, i.c.v.; $n = 6$) in mean arterial blood pressure (MAP), heart rate (HR), renal nerve activity (RNA) and phrenic nerve activity (PNA). All values are the mean with s.e.mean shown by the vertical bars. $*P<0.05$ and $**P<0.01$. Comparisons are made against the appropriate control.

animals pretreated with the 5-HT_2/5-HT_{1C} receptor antagonists cinanserin or LY 53857 (Rubin *et al.*, 1964; Cohen *et al.*, 1983; 1985; see Hoyer, 1991)], caused renal sympathoexcitation, tachycardia and a rise in blood pressure. As DP-5-CT is a highly selective agonist for 5-HT_{1A} receptors (Mir *et al.*, 1987; Doods *et al.*, 1988; see Schoeffter & Hoyer, 1988) and the sympathoexcitation, tachycardia and rise in blood pressure caused by i.c.v. administration of DP-5-CT are blocked by the 5-HT_{1A} receptor antagonists, methiothepin (Fozard *et al.*, 1987; Schoeffter & Hoyer, 1988) and spiroxatrine, a selective antagonist for this subtype of the 5-HT receptor (Nelson & Taylor, 1986; see Hoyer, 1991), it is concluded that such responses are due to the activation of 5-HT_{1A} receptors. Furthermore, 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist (Middlemiss & Fozard, 1983; Fozard *et al.*, 1987) and 5-CT, a non-selective 5-HT_1 receptor agonist (Schoeffter & Hoyer, 1988; see Hoyer, 1991) also caused sympathoexcitation, tachycardia and a rise in blood pressure supporting the above conclusion. The observation that the rise in blood pressure, sympathoexcitation and tachycardia caused by 5-HT in the presence of a 5-HT_2/5-HT_{1C} antagonist could be blocked by the addition of spiroxatrine, demonstrates that the sympathoexcitation caused by 5-HT is also due to activation of 5-HT_{1A} receptors.

The above data indicating the activation of 5-HT_{1A} receptors, reached by i.c.v. administration, causes sympathoexcitation contrast with the findings from previous studies using 8-OH-DPAT and other non-structurally related 5-HT_{1A} agonists. These agonists given i.v. have been demonstrated to cause a centrally mediated decrease in blood pressure and sympathoinhibition in rats, cats, rabbits and dogs (see Ramage, 1990). However, in the rat there is some evidence that activation of 5-HT_{1A} receptors can also cause a pressor response and/or sympathoexcitation. In conscious spontaneously hypertensive rats i.v. 8-OH-DPAT caused an initial tachycardia and rise in blood pressure which was then followed by a bradycardia and hypotension (Fozard *et al.*, 1987). 8-OH-DPAT (i.v.) in conscious and anaesthetized rats mediates the release of adrenaline by central sympathoexcitation of the adrenal glands (Chaouloff & Jeanrenaud, 1987; Chaouloff *et al.*, 1990a,b; Bagdy *et al.*, 1989; Bouhelal & Mir, 1990). Microinjection of 5-HT_{1A} agonists into the raphé obscurus causes a pressor response (Dreteler *et al.*, 1991). Furthermore, i.c.v. administration of low doses of 8-OH-DPAT in conscious rats also causes a pressor response which is attenuated by methiothepin (Dedeoglu & Fisher, 1991). These combined data, at the least in the rat, demonstrate that activation of 5-HT_{1A} receptors can cause sympathoexcitation as well sympathoinhibition. In the present study and that of Dedeoglu & Fisher (1991), higher doses of 8-OH-DPAT administered i.c.v. tended to cause falls in blood pressure. A possible explanation for this observation is that the sympathoexcitation is masked by the sympathoinhibitory action of 8-OH-DPAT. A high dose of 8-OH-DPAT when given i.c.v. may diffuse to the mid and hind brain of the rat where activation of 5-HT_{1A} receptors is known to cause a fall in blood pressure and/or sympathoinhibition, in areas such as the dorsal raphé (Connor & Higgins, 1990), raphé magnus and pallidus (Valenta & Singer, 1990) and the rostral ventrolateral medulla (Nosjean & Guynet, 1991). However, it is possible that high doses of 8-OH-DPAT could have a non-selective action at receptors other than 5-HT_{1A} receptors (see Dedeoglu & Fisher, 1991).

5-HT has previously been shown to regulate a number of neuroendocrine responses including the release of vasopressin in conscious rats (Steardo & Iovino, 1986; see Van de Kar, 1991). Furthermore, in anaesthetized rats the pressor response to i.c.v. 5-HT is blocked by pretreatment with the vasopressin V_1-receptor antagonist d(CH_2)$_5$Tyr(Me)AVP (Inoue & Buñag, 1989), although the associated bradycardia is only attenuated in the first 5 min. In the present study, pretreatment with d(CH_2)$_5$Tyr(Me)AVP, at a dose which has previously been shown to abolish the pressor response to injected vasopressin (Buñag & Miyajima, 1984), only attenuated the duration of the rise in blood pressure caused by i.c.v. 5-HT. This difference may be explained by the different anaesthetic used, in the present study α-chloralose was used while in that of Inoue & Buñag (1989) urethane was used. Interestingly, in the present study the bradycardia and sympathoinhibition caused by 5-HT were reversed to tachycardia and sympathoexcitation in the presence of d(CH_2)$_5$Tyr(Me)AVP. A similar observation has been made in conscious rats (Pergola & Alper, 1991) and in that study the pressor response to i.c.v. 5-HT was completely blocked by combined α_1-adrenoceptor and vasopressin V_1-receptor blockade. In conscious rats in which the sinoaortic nerves had been cut (Pergola & Alper, 1991) i.c.v. 5-HT, although causing a pressor response, produced a marked tachycardia. Taken together these data indicate that i.c.v. 5-HT in conscious and anaesthetized rats causes the release of vasopressin. The peripherally mediated pressor response induces a baroreceptor mediated sympathoinhibition and bradycardia which masks the ability of 5-HT to cause sympathoexcitation and tachycardia through activation of 5-HT_{1A} receptors. It is of interest that a low dose of 5-HT (4 nmol kg^{-1}) given i.c.v. caused a pressor response associated with a tachycardia in conscious rats, whereas a higher dose (120 nmol kg^{-1}) produced a pressor response and a biphasic effect on heart rate, bradycardia followed by tachycardia (Dedeoglu & Fisher, 1991; Anderson *et al.*, 1992a). Presumably the low dose of 5-HT produced only the sympathoexcitatory component of the 5-HT response. Therefore the pattern of response caused by i.c.v. administration of 5-HT is dependent on the dose of 5-HT given, and in anaesthetized animals, the anaesthetic used.

The present data show that the release of vasopressin by i.c.v. 5-HT is mediated by 5-HT_2 or 5-HT_{1C} receptors, as a similar effect is obtained by pretreatment with the 5-HT_2/5-HT_{1C} receptor antagonists, cinanserin and LY 53857 (i.c.v.) to that with the vasopressin V_1-receptor antagonist. This conclusion is supported by other studies in conscious rats (Brownfield *et al.*, 1988; Pergola & Alper, 1991; see Van de Kar, 1991).

The selective 5-HT_2/5-HT_{1C} receptor agonist DOI given i.c.v. or i.v. caused a rise in blood pressure, bradycardia and sympathoinhibition. However, these effects were attenuated by the peripherally acting 5-HT_2/5-HT_{1C} receptor antagonist BW501C67 (Mawson & Whittington, 1970; Fuller *et al.*, 1986; BW501C67 has a pK_D of 9.5 at 5-HT_2 and a pK_D of 8.5 at 5-HT_{1C} receptors, G.R. Martin unpublished observations), whereas the response produced by i.c.v. administration of 5-HT on these variables was essentially unaffected by i.v. BW501C67. Therefore, the effects of DOI on blood pressure, heart rate and renal nerve activity can be attributed to activation of peripheral 5-HT_2 receptors on vascular smooth muscle (Dabire *et al.*, 1989; Alper, 1990) and not by activation of central 5-HT_2 or 5-HT_{1C} receptors to release vasopressin. In this context, DOI given i.v. has been shown not to cause the release of vasopressin (Bagdy *et al.*, 1992; see Van de Kar, 1991). Interestingly, in the anaesthetized cat, activation of central 5-HT_2 or 5-HT_{1C} receptors has been shown to cause sympathoexcitation (McCall & Harris, 1988; Vayssettes-Courchay *et al.*, 1991; Shepheard *et al.*, 1991; Ramage *et al.*, 1991). In the present study and in previous studies (Alper, 1990; Vayssettes-Courchay *et al.*, 1990) there is no evidence for a centrally mediated increase in sympathetic tone in the rat. Therefore, it appears that the cat and rat are different in this respect.

In previous studies activation of 5-HT pathways has been demonstrated to increase central respiratory drive (Holtman *et al.*, 1986a,b; Dreteler *et al.*, 1991). Furthermore, central administration of 8-OH-DPAT has been shown to increase respiratory rate (Gillis *et al.*, 1989) and phrenic nerve activity (Sporton *et al.*, 1991), a measure of central inspiratory drive. The present results also demonstrate that 8-OH-DPAT can cause an increase in central inspiratory drive. However, the

involvement of 5-HT_{1A} receptors in this action of 8-OH-DPAT is doubtful as both DP-5-CT and 5-CT failed to have any effect on phrenic nerve activity.

The precise site/sites in the brain where 5-HT is acting to cause these cardiovascular effects remain to be determined but the rapid onset of response would suggest a brain area close to the lateral or 3rd ventricles. Angiotensin II administered i.c.v. has been shown to cause a rise in blood pressure and bradycardia in conscious rats and this has been attributed to the release of vasopressin through activation of angiotensin receptors located in the subfornical organ (Iovino & Steardo, 1984; see Hartle & Brody, 1984). Therefore, it is possible that the release of vasopressin caused by 5-HT may occur following activation of 5-HT receptors located in the subfornical organ. Smits & Struyker-Boudier (1976) have demonstrated that microinjection of 5-HT into the anterior hypothalamus/pre-optic area can cause an increase in blood pressure. This area is situated close to the 3rd ventricle and 5-HT containing neurones have been shown to project to this site from the dorsal raphé nucleus (see Coote, 1990). Thus, 5-HT may be acting at the level of the hypothalamus to produce the cardiovascular response observed following i.c.v. injection. Further microinjection studies are required to determine the precise site of action of 5-HT.

In conclusion, the present study demonstrates that i.c.v. administration of 5-HT causes sympathoexcitation by activation of 5-HT_{1A} receptors and the release of vasopressin through activation of 5-HT_2 or 5-HT_{1C} receptors.

I.K.A. is in receipt of a SERC CASE studentship with Wellcome Research Laboratories, Beckenham, Kent. We would like to thank Mr S. Wilkinson for valuable technical assistance. We are grateful to the Royal Society for providing funds to purchase a surgical microscope.

References

ALPER, R.H. (1990). Hemodynamic and renin responses to (±)-DOI, a selective 5-HT_2 receptor agonist, in conscious rats. *Eur. J. Pharmacol.*, **175**, 323–332.

ANDERSON, I.K. (1991). Evidence to suggest that activation of forebrain 5-HT_{1A} receptors causes sympathoexcitation in anesthetized rats. *Br. J. Pharmacol.*, **104**, 65P.

ANDERSON, I.K., GARDINER, S.M., WIDDOP, R.E., BENNETT, T., MARTIN, G.R. & RAMAGE, A.G. (1992a). Regional haemodynamic effects of 5-HT i.c.v. in conscious Long-Evans and Brattleboro rats. *Br. J. Pharmacol.*, **107**, 14P.

ANDERSON, I.K., MARTIN, G.R. & RAMAGE, A.G. (1992b). Evidence to suggest that activation of central 5-$HT_{2/1C}$ receptors causes the release of vasopressin in anaesthetized rats. *Br. J. Pharmacol.*, **105**, 30P.

BAGDY, G., SVED, A.F., MURPHY, D.L. & SZEMEREDI, K. (1992). Pharmacological characterization of serotonin receptor subtypes involved in vasopressin and plasma renin activity responses to serotonin agonists. *Eur. J. Pharmacol.*, **210**, 285–289.

BAGDY, G., SZEMEREDI, K. & MURPHY, D.L. (1989). Marked increases in plasma catecholamine concentrations preceded hypotension and bradycardia caused by 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) in conscious rats. *J. Pharm. Pharmacol.*, **41**, 270–272.

BOUHELAL, R. & MIR, A.K. (1990). Role of the adrenal gland in the metabolic and cardiovascular effects of 8-OH-DPAT. *Eur. J. Pharmacol.*, **181**, 173–179.

BROWNFIELD, M.S., GREATHOUSE, J., LORENS, S.A., ARMSTRONG, J., URBAN, J.H. & VAN DE KAR, L.D. (1988). Neuropharmacological characterization of serotonergic stimulation of vasopression secretion in conscious rats. *Neuroendocrinology*, **47**, 277–283.

BUÑAG, R.D. & MIYAJIMA, E. (1984). Sympathetic hyperactivity elevates blood pressure during acute cerebroventricular infusions of hypertonic salt in rats. *J. Cardiovasc. Pharmacol.*, **6**, 844–851.

CHAOULOFF, F., BAUDRIE, V. & LAUBIE, D. (1990a). Evidence that the 5-HT_{1A} receptor agonists buspirone and ipsapirone activate adrenaline release in the conscious rat. *Eur. J. Pharmacol.*, **177**, 107–110.

CHAOULOFF, F. & JEANRENAUD, B. (1987). 5-HT_{1A} and alpha-2 adrenergic receptors mediate the hyperglycaemic and hypoinsulinaemic effects of 8-hydroxy-2-(di-n-propylamino)-tetralin in conscious rats. *J. Pharmacol. Exp. Ther.*, **243**, 1159–1166.

CHAOULOFF, F., LAUBIE, D. & BAUDRIE, V. (1990b). Ganglionic transmission is a prerequisite for the adrenaline-releasing and hyperglycaemic effects of 8-OH-DPAT. *Eur. J. Pharmacol.*, **185**, 11–18.

COHEN, M.L., FULLER, R.W. & KURZ, K.D. (1983). LY 53857, a selective and potent serotonergic (5-HT_2) receptor antagonist, does not lower blood pressure in the spontaneously hypertensive rat. *J. Pharmacol. Exp. Ther.*, **227**, 327–332.

COHEN, M.L., KURZ, K.D., MASON, N.R., FULLER, R.W., MARZONI, G.P. & GARBRECHT, W.L. (1985). Pharmacological activity of the isomers of LY 53857, potent and selective 5-HT_2 receptor antagonists. *J Pharmacol. Exp. Ther.*, **235**, 319–323.

CONNOR, H.E. & HIGGINS, G.A. (1990). Cardiovascular effects of 5-HT_{1A} receptor agonists injected into the dorsal raphe nucleus of conscious rats. *Eur. J. Pharmacol.*, **182**, 63–72.

COOTE, J.H. (1990). The central antihypertensive action of 5-hydroxytryptamine: the location of site of action. In *Cardiovascular Pharmacology of 5-Hydroxytryptamine; Prospective Therapeutic Applications*. ed. Saxena, P.R., Wallis, D.I., Wouters, W. & Bevan, P. pp. 259–266. The Netherlands: Kluwer.

DABIRE, H., CHAOUCHE-TEYARA, K., CHERQUI, C., FOURNIER, B. & SCHMITT, H. (1989). DOI is a mixed agonist-antagonist at postjunctional 5-HT_2 receptors in the pithed rat. *Eur. J. Pharmacol.*, **170**, 109–111.

DALTON, D.W. (1986). The cardiovascular effects of centrally administered 5-hydroxytryptamine in the conscious normotensive and hypertensive rat. *J. Auton. Pharmacol.*, **6**, 67–75.

DEDEOGLU, A. & FISHER, L.A. (1991). Central nervous actions of serotonin and a serotonin$_{1A}$ receptor agonist: Cardiovascular excitation at low doses. *J. Pharmacol. Exp. Ther.*, **257**, 425–432.

DOODS, H.N., BODDEKE, H.W.G.M., KALKMAN, H.O., HOYER, D., MATHY, M.-J. & VAN ZWIETEN, P.A. (1988). Central 5-HT_{1A} receptors and the mechanism of the central hypotensive effect of (+) 8-OH-DPAT, DP-5-CT, R28935, and urapidil. *J. Cardiovasc. Pharmacol.*, **11**, 432–437.

DRETELER, G.H., WOUTERS, W., SAXENA, P.R. & RAMAGE, A.G. (1991). Pressor effects following microinjection of 5-HT_{1A} receptor agonists into the raphe obscurus of the anaesthetized rat. *Br. J. Pharmacol.*, **102**, 317–322.

FOZARD, J.R., MIR, A.K. & MIDDLEMISS, D.N. (1987). Cardiovascular response to 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT): Site of action and pharmacological analysis. *J Cardiovasc. Pharmacol.*, **9**, 328–347.

FULLER, R.W., KURZ, K.D., MASON, N.R. & COHEN, M.L. (1986). Antagonism of a peripheral vascular but not an apparently central serotonergic response by xylamidine and BW501C67. *Eur. J. Pharmacol.*, **125**, 71–77.

GILLIS, R.A., HILL, K.J., KIRBY, J.S., QUEST, J.A., HOMASH, P., NORMAN, W.P. & KELLAR, K.J. (1989). Effect of activation of central nervous system serotonin 1A receptors on cardiorespiratory function. *J. Pharmacol. Exp. Ther.*, **248**, 851–857.

HARTLE, D.K. & BRODY, M.J. (1984). The angiotensin II pressor system of the rat forebrain. *Circ. Res.*, **54**, 355–366.

HOLTMAN, J.R., ANASTASI, N.C., NORMAN, W.P. & DRETCHEN, K.L. (1986a). Effect of electrical and chemical stimulation of the raphe obscurus on phrenic nerve activity in the cat. *Brain Res.*, **362**, 214–220.

HOLTMAN, J.R., DICK, T.E. & BERGER, A.J. (1986b). Involvement of serotonin in the excitation of phrenic motoneurones evoked by stimulation of the raphe obscurus. *J. Neuroscience*, **6**, 1185–1193.

HOYER, D. (1991). The 5-HT receptor family: ligands, distribution and receptor-effector coupling. In *5-HT_{1A} Agonists, 5-HT_3 Antagonists and Benzodiazepines: their Comparative Behavioural Pharmacology*. ed. Rodgers, R.J. & Cooper, S.J. pp. 31–57. Chichester: John Wiley & Sons Ltd.

INOUE, A. & BUÑAG, R.D. (1989). Sympathetic inhibition and vasopressin mediation during centrally induced responses to serotonin in rats. *J. Cardiovasc. Pharmacol.*, **13**, 902–907.

IOVINO, M. & STEARDO, L. (1984). Vasopressin release to central and peripheral angiotensin II in rats with lesions of the subfornical organ. *Brain Res.*, **322**, 365–368.

KRISTIC, M.K. & DJURKOVIC, D. (1976) Hypertension mediated by activation of the rat brain 5-hydroxytryptamine receptor sites. *Experientia*, **32**, 1187–1189.

KRISTIC, M.K. & DJURKOVIC, D. (1980). Analysis of cardiovascular responses to central administration of 5-hydroxytryptamine in rats. *Neuropharmacol.*, **19**, 455–463.

LAMBERT, G.A., FRIEDMAN, E. & GERSHON, S. (1975). Centrally mediated cardiovascular responses to 5-HT. *Life Sci.*, **17**, 915–920.

LAMBERT, G.A., FRIEDMAN, E., BUCHWEITZ, E. & GERSHON, S. (1978). Involvement of 5-hydroxytryptamine in the central control of respiration, blood pressure and heart rate in the anaesthetized rat. *Neuropharmacol.*, **17**, 807–813.

MAWSON, C. & WHITTINGTON, H. (1970). Evaluation of the peripheral and central antagonistic activities against 5-hydroxytryptamine of some new agents. *Br. J. Pharmacol.*, **39**, 223P.

MCCALL, R.B. & HARRIS, L.T. (1988). 5-HT_2 receptor agonists increase spontaneous sympathetic nerve discharge. *Eur. J. Pharmacol.*, **151**, 113–116.

MIDDLEMISS, D.N. & FOZARD, J.R. (1983). 8-hydroxy-2-(di-n-propylamino) tetralin discriminates between subtypes of the 5-HT_1 recognition site. *Eur. J. Pharmacol.*, **90**, 151–153.

MIR, A.K., HIBERT, M. & FOZARD, J.R. (1987). Cardiovascular effects of N,N-dipropyl-5-carboxamidotryptamine, a potent and selective 5-HT_{1A} receptor ligand. In *Neuronal Messengers in Vascular Function*. ed. Nobin, A., Owman, C. & Arneklo-Nobin, B. pp. 21–29. Amsterdam: Elsevier.

NELSON, D.L. & TAYLOR, E.W. (1986). Spiroxatrine: a selective serotonin$_{1A}$ receptor antagonist. *Eur. J. Pharmacol.*, **129**, 307–314.

NOSJEAN, A. & GUYENET, P.G. (1991). Role of the ventral lateral medulla in the sympatholytic effect of 8-OH-DPAT in rats. *Am. J Physiol.*, **260**, R600–R609.

PERGOLA, P.E. & ALPER, R.H. (1991). Vasopressin and autonomic mechanisms mediate cardiovascular actions of central serotonin. *Am. J. Physiol.*, **260**, R1188–R1193.

PERGOLA, P.E. & ALPER, R.H. (1992). Effects of central serotonin on autonomic control of heart rate in intact and baroreceptor deficient rats. *Brain Res.*, **582**, 215–220.

RAMAGE, A.G. (1985). The effects of ketanserin, methysergide and LY 53857 on sympathetic nerve activity. *Eur. J. Pharmacol.*, **113**, 295–303.

RAMAGE, A.G. (1990). Influence of 5-HT_{1A} receptor agonists on sympathetic and parasympathetic nerve activity. *J. Cardiovasc. Pharmacol.*, **15**, (suppl. 7), S75–S85.

RAMAGE, A.G., SHEPHEARD, S.L., JORDAN, D. & KOSS, M.C. (1991). Examination of the sympathoexcitatory action of the 5-HT_2 agonist, 1-(2,5-dimethoxy-4-idophenyl)-2-aminopropane (DOI), on cardiac sympathetic nerve activity in the anaesthetized cat. *J Physiol.*, **438**, 84P.

RAMAGE, A.G. & WILKINSON, S.J. (1989). Evidence that different regional sympathetic outflows vary in their sensitivity to the sympathoinhibitory actions of putative 5-HT_{1A} and α_2 adrenoceptor agonists in anaesthetised cats. *Br. J. Pharmacol.*, **98**, 1157–1164.

RUBIN, B., PIALA, J.J., BURKE, J.C. & CRAVER, B.N. (1964). A new potent and specific serotonin inhibitor (SQ 10,643) 2'-(3-dimethylaminopropylthio) cinnamanilide hydrochloride: anti-serotonin activity on the uterus and on gastrointestinal, vascular and respiratory systems of animals. *Arch. Int. Pharmacodyn.*, **152**, 132–143.

SCHOEFFTER, P. & HOYER, D. (1988). Centrally acting hypotensive agents with affinity for 5-HT_{1A} binding sites inhibit forskolin-stimulated adenylate cyclase activity in calf hippocampus. *Br. J. Pharmacol.*, **95**, 975–985.

SHEPHEARD, S.L., JORDAN, D. & RAMAGE, A.G. (1991). Investigation of the effects of IVth ventricular administration of the 5-HT_2 agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), on autonomic outflow in the anaesthetized cat. *Br. J. Pharmacol.*, **104**, 367–372.

SMITS, J.F. & STRUYKER-BOUDIER, H.A. (1976). Intrahypothalamic serotonin and cardiovascular control in the rat. *Brain Res.*, **111**, 422–427.

SOKAL, R.R. & ROHLF, F.J. (1969). *Biometry: The Principles and Practice of Statistics in Biological Research*. San Francisco, CA: Freeman.

SPORTON, S.C.E., SHEPHEARD, S.L., JORDAN, D. & RAMAGE, A.G. (1991). Microinjections of 5-HT_{1A} agonists into the dorsal motor vagal nucleus produce a bradycardia in the atenolol-pretreated anaesthetised rat. *Br. J. Pharmacol.*, **104**, 466–470.

STEARDO, L. & IOVINO, M. (1986). Vasopressin release after enhanced serotonergic transmission is not due to activation of the renin-angiotensin system. *Brain Res.*, **382**, 145–148.

SUKAMOTO, T., YAMAMOTO, T., WATANABE, S. & UEKI, S. (1984). Cardiovascular responses to centrally administered serotonin in conscious normotensive and spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **100**, 173–179.

VALENTA, B. & SINGER, E.A. (1990). Hypotensive effects of 8-hydroxy-2-(di-n-propylamino)tetralin and 5-methylurapidil following stereotaxic microinjection into the ventral medulla of the rat. *Br. J. Pharmacol.*, **99**, 713–716.

VAN DE KAR, L.D. (1991). Neuroendocrine pharmacology of serotonergic (5-HT) neurons. *Annu. Rev. Pharmacol. Toxicol.*, **31**, 289–320.

VAYSSETTES-COURCHAY, C., BOUYSSET, F., VERBEUREN, T.J., LAUBIE, M. & SCHMITT, H. (1990). The cardiovascular effects of quipazine are mediated by peripheral 5-HT_2 and 5-HT_3 receptors in anaesthetised rats. *Eur. J. Pharmacol.*, **192**, 389–395.

VAYSSETTES-CHOURCHAY, C., BOUYSSET, F., VERBEUREN, T.J., LAUBIE, M. & SCHMITT, H. (1991). Quipazine-induced hypotension in anaesthetized cats is mediated by central and peripheral 5-HT_2 receptors: role of the ventrolateral pressor area. *Eur. J. Pharmacol.*, **192**, 389–395.

(Received May 11, 1992
Revised July 31, 1992
Accepted August 3, 1992)

Br. J. Pharmacol. (1992), **107**, 1029–1036

Guinea-pig treatment with pertussis toxin suppresses macrophage-dependent bronchoconstriction by fMLP and fails to inhibit the effects of PAF

[1]Chakir Kadiri, Dominique Leduc, Jean Lefort, *Atsushi Imaizumi & [2]B. Boris Vargaftig

Unité de Pharmacologie cellulaire, Unité Associée Institut Pasteur-INSERM no. 285, 25, Rue du Dr, Roux, 75015, Paris, France and *Teijin Research Institute, Tokyo, Japan

1 Bronchoconstriction and thromboxane B_2 (TxB_2) release following the intra-tracheal administration of the secretagogue N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) to lungs from pertussis toxin-treated guinea-pigs *in vivo* and *in vitro* were inhibited as compared to saline-treated animals, under conditions where the responses to PAF were modified less effectively.

2 The cell target accounting for bronchoconstriction by fMLP and for inhibition by pertussis toxin is located in the airways and is probably the alveolar macrophage. Indeed (a) fMLP-induced superoxide anions and TxB_2 formation by alveolar macrophages were inhibited by pertussis toxin given *in vivo*; (b) G_i proteins of membranes from alveolar macrophages were ADP-ribosylated *in vivo* by pertussis toxin and (c) bronchoconstriction and TxB_2 release in response to the intra-tracheal administration of fMLP to lungs from pertussis toxin-treated animals were restored when alveolar macrophages from control guinea-pigs were transferred into the airways of pertussis toxin-treated animals before lung isolation.

3 Pertussis toxin administered to guinea-pigs *in vivo*, reduced the subsequent TxB_2 formation and superoxide anion release by alveolar macrophages stimulated with PAF, but failed to inhibit PAF-induced bronchoconstriction.

4 Formation of TxB_2 by alveolar macrophages following the intra-tracheal administration of fMLP accounts for bronchoconstriction and requires pertussis toxin-sensitive G_i proteins. PAF operates via a different mechanism, which is independent of G_i-like protein and involves mediators other than TxB_2 and superoxide anions.

Keywords: Alveolar macrophages; bronchoconstriction; N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP); G proteins; PAF; pertussis toxin

Introduction

The *in vivo* effects of inflammatory mediators involve different mechanisms and cells, and in particular their route of administration determines what cell targets are first encountered. Thus i.v. PAF triggers bronchoconstriction in the guinea-pig by a platelet-dependent and cyclo-oxygenase-independent mechanism (Vargaftig *et al.*, 1980), whereas bronchoconstriction following its intra-tracheal administration is not platelet-dependent and is inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) (Lefort *et al.*, 1984). The peptide secretagogue N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) induces bronchoconstriction, leucopenia and thrombocytopenia when injected i.v. to the guinea-pig (Boukili *et al.*, 1986; 1989). Bronchoconstriction and the *in vitro* stimulation of tracheal smooth muscle are inhibited by NSAIDs, whereas leucopenia is unaffected and thrombocytopenia is enhanced (Bureau *et al.*, 1992). Since neutrophil depletion does not prevent bronchoconstriction by fMLP, the intravascular activation and recruitment to lungs of circulating leukocytes does not account for bronchoconstriction (Boukili *et al.*, 1986). Bronchoconstriction and leukocyte activation by i.v. fMLP are suppressed by pertussis toxin (Imaizumi *et al.*, 1992), suggesting the involvement of pertussis toxin-sensitive G proteins, which transduce the signals to phospholipase C (Lad *et al.*, 1985; Fu *et al.*, 1988; Crouch & Lapetina, 1988) and to Ca^{2+} channels (Hescheler *et al.*, 1987) and thus induce cell activation.

In general, the intra-tracheal generation and administration of mediators may be more relevant for the pathology of human pulmonary allergic diseases than the i.v. route. The intra-tracheal instillation of fMLP to the guinea-pig is followed by bronchoconstriction, which is inhibited by NSAIDs (Boukili *et al.*, 1989). fMLP is a powerful stimulator of the release of arachidonate by alveolar macrophages *in vitro*, via a pertussis toxin-sensitive process (Kadiri *et al.*, 1990). This has now led us to investigate the involvement of pertussis toxin-sensitive targets with the effects of the two inflammatory agents fMLP and PAF given into the trachea *in vivo* and *in vitro* and to study whether alveolar macrophages account for bronchoconstriction by fMLP and if so, via which mechanisms.

Methods

Pertussis toxin pretreatment

Hartley guinea-pigs (400–500 g, Elevages Lebau, France) were given either pertussis toxin (20 $\mu g\,kg^{-1}$) or its vehicle (0.9% NaCl) via the popliteal vein 72 h before the *in vivo* or *ex vivo* experiments. This schedule of administration was determined by Imaizumi *et al.* (1992).

In vivo *bronchoconstriction*

To evaluate the increase in pulmonary insufflation pressure (designated bronchoconstriction), guinea-pigs were anaesthetized with sodium pentobarbitone (40 $mg\,kg^{-1}$, i.p.), ventilated through the cannulated trachea with a Palmer miniature pump (tidal volume 1 $mg\,100\,g^{-1}$, frequency 60 strokes min^{-1}), spontaneous breathing being suppressed with 2 mg kg^{-1} pancuronium, i.p. Catheters were inserted into the jugular vein for drug administration. To perform the intra-tracheal instillation of drugs, a catheter was inserted into the

[1] Present address: Rectorat de l'Université Mohamed 1er., BP 524, Oujda, Marroco.
[2] Author for correspondence.

trachea, which required a 10–20 s interruption of the ventilation. Bronchoconstriction was monitored via a T cannula inserted between the pump and the trachea and connected to a Statham pressure transducer (P23Db), the signal being displayed on a Beckman recorder (B511A). The intensity of bronchoconstriction was measured at the peak response to fMLP or PAF and was expressed as % of the total constriction obtained by occluding the cannula at the end of the experiment.

Experimental design for lung perfusion

Guinea-pigs were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.), tracheae were cannulated, and the animals were ventilated as above. A thoracotomy was performed, the lungs were removed, placed in a plastic chamber, ventilated (60 strokes min^{-1}) and perfused (10 ml min^{-1}, 37°C) with a gassed (95% O_2/5% CO_2) Krebs solution containing 0.25% (wt.vol^{-1}) bovine serum albumin (BSA) and mepyramine (3 μM). The changes in resistance to inflation (bronchoconstriction) were continuously recorded through a T cannula inserted between the outlet of the respiratory pump and the trachea with a Statham transducer (P23Db). After 10 min, fMLP (0, 1 and 10 μg) or PAF (10 and 100 ng) were administered into the trachea via a lateral tube at a 20 min interval in 0.1 ml. The stock solution of fMLP (1 mg ml^{-1} of dimethylsulphoxide) and of PAF (10 μg ml^{-1} of a solution of 0.25% BSA in 0.9% NaCl), were diluted in 0.9% NaCl before use. The lungs were perfused with a peristaltic pump at 10 ml min^{-1}. One minute fractions of the lung effluent were collected at 4°C before and during the first 6 min after the injections for the determination of TxB_2 content. Aliquots of 1 ml from each sample were centrifuged at 3000 r.p.m. for 10 min at 4°C and the supernatant was stored at −20°C until further analysis. Total occlusion of the trachea was determined by mechanical clamping at the end of the each experiment.

Purification of alveolar macrophages

Alveolar macrophages were prepared from bronchoalveolar lavage (BAL) fluid obtained by the instillation and gentle recovery of 10 ml fractions of 5 ml each of saline into the lungs via the trancheal cannula (Bachelet *et al.*, 1989). They were isolated on a metrizamide gradient according to Vadas *et al.* (1979). Macrophages were collected from the top of the gradient, washed once in Hanks buffer, counted and their number was adjusted to 3×10^6 cells ml^{-1} in RPMI 1640. Differential cell counts performed on cytocentrifuged preparations stained with May-Grunwald Giemsa dye (Diff-Quick, American Scientific Products, Macgaw Park, IL, U.S.A.) showed no differences in number and type of cells recovered from the BAL of control or of pertussis toxin-treated animals. Indeed, the total cell number was of $59.4 \pm 11.6 \times 10^6$ and $48.3 \pm 15.4 \times 10^6$ in BAL from control and from pertussis toxin-treated guinea-pigs, respectively (means ± s.e.mean, $n = 10$). BAL from control guinea-pigs contained 66.5 ± 7.7% macrophages, 14.8 ± 11.8% lymphocytes, 0.25 ± 0.4% neutrophils and 18.5 ± 6.9% eosinophils, whereas BAL from pertussis toxin-treated guinea-pigs showed 68.5 ± 14.2% macrophages, 9.8 ± 6% lymphocytes, 1.5 ± 1.7% neutrophils and 20.2 ± 7% eosinophils. The macrophages purified on metrizamide gradient were 95% pure and their viability assessed by the trypan blue exclusion test was always above 90%.

Macrophage preparation and stimulation

Alveolar macrophages were allowed to adhere for 1 h to plastic Petric dishes in RPMI 1640 containing 3% foetal bovine serum at 37°C and 5% CO_2. The adherent cells were washed twice with RPMI and stimulated with fMLP or PAF at the indicated concentrations in 1 ml RPMI at a pH of 7.4. After stimulation, the media were collected and centrifuged at 600 *g* for 10 min to remove nonadherent cells. Aliquots were used to evaluate the TxB_2 content. The adherent cells were scraped off, disrupted by sonication, and the homogenate used for protein determination (Bradford, 1976).

Membrane isolation from alveolar macrophages

Cells from pertussis toxin-treated guinea-pigs or from controls were sonicated with a Sonimasse sonicator for 15 s in 40 mM Tris-HCl buffer, pH 7.4, containing 0.25 M saccharose, 0.1 μM phenylmethylsulphonylfluoride (PMSF), 0.5 mg ml^{-1} leupeptine, and 0.5 mg ml^{-1} aprotinin. The homogenate was then centrifuged at 600 *g* for 10 min at 4°C. The supernatant was further centrifuged at 30 000 *g* for 1 h at 4°C, the membrane pellets were resuspended at 0.5–1 mg of protein ml^{-1} in the same buffer and frozen at −70°C.

ADP-ribosylation of macrophage membranes by pertussis or cholera toxins

Cholera or pertussis toxins were first activated at 37°C for 10 min with 40 mM dithiothreitol and incubated with alveolar macrophage membranes (100 μg) for 45 min at 37°C. In the case of pertussis toxin, the medium contained 40 mM Tris buffer pH 7.4, 10^7 c.p.m. of [a-^{32}P]-nicotine amide dinucleotide ([a-^{32}P]-NAD, 30 Ci $mmol^{-1}$, Amersham), 3 μM NAD, 0.2 mM ATP, 100 mM NaCl, 0.5 mg ml^{-1} leupeptine, 0.5 mg ml^{-1} aprotinin, 0.1 mM PMSF, 10 mM phosphoenolpyruvate, 1 μg ml^{-1} myokinase, 5 μg ml^{-1} pyruvate kinase and 10 mM thymidine in a final volume of 200 μl. The membranes were incubated with cholera toxin for 10 min at 37°C in 100 mM phosphate buffer pH 7.4, 10^7 c.p.m. of [a-^{32}P]-NAD (30 Ci $mmol^{-1}$, Amersham), 3 μM NAD, 1 mM ATP, 1 mM GTP, 5 mM $MgCl_2$, 0.1 mM PMSF, 0.5 mg ml^{-1} leupeptine and 0.5 mg ml^{-1} aprotinine. Sequential additions of 4 μl of phosphate buffer containing 50 mM ATP and 50 mM GTP were performed every 2 min. ADP-ribosylation was stopped with 200 μl of cold Tris buffer and samples were centrifuged for 10 min at 17 000 *g*. The pellets were then washed twice in Tris buffer, resuspended in 40 μl of water and 20 μl of a buffer containing 0.4 M Tris buffer pH 8.8, 10 mM EDTA, 0.02% blue bromophenol, 0.8 M sucrose and 6% sodium dodecyl sulphate (SDS), dithiothreitol at 15 mM was added followed by boiling at 100°C for 3 min. After being alkylated with iodoacetamide 50 mM for 15 min at 20°C, each sample was subjected to SDS-polyacrylamide gel (10%) electrophoresis. The gel was stained with Coomassie Brilliant, destained, dried, and exposed to a Kodak film for 72 h with an intensifying screen at −70°C.

Superoxide anion generation

BAL cells or purified macrophages were resuspended in MEM medium supplemented with 20 mM HEPES to a final concentration of 3×10^6 cells ml^{-1} and were left for 2 h at 37°C for equilibration; 500 μl of the suspension were transferred to a 1 ml spectrophotometer cuvette at 37°C and 80 μM horse ferricytochrome c was added. The reaction was started by adding fMLP or PAF in 10 μl and the absorbance changes were recorded at 550 nm at 20 s intervals for 3 min in a Kontron spectrophotometer type Uvikon 810 against a blank assay containing MEM with superoxide dismutase (SOD) at 25 μg ml^{-1}. In order to check the specific superoxide reduction of cytochrome c, control cells were pre-treated with SOD at 25 μg ml^{-1} before stimulation and the amounts of cytochrome c were subtracted from the total reduced cytochrome obtained in absence of SOD. The amounts of reduced cytochrome c were calculated using an extinction coefficient of 21.1 mM^{-1} cm^{-1}.

Thromboxane B_2 assay

The basic procedure was that of Sors *et al.* (1978). Briefly, 100 µl of the lung effluents or adherent macrophages media were incubated overnight at 4°C with ^{125}iodine-labelled thromboxane B_2 (TxB_2) and anti-TxB_2 antiserum in a phosphate buffer (10 mM, pH 7.4) containing bovine γ-globulin (0.3% wt.vol^{-1}). The next day, bound and free ligands were separated by adding a solution of polyethyleneglycol 6000 (30% in distilled water), followed by centrifugation for 10 min at 1500 *g* and at 4°C. The supernatants were decanted, and radioactivity present in the pellet, which corresponded to the bound fraction, was counted for 1 min in a γ counter. The monoclonal antibody employed was less than 0.002% crossreactive with prostaglandin D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and arachidonic acid. The sensitivity of the assay was approximately 2 pg of immunoreactive TxB_2 in 0.1 ml sample.

Evaluation of cyclic AMP

Amounts of adenosine 3':5'-cyclic monophosphate (cyclic AMP) contained in alveolar macrophages were measured by radioimmunoassay. The samples were treated with perchloric acid 1 N and following appropriate dilution in 0.05 M acetate buffer at pH 6.4, acetylated and assayed for cyclic AMP according to Cailla *et al.* (1973). The total amount of cyclic AMP was expressed in pmol/10^6 cell per min.

Reagents

Pertussis and cholera toxins were a generous gift of Dr A. Ginnaga (The Chemo-sero-therapeutic Research Institute, Japan) or from Seikagaku Kogyo Co., Japan. Sodium pentobarbitone (Clin Midy, France); fMLP, BSA fraction V, TxB_2, ATP, GTP, NAD, PMSF, thymidine, ferricytochrome c, dithiothreitol, phosphoenolpyruvate (Sigma, St. Louis, MO, U.S.A.); perchloric acid, polyethyleneglycol 6000 (Merck, Darmstadt, Germany); leupeptine, myokinase, pyruvate kinase, aprotinin (Boehringer Mannheim, Germany); RPMI 1640, MEM, foetal calf serum (Gibco, UK); PAF (Bachem, Switzerland); mepyramine maleate (Rhone-Poulenc, France) chicken ovalbumin (Miles, Naperville, IL, U.S.A.); radiolabelled TxB_2 and anti-serum was obtained from the URIA, Institut Pasteur-INSERUM U 207, France; [^{32}P]-NAD, ^{125}I labelled protein (NEN research products, Boston-USA); nitrocellulose (Biorad, U.S.A.).

Data analysis

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

Results

Effects of pertussis toxin on in vivo *bronchoconstriction induced by the intra-tracheal administration of fMLP and PAF*

As seen in Figure 1, the intra-tracheal instillation of 0.1 µg of fMLP induced a moderate bronchoconstriction, which was more intense at 10 µg. Bronchoconstriction by 0.1 µg fMLP was reduced and by 10 µg was suppressed by pertussis toxin treatment. One µg of PAF was as effective as 10 µg of fMLP in inducing bronchoconstriction and was not significantly affected by pertussis toxin.

The effects of pertussis toxin on fMLP- and PAF-induced bronchoconstriction and release of thromboxane B_2 from isolated lungs

Guinea-pigs were injected i.v. with 20 µg kg^{-1} of pertussis toxin and isolated lungs were prepared 72 h later. Administered intra-tracheally to the perfused lungs at 0.1 and 10 µg, fMLP induced bronchoconstriction of 22.5 ± 3% and 42.9 ± 8%, respectively, of the values obtained after the total occlusion of the trachea for control lungs, and of 6 ± 4% and 9.6 ± 6.0% respectively, for those from pertussis toxin-treated animals (Figure 2a). Pertussis toxin also reduced the release of TxB_2 into the lung effluent following the intra-tracheal administration of fMLP (Figure 3a). The amounts of TxB_2 formed were 3.11 ± 1.3 ng ml^{-1} and 7.34 ± 2.6 ng ml^{-1} respectively for controls, and 1.3 ± 0.6 ng ml^{-1} and 1.6 ± 0.8 ng ml^{-1}, respectively, for lungs from pertussis toxin-treated animals, for 0.1 and 10 µg of fMLP.

In contrast, no differences were observed between the intensity of bronchoconstriction in response to PAF in lungs from controls, as compared to those from pertussis toxin-treated animals (Figure 2b). Thus, bronchoconstriction induced by 10 and 100 ng of PAF, reached 30.2 ± 13.5% and 49.5 ± 9% of the values obtained after the total occlusion of

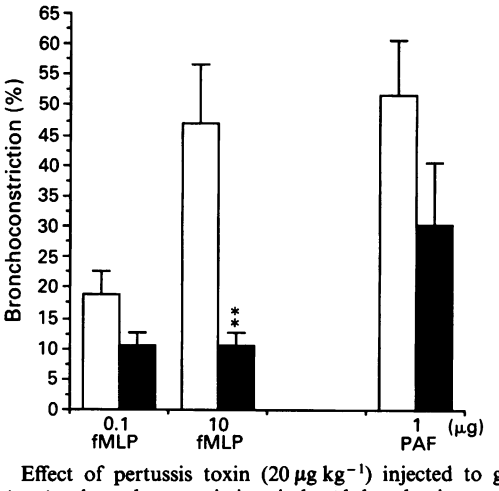

Figure 1 Effect of pertussis toxin (20 µg kg^{-1}) injected to guinea-pigs on *in vivo* bronchoconstriction induced by the intra-tracheal instillation of fMLP (0.1 and 10 µg) and PAF (1 µg) to control (open columns) or to pertussis toxin-treated guinea-pigs (solid columns). Results are mean ± s.e.mean (vertical bars) of 5 experiments. $^{**}P<0.01$.

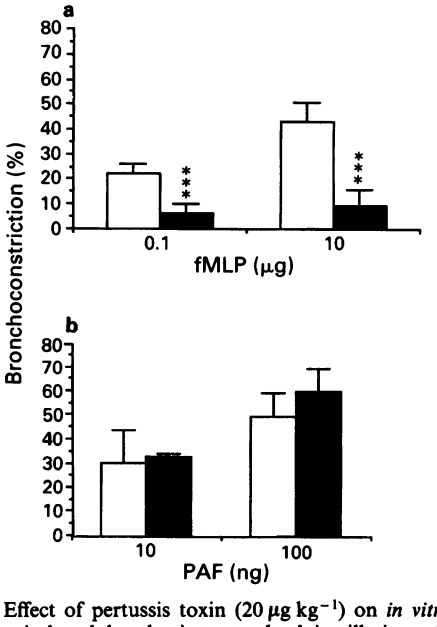

Figure 2 Effect of pertussis toxin (20 µg kg^{-1}) on *in vitro* bronchoconstriction induced by the intra-tracheal instillation of fMLP (a) and of PAF (b) to isolated lungs from control (open columns) and pertussis toxin-treated guinea-pigs (solid columns). Results are mean ± s.e.mean (vertical bars) of 5 experiments. $^{**}P<0.001$.

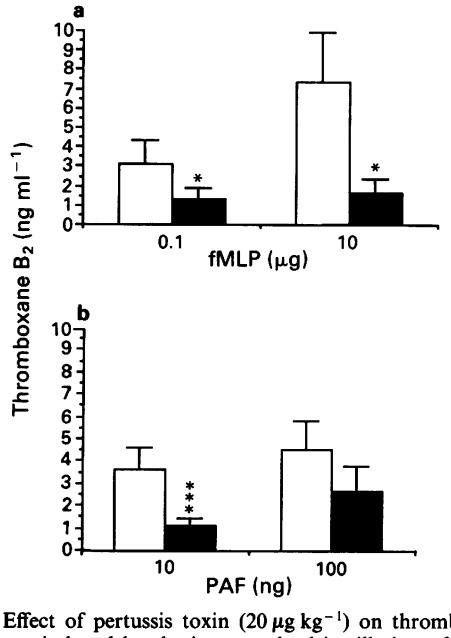

Figure 3 Effect of pertussis toxin (20 μg kg^{-1}) on thromboxane B_2 (TxB_2) release induced by the intra-tracheal instillation of fMLP (a) and of PAF (b) to isolated lungs from control (open columns) and pertussis toxin-treated guinea-pigs (solid columns). Results are mean ± s.e.mean (vertical bars) of 7 experiments.
**$P<0.05$; ***$P<0.001$.

the trachea, respectively for controls and of 33.0 ± 1% and 60 ± 10% for pertussis toxin-treated animals. TxB_2, measured in the lung effluent after the intra-tracheal administration of 10 ng of PAF, was reduced from 3.6 ± 1.0 ng ml^{-1} in control lungs to 1.10 ± 0.4 ng ml^{-1} in those from pertussis toxin-treated guinea-pigs ($P<0.001$). The TxB_2-releasing effect of 100 ng of PAF was not reduced significantly by pertussis toxin (4.5 ± 1.3 ng ml^{-1} in controls vs. 2.7 ± 1 ng ml^{-1} in pertussis toxin-treated lungs) (Figure 3b).

Effects of pertussis toxin on superoxide anion, TxB_2 and cyclic AMP production by BAL cells and by purified alveolar macrophages

Superoxide anion production and TxB_2 released by the whole cell population from the BAL of control guinea-pigs were increased by 0.01–1 μM of fMLP and were absent in cells from pertussis toxin-treated animals (Figures 4a and b). As seen in Figure 4a, the amounts of superoxide anions produced by the whole BAL cell populations exposed to PAF were very low and accordingly, the effects of pertussis toxin could not be evalauted. By contrast, TxB_2 was released by PAF-treated cells, and this was suppressed in those from pertussis toxin-treated animals (Figure 4b).

Alveolar macrophages stimulated with 1 μM fMLP produced less superoxide anions (3 ± 1.3 nmol for 10^6 cells per 3 min and 26 ± 12 nmol for 10^6 cells per 3 min; Figure 4a) and less TxB_2 (76 ± 20 ng ml^{-1} of protein per 5 min vs 255 ± 141 ng ml^{-1} of protein per 5 min; Figure 5), respectively, when cells from pertussis toxin-treated and control animals were compared. Macrophages stimulated with PAF produced 4 ± 1.2 nmol 10^{-6} cells per 3 min and 17 ± 4 nmol 10^{-6} cells per 3 min of superoxide anions when they were from pertussis toxin-treated and control animals, respectively (Figure 5a). TxB_2 release was of 41 ± 5 ng ml^{-1} of protein per 5 min and of 215 ± 150 ng mg^{-1} of protein per 5 min from pertussis toxin-treated and control animals, respectively (Figure 5b).

The basal content of cyclic AMP of alveolar macrophages from pertussis toxin-treated and control animals were not significantly different. By contrast, the increase in the cyclic AMP content in cells stimulated with PGE_2 (1 μM, final concentration in the medium) was significantly more pronounced ($P<0.05$) in macrophages from pertussis toxin-treated animals as compared to those from controls (Table 1). When macrophages were stimulated with fMLP or PAF, the cyclic AMP levels were reduced below the levels of detection.

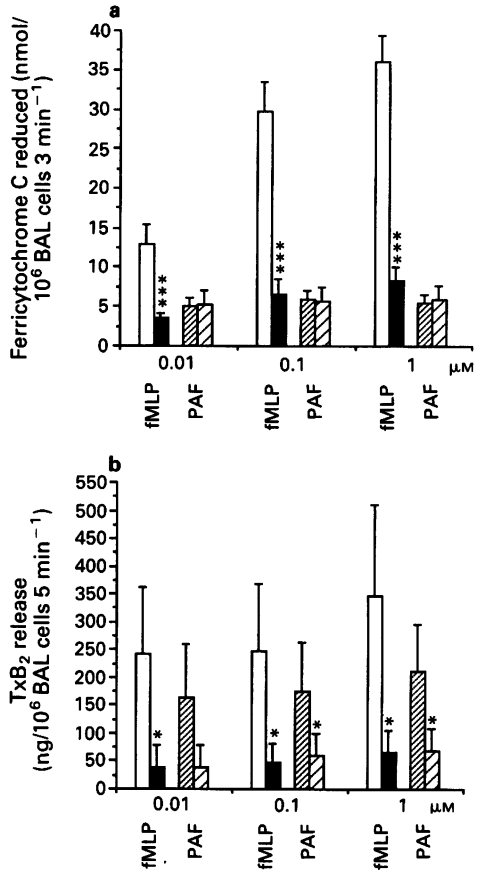

Figure 4 Effect of pertussis toxin (20 μg kg^{-1}) on superoxide anion production (a) and thromboxane B_2 (TxB_2) formation (b) by BAL cells stimulated *in vitro* with fMLP and PAF. The total cell population of the BAL was stimulated for 5 min with fMLP (open columns for controls and solid columns for pertussis toxin-treated animals) or PAF (closely hatched columns for controls and widely hatched columns for pertussis toxin-treated animals) at the concentrations indicated, to induce superoxide anion production. (b) Cells as in (a) were stimulated with fMLP or with PAF to induce TxB_2 release. Key as above. Results are mean ± s.e.mean (vertical bars) of 6 experiments.
*$P<0.05$; ***$P<0.01$.

Effect of the in vitro *exposure to pertussis toxin on superoxide and TxB_2 release by BAL cells and alveolar macrophages*

Pertussis toxin applied *in vitro* suppressed fMLP- and PAF-induced superoxide anion production by BAL cells (Figures 6a and 6b). Fifty percent inhibition of superoxide anion formation by BAL cells stimulated with 1 μM fMLP or PAF was obtained with 10 ng ml^{-1} and 3 ng ml^{-1} of the toxin, respectively. The 50% inhibition of TxB_2 release was reached with 12 and 6 ng ml^{-1} of pertussis toxin, for PAF and fMLP, respectively.

Similar results were obtained with purified alveolar macrophages (Figure 7), a dose-dependent inhibition of PAF- and fMLP-stimulated TxB_2 release being noted when the macrophages were treated with pertussis toxin. However, 50% inhibition of PAF-stimulated TxB_2 release occurred at 20 ng ml^{-1} of pertussis toxin but required only 4 ng ml^{-1} for fMLP. Maximal inhibition by pertussis toxin of TxB_2 release by fMLP and by PAF was similar.

ADP-ribosylation by pertussis toxin and cholera toxins in alveolar macrophages

To ascertain that systemic pertussis toxin induced the expected ADP-ribosylation of G proteins, as described *in vitro* (Gilman, 1987), macrophage membranes were prepared from pertussis toxin-treated and control animals. When pertussis toxin was used *in vitro* for the ADP-ribosylation of control membranes, the proteins analysed by SDS-polyacrylamide gel and autoradiography showed a major radiolabelled band corresponding to the 40-kDa protein. The immunoblot con-

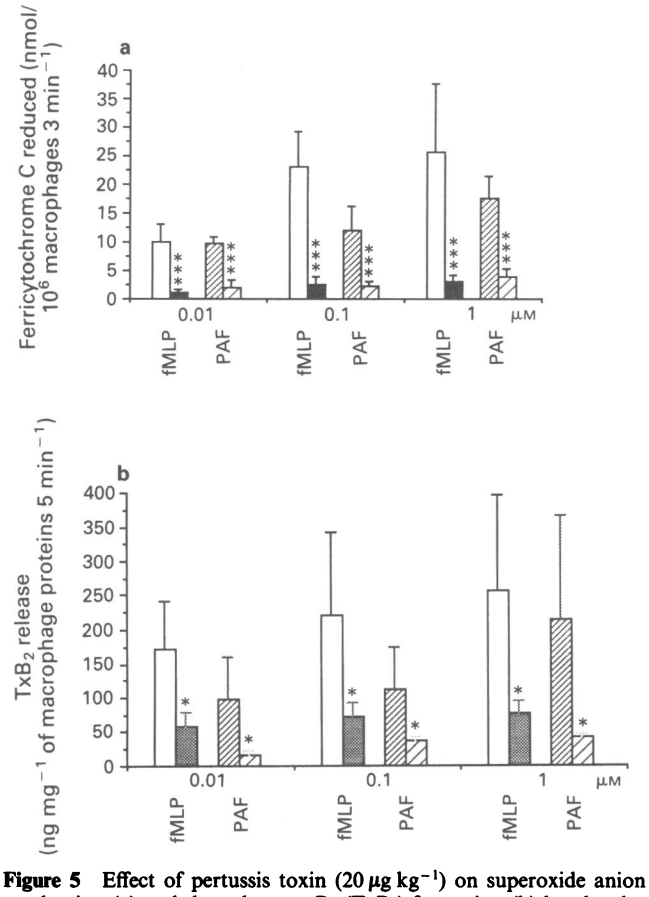

Figure 5 Effect of pertussis toxin (20 μg kg^{-1}) on superoxide anion production (a) and thromboxane B_2 (TxB_2) formation (b) by alveolar macrophages stimulated *in vitro* with fMLP and PAF. (a) Alveolar macrophages were stimulated for 5 min with fMLP (open columns for controls and solid columns for pertussis toxin-treated animals) or with PAF (closely hatched columns for controls and widely hatched columns for pertussis toxin-treated animals) at the concentrations indicated to induce superoxide anion production. (b) Cells as above stimulated for 5 min with 1 μM fMLP or PAF to induce thromboxane B_2 release. Keys as above. The results are mean ± s.e.mean (vertical bars) of 6 experiments.
*$P<0.05$; **$P<0.01$, ***$P<0.001$.

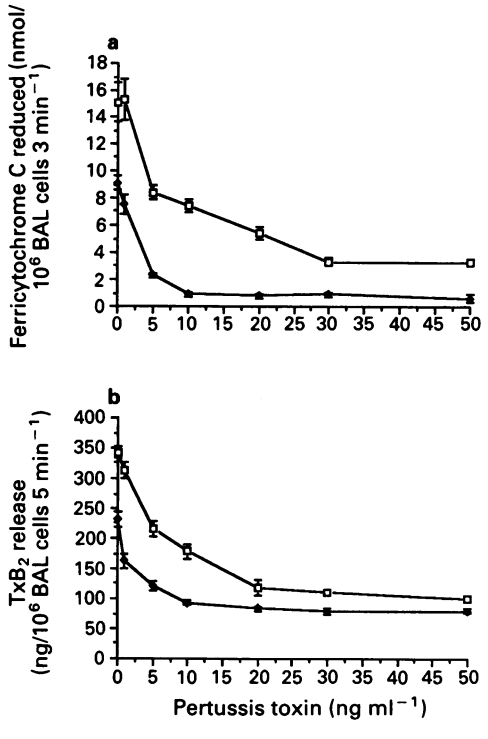

Figure 6 Effect of pertussis toxin applied *in vitro* to BAL cells on fMLP- and PAF-stimulated superoxide anion production (a) and thromboxane B_2 (TxB_2) release (b). BAL cells were pretreated for 3 h with pertussis toxin at the indicated concentrations and then stimulated with 1 μM fMLP (□) or with 1 μM PAF (◆), for 3 min in case of superoxide anions and for 5 min in case of TxB_2.

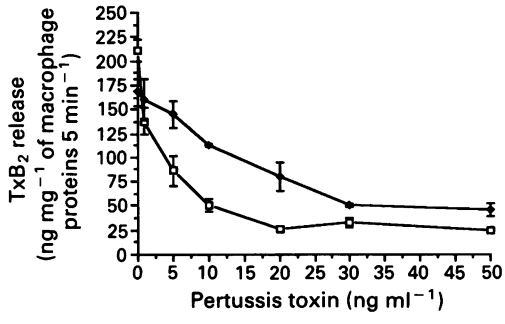

Figure 7 Effect of pertussis toxin applied *in vitro* to alveolar macrophages cells on fMLP- and PAF-stimulated superoxide anion production (a) and thromboxane B_2 (TxB_2) release (b). Aveolar macrophages were pretreated for 3 h with pertussis toxin at the indicated concentrations and then stimulated for 5 min with 1 μM fMLP (□) or with 1 μM PAF (◆). Results are mean ± s.e.mean (vertical bars) of 6 experiments.

Table 1 Interference of *in vivo* treatment with pertussis toxin with the basal and prostaglandin E_2 (PGE_2)-stimulated cyclic AMP levels in alveolar macrophages

	Cyclic AMP (pmol/10^6 cells per min)	
	Basal	*+ PGE_2* (1 μM)
Alveolar macrophages from:		
Control animals	1.2 ± 0.5 ($n = 4$)	3.2 ± 0.2 ($n = 4$)
Pertussis toxin-treated animals	1.7 ± 0.7 ($n = 5$)	5.4 ± 1.3* ($n = 5$)

Alveolar macrophages were isolated from pertussis toxin-treated guinea-pigs and from controls. Cyclic AMP was measured after 5 min incubation at 37°C. In the indicated cases, PGE_2 was used at 1 μM and the incubation was stopped by adding perchloric acid 1 N. Cyclic AMP was measured by the method of Cailla *et al.* (1973). Number of experiments shown in parentheses.
*$P<0.05$.

ducted with specific antibodies showed that this band corresponds to the αi subunit of the G_i protein (result not shown). The ADP-ribosylation was stable and was thus retained in the protein. Under those conditions, no radioactive ADP-ribose was further incorporated into the G proteins when the membranes were incubated *in vitro* with pertussis toxin in the presence of [^{32}P]-NAD (Iiri *et al.*, 1989). Pertussis toxin was also less effective in catalysing the ADP-ribosylation of the alveolar macrophage membranes from pertussis toxin-treated animals than from controls, indicating that indeed pertussis toxin exerts its effects *in vivo* on alveolar macrophages (Figure 8). When cholera toxin was used in the same conditions as pertussis toxin, no difference was observed in the labelling of the 42-kDa protein corresponding to the αs subunit of the G_s protein. In contrast, alveolar macrophage membranes isolated from pertussis toxin-treated animal showed a new radiolabelled band corresponding to a Mr of 114.5-kDa. The same radiolabelled band was seen when the ADP-ribosylation reaction was conducted without toxins, but in the presence of GTP, indicating that it resulted from endogenous ADP-ribosylation.

Recovery by macrophage transfer to the lungs of the ability of fMLP to induce bronchoconstriction and to release thromboxane B_2

Finally, to confirm that alveolar macrophages are the target cells for pertussis toxin, we instilled intra-tracheally to anaesthesized pertussis toxin-treated guinea-pigs, 6×10^6 macrophages collected from control guinea-pigs. Lungs were prepared after 20 min and fMLP was injected intra-tracheally. As a control (Figure 9), inhibition of bronchoconstriction persisted when this replenishing was performed with macrophages pretreated for 3 h with 1 μg ml^{-1} pertussis toxin. In contrast, bronchoconstriction in response to 0.1 and 10 μg of fMLP was recovered by 71 ± 11% and 57 ± 11%, respectively when the replenishing was performed with cells from control animals (Figure 9a). In addition, TxB_2 release in lung effluent (Figure 6b) was fully recovered.

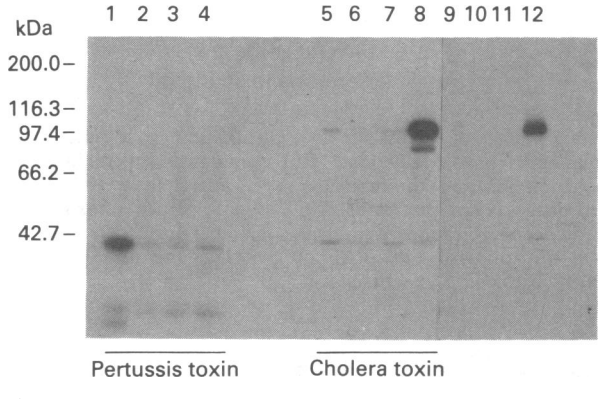

Figure 8 Effect of *in vivo* or *in vitro* treatment with pertussis toxin on the ADP-ribosylation of G proteins of alveolar macrophages. ADP-ribosylation of G proteins (100 μg of membrane proteins from alveolar macrophages) by pertussis and cholera toxins. Alveolar macrophages from pertussis toxin-treated guinea-pigs (lanes 4, 8, 11, 12) were compared to those from controls (lanes 1–5) or were treated with pertussis toxin *in vitro* during 1 h (lanes 2, 6, 9) or 3 h (lanes 3, 7, 10). ADP-ribosylation with pertussis toxin (1,2,3,4) was allowed for 45 min, for 10 min with cholera toxin (5,6,7,8) and in toxin absence (9,10,11,12) in the presence of [^{32}P]-NAD at 37°C before sodium dodecyl sulphate-polyacrylamide gel electrophoresis and autoradiography, as described in Methods.

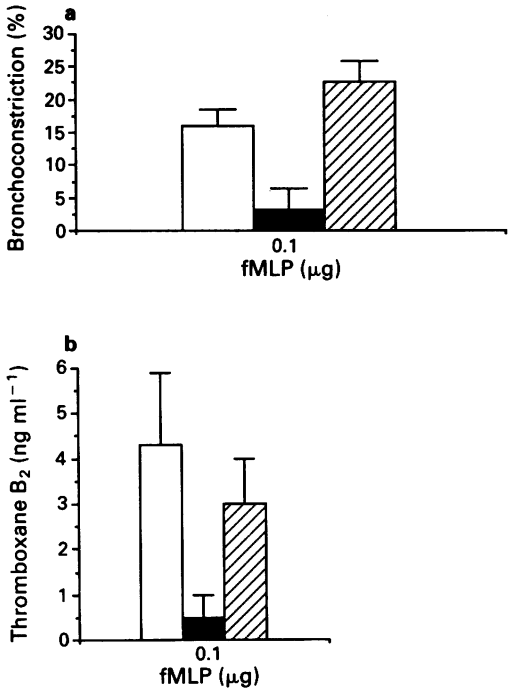

Figure 9 Effect of macrophage transfer to the isolated lungs from pertussis toxin-treated guinea-pigs on bronchoconstriction (a) and thromboxane B_2 (TxB_2) release (b) induced by the intra-tracheal instillation of fMLP. Perfused lungs provided by pertussis-treated guinea-pigs were instilled intra-tracheally with 6×10^6 macrophages pretreated 3 h *in vitro* with 1 μg ml^{-1} of pertussis toxin (solid column) or its solvent (open column). Bronchoconstriction and TxB_2 release by normal lungs are represented by hatched columns. ***$P < 0.001$.

Discussion

The purpose of this study was to determine, by use of pertussis toxin, the target cell which accounts for bronchoconstriction following the intra-tracheal administration of fMLP to the guinea-pig. As a background, we knew that pertussis toxin suppresses bronchconstriction induced by i.v. fMLP (Imaizumi *et al.*, 1992). We initially demonstrated that pertussis toxin also suppresses bronchoconstriction by the intra-tracheal administration of fMLP *in vivo*, but fails to modify the responses to 5-hydroxytryptamine (5-HT) (not shown) and to PAF (Figure 1). Thus, at the dose used, pertussis toxin is not toxic to the tissues involved with activation and final bronchoconstriction. Interestingly, even though pertussis toxin applied *in vitro* to alveolar macrophages prevents their activation by fMLP and PAF (Figures 6 and 7), the *in vivo* effects were selective to the former.

Bronchoconstriction induced by fMLP administered to lungs *in vivo* and *in vitro* was suppressed by pertussis toxin, suggesting that its target is present in the lung itself. Since fMLP was administered directly into the airways, its target is likely to be a component of the BAL population. In confirmation, release of superoxide anions and of TxB_2 from fMLP-stimulated BAL cells and alveolar macrophages, the major cell population of the BAL, collected from pertussis toxin-treated animals, was reduced strongly.

TxB_2 release and superoxide anion production after cell stimulation by PAF and fMLP correlate with phospholipase C activation (Homma *et al.*, 1985; Prpic *et al.*, 1988), leading to the production of diacylglycerol, inositol triphosphate and to increased free intracellular Ca^{2+} levels (Naccache *et al.*, 1985; Kikuchi *et al.*, 1986), with protein kinase C translocation (Horn & Karnovsky, 1986; O'Flaherty & Nishihira, 1987). These changes are blocked by pertussis toxin in different cells via the ADP-ribosylation of G proteins (Lad *et al.*,

1985; Schlondorff *et al.*, 1989), and the consequent uncoupling of the receptor-effector pathways (Gilman, 1987). G_i proteins are responsible for phospholipase C activation in the case of thrombin receptor (Crouch & Lapetina, 1988). Nevertheless, according to the cell type and to the agonists concerned, pertussis toxin affects these mechanisms differently (Fu *et al.*, 1988), indicating that G_i proteins are not the only G proteins involved in the control of phospholipase C and, as a consequence, in TxB_2 and superoxide anion production. Other G proteins, with a small molecular weight, are a likely target for pertussis toxin, which would explain the recently described effects of Yamamoto *et al.* (1989).

Three lines of evidence indicate that pertussis toxin *in vivo* interacts directly with the cells present in the guinea-pig airways. Firstly, fMLP-induced TxB_2 release from BAL cells and alveolar macrophages exposed *in vitro* to pertussis toxin or collected from pertussis toxin-induced animals was reduced. Secondly, under conditions where no differences in basal cyclic AMP levels were noted between alveolar macrophages from pertussis toxin-treated or untreated guinea-pigs (in agreement with Krzanowski *et al.*, 1976), a significant increase in the cyclic AMP response of macrophages from pertussis toxin-treated animals exposed to PGE_2, as compared to controls, was obtained. In addition, cyclic AMP levels decreased strongly and could not be measured when the macrophages from both control or pertussis toxin-treated animals were stimulated. Since it is recognized that pertussis toxin, ADP-ribosylating the G_i proteins, removes negative control for adenylyl cyclase (Gilman, 1989), it was likely that ADP-ribosylation of G_i protein had occurred *in vivo* and, as a consequence, that the control of the adenylyl cylcase had been removed. Accordingly, this rules out the possibility that pertussis toxin inhibits the production of TxB_2 and superoxide anions by increasing cyclic AMP content and, as a consequence, by the activation of protein kinase A, which regulates negatively the production of arachidonate, the precursor of eicosanoids (Kadiri *et al.*, 1989). As a consequence, G_i protein can control negatively adenylyl cyclase and positvely the pathway involved in TxB_2 release and superoxide anion production stimulated by PAF and by fMLP. Thirdly, a 40-kDa protein substrate was identified with specific antibodies as the α_i subunit of G_i protein in isolated aveolar macrophage membranes after *in vitro* ADP-ribosylation with pertussis toxin, as recognized for HL60 cells (Polakis *et al.*, 1988; Iiri *et al.*, 1989). Since this ADP-ribosylation was significantly decreased on the membranes of macrophages from animals treated with pertussis toxin *in vivo* and re-exposed to it *in vitro*, it is clear that macrophages from pertussis toxin-treated guinea-pigs had been indeed ADP-ribosylated *in vivo*. The reduction of G_i protein ADP-ribosylation cannot be accounted for by down-regulation of G_i protein, a mechanism described for G_S only (Chang & Bourne, 1989). No differences in the ADP-ribosylation were observed between membranes from controls and from pertussis toxin-treated animals exposed to cholera toxin *in vitro*, indicating that the ADP-ribosylation with pertussis toxin was specific for G_i proteins. However, endogenous ADP-ribosylation of a protein with an apparent Mr of 114 kDa was seen in presence of GTP, but only in membranes from macrophages of pertussis toxin-treated animals and not after *in vitro* exposure to pertussis toxin. Indeed, enzymes which induced endogenous ADP-ribosylation of macromolecules were described in other cell systems (reviewed by Hayaishi & Ueda, 1977).

The relationship between ADP-ribosylation of alveolar macrophages by pertussis toxin *in vivo* and inhibition of fMLP-induced bronchoconstriction was next examined. When alevolar macrophages from untreated guinea-pigs, were reinjected intra-tracheally to lungs from pertussis toxin-treated guinea-pigs, inhibition of bronchoconstriction by fMLP was largely reversed. It is thus clear that pertussis toxin injected *in vivo* interacts with alveolar macrophages, leading to a marked inhibition of fMLP-induced bronchoconstriction and that pertussis toxin-sensitive G proteins play an important role in the control of the production of the mediators which account for the effects of fMLP. It is likely that these mediators are arachidonate derivatives, since *in vivo* bronchoconstriction by i.v. or intra-tracheal fMLP is suppressed by indomethacin (Boukili *et al.*, 1986; 1989).

PAF was selected in our study because of its potential relevance for allergen-induced bronchoconstriction (reviewed by Braquet *et al.*, 1987). Bronchoconstriction by intra-tracheal PAF in untreated guinea-pigs is suppressed by aspirin (Lefort *et al.*, 1984) and accordingly, is most probably accounted for by the *in vivo* liberation of eicosanoids. Formation of TxB_2 following the intra-tracheal administration of PAF was reduced by pertussis toxin, but less efficiently than in case of fMLP, even though the production of TxB_2 by BAL cells was suppressed by pertussis toxin *in vitro* (Figure 6b), and by macrophages *ex vivo* (Figure 5b) and *in vitro* (Figure 7). Since bronchoconstriction by intra-tracheal PAF was not inhibited *in vivo* (Figure 1) nor *in vitro* (Figure 2) by pertussis toxin, whereas bronchoconstriction by fMLP was suppressed, it is likely that the targets for fMLP- and for PAF-induced bronchoconstriction differ, the alveolar macrophage having been identified for the former, but not for the latter. Similar results were shown with superoxide anion production by BAL cells (Figure 4a and 6a) and by macrophages (Figure 5a) stimulated with PAF. The responses to PAF of macrophages from pertussis toxin-treated guinea-pigs were thus in general less affected than those to fMLP, even though their *in vitro* exposure to pertussis toxin was fully suppressive, as shown previously with respect to release of arachidonate (Kadiri *et al.*, 1990). Accordingly, TxB_2 and superoxide anions produced by alveolar macrophages are not important for bronchoconstriction by intra-tracheal PAF, but the possibility remains that other mediators produced by the alveolar macrophages (reviewed by Nathan, 1987) may account for it.

In conclusion, bronchoconstriction by intra-tracheal fMLP follows the activation of alveolar macrophages, via a pertussis toxin-sensitive G protein. Bronchoconstriction by PAF involves a different mechanism, since it persists when the production by BAL cells and macrophages of superoxide anions and TxB_2 is suppressed.

References

BACHELET, M., MASLIAH, J., MALANCHÈRE, E., LEFORT, J., BÉRÉZIAT, G. & VARGAFTIG, B.B. (1989). Reduced responsiveness of bronchoalveolar cells from sensitized guinea-pigs to the cyclic AMP-stimulating effect of prostaglandin E_2 and β-adrenoceptor agonists. *Pulm. Pharmacol.*, **2**, 41–44.

BOUKILI, M.A., BUREAU, M., LAGENTE, V., LEFORT, J., LELLOUCH-TUBIANA, A., MALANCHERE, E. & VARGAFTIG, B.B. (1986). Pharmacological modulation of the effects of N-formyl-L-methionyl-L-leucyl-L-phenylalanine in guinea-pigs: involvement of the arachidonic acid cascade. *Br. J. Pharmacol.*, **89**, 349–359.

BOUKILI, M.A., BUREAU, M., LELLOUCH-TUBIANA, A., LEFORT, J., SIMON, M.T. & VARGAFTIG, B.B. (1989). Alveolar macrophages and eicosanoids but not neutrophils, mediate bronchoconstriction induced by fMLP in the guinea-pig. *Br. J. Pharmacol.*, **98**, 61–70.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

BRAQUET, P., TOUQUI, L., SHEN, T.Y. & VARGAFTIG, B.B. (1987). Perspectives in platelet-activating factor research. *Pharmacol. Rev.*, **39**, 97–145.

BUREAU, M., DE CLERCK, F., LEFORT, J., ARRETO, C.D. & VARGAFTIG, B.B. (1992). Thromboxane A2 accounts for bronchoconstriction but not for platelet sequestration and microvascular albumin exchanges induced by fMLP in the guinea-pig lungs. *J. Pharmacol. Exp. Ther.*, **260**, 832–841.

CAILLA, H.L., CROS, G.S., JOLUE, J.P., DELAAGE, M.A. & DEPIEDS, R.C. (1973). Comparison between rat and rabbit anticyclic AMP antibodies specificity toward acyl derivatives of cyclic AMP. *Anal. Biochem.*, **56**, 383–393.

CHANG, F.-H. & BOURNE, H.R. (1989). Cholera toxin induces cAMP-independent degradation of Gs. *J. Biol. Chem.*, **264**, 5352–5357.

CROUCH, M. & LAPETINA, E.G. (1988). A role for Gi in control of thrombin receptor-phospholipase C coupling in human platelets. *J. Biol. Chem.*, **263**, 3363–3371.

FU, T., OKANO, Y. & NOZAWA, Y. (1988). Bradykinin-induced generation of inositol 1,4,5-triphosphate in fibroblasts and neuroblastoma cells: effects of pertussis toxin, extracellular calcium, and down regulation of protein kinase C. *Biochem. Biophys. Res. Commun.*, **157**, 1429–1435.

GILMAN, A.G. (1987). G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.*, **56**, 615–649.

GILMAN, A.G. (1989). G proteins and regulation of adenylyl cyclase. *J. Am. Med. Ass.*, **262**, 1819–1825.

HAYAISHI, O. & UEDA, K. (1977). Poly(ADP-ribose) and ADP-ribosylation of proteins. *Annu. Rev. Biochem.*, **46**, 95–96.

HESCHERLER, J., ROSENTHAL, W., TRAUTWEIN, S. & SCHULTZ, G. (1987). The GTP-binding protein, Go, regulates neuronal calcium channels. *Nature*, **325**, 445–447.

HOMMA, Y., HASHIMOTO, Y.N. & TKENAWA, T. (1985). Evidence for differential activation of arachidonic acid metabolism in formyl-peptide- and macrophages-activation-factor-stimulated guinea-pig macrophages. *Biochem. J.*, **229**, 643–651.

HORN, W. & KARNOVSKY, M.L. (1986). Features of translocation of protein kinase C in neutrophils stimulated with the chemotactic peptide f-Met-Leu-Phe. *Biochem. Biophys. Res. Commun.*, **139**, 1169–1175.

IIRI, T., TOHKIN, M., MORISHIMA, N., OHOKA, Y., UI, M. & KADATA, T. (1989). Chemotactic peptide receptor-supported ADP-ribosylation of a pertussis toxin substrat GTP-binding protein by cholera toxin in neutrophil-type HL-60 cells. *J. Biol. Chem.*, **264**, 21394–21400.

IMAIZUMI, A., LEFORT, J., LEDUC, D. & VARGAFTIG, B.B. (1992). Pertussis toxin induces bronchopulmonary hyperreactivity to histamine and serotonin in guinea-pigs, while antagonising the effects of fMLP. *Eur. J. Pharmacol.*, **212**, 177–186.

KADIRI, C., MASLIAH, J., BACHELET, M., VARGAFTIG, B.B. & BEREZIAT, G. (1989). Phospholipase A_2-mediated release of arachidonic acid in stimulated guinea-pig alveolar macrophages: interaction with lipid mediators and cyclic AMP. *J. Cell. Biochem.*, **40**, 157–164.

KADIRI, C., CHERQUI, C., MASLIAH, J., RYBKINE, T., ETIENNE, J. & BEREZIAT, J. (1990). Mechanism of N-formyl-metionyl-leucyl-phenyl-alanine- and platelet-activation factor-induced arachidonic acid release in guinea pig alevolar macrophages: involvement of GTP-binding protein and role of protein kinase A and protein kinase C. *Mol. Pharmacol.*, **38**, 418–425.

KIKUCHI, A., KOZAWA, O., KAIBUCHI, K., KADATA, T., UI, M. & TAKAI, Y. (1986). Direct evidence for involvement of guanine nucleotide binding protein in chemotactic peptide-stimulated formation of inositol biphosphate and triphosphate in differentiating human leukemic (HL60) cells. *J. Biol. Chem.*, **261**, 11558–11562.

KRZANOWSKY, J.J., POLSON, J.B. & SZENTIVANYI, A. (1976). Pulmonary patterns of adenosine-3',5'-cyclic monophosphate accumulation in response to adrenergic or histamine stimulation in *Bordetella Pertussis*-sensitized mice. *Biochem. Pharmacol.*, **25**, 1631–1637.

LAD, P., OLSON, C.V. & SMILEY, P.A. (1985). Association of N-formyl-Met-Leu-Phe receptor in human neutrophils with a GTP-binding protein sensitive to Pertussis toxin. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 869–873.

LEFORT, J., ROTILIO, D. & VARGAFTIG, B.B. (1984). The platelet-independent release of thromboxane A_2 by Paf-acether from guinea-pig lungs involves mechanisms distinct from those of leukotriene. *Br. J. Pharmacol.*, **82**, 565–575.

NACCACHE, P.H., MOLSKI, M.M., VOLPI, M., BECKER, E.L. & SHA'AFI, R.I. (1985). Unique inhibitory profile of platelet activating factor induced calcium mobilization, polyphosphoinositide turnover and granule enzyme secretion in rabbit neutrophils towards pertussis toxin and phorbol ester. *Biochem. Biophys. Res. Commun.*, **130**, 677–684.

NATHAN, C.F. (1987). Secretory products of macrophages. *J. Clin. Invest.*, **79**, 319–326.

O'FLAHERTY, J.T. & NISHIHIRA, J. (1987). Arachidonate metabolites, platelet-activation factor and mobilization of protein kinase C in human polymorphonuclear neutrophils. *J. Immunol.*, **138**, 1889–1895.

POLAKIS, P.G., UHING, R.J. & SNYDERMAN, R. (1988). The formylpeptide chemoattractant receptor copurifies with a GTP-binding protein containing a distinct 40-KDa Pertussis toxin substrate. *J. Biol. Chem.*, **263**, 4969–4976.

PRPIC, V., UHING, R.J., WEIEL, E.J., JAKOI, L., GAWDI, G., HERMAN, B. & ADAMS, D.O. (1988). Biochemical and functional responses stimulated with platelet activating factor in murin peritoneal macrophages. *J. Cell. Biol.*, **107**, 363–372.

SCHLONDORF, D., SINGHAL, P., HASSID, A., SATRIANO, J.A. & DACANDIDO, S. (1989). Relationship of GTP-binding proteins, phospholipase C, and PGE_2 synthesis in rat glomerular mesangial cells. *Am. J. Physiol.*, **256**, F171–F178.

SORS, H., PRADELLES, P. & DRAY, F. (1978). Analytical methods for thromboxane B_2 measurement and validation on radioimmunoassay by gaz liquid chromatography-mass spectrometry. *Prostaglandins*, **16**, 227–290.

VADAS, M.A., DAVID, J.R., BUTTERWORTH, A., PISANI, N.T. & SIONGOK, T.A. (1979). A new method for the purification of human eosinophils and neutrophils, and a comparison to the ability of these cells to damage schistosomula of Schistosoma mansoni. *J. Immunol.*, **122**, 1228–1238.

VARGAFTIG, B.B., LEFORT, J., CHIGNARD, M. & BENVENISTE, J. (1980). Platelet-activating factor induces a platelet-dependent bronchoconstriction unrelated to the formation of prostaglandin derivates. *Eur. J. Pharmacol.*, **65**, 185–192.

YAMAMOTO, K., TANIMOTO, T., KIM, S., KIKUCHI, A. & TAKAI, Y. (1989). Small molecular weight GTP- binding proteins and signal transduction. *Clin. Chem. Acta*, **185**, 347–356.

(Received March 9, 1992
Revised July 24, 1992
Accepted August 3, 1992)

Br. J. Pharmacol. (1992), **107**, 1037–1041

Mechanisms of the biphasic responses to endothelin-3 in dog coronary arteries

[1]Tomio Okamura, Tetsuya Matsumoto, *Fumihiko Ikemoto & Noboru Toda

Department of Pharmacology, Shiga University of Medical Sciences, Seta, Ohtsu 520–21, Japan and *Tsukuba Research Institute, Banyu Pharmaceutical Co. Ltd., 3 Okubo, Tsukuba 300-33, Japan

1 Endothelin-3 (ET-3) elicited relaxations at low concentrations (up to 10^{-8} M) and contractions at higher concentrations in dog isolated coronary arteries precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The relaxation by ET-3 was not affected by endothelium denudation nor treatment with N^G-nitro-L-arginine, but was abolished or reversed to a contraction by treatment with indomethacin and markedly suppressed by tranylcypromine, a PGI_2 synthetase inhibitor, or diphloretin phosphate, a prostaglandin receptor antagonist. ET-1 produced only concentration-dependent contractions.

2 BQ-123, a new selective ET_A receptor antagonist, caused relaxation of the strips contracted with ET-3 in a dose-dependent manner and prevented the ET-3-induced contraction but did not affect the contraction produced by $PGF_{2\alpha}$. The relaxation caused by ET-3 was enhanced by treatment with BQ-123.

3 It is concluded that the relaxations elicited by ET-3 in dog coronary arteries are mediated via liberation of PGI_2 by activation of non-ET_A receptors, located in subendothelial tissues, possibly smooth muscle cells, whereas the peptide-induced contractions are mediated via ET_A receptors.

Keywords: Endothelin; ET_A receptor; vasorelaxation; dog coronary artery; endothelium; PGI_2

Introduction

Endothelin (ET) was first described as an endothelium-derived, vasoconstrictor peptide (Yanagisawa *et al.*, 1988), but has also been demonstrated to have a vasodilator action. ET-1 or ET-3 has been reported to cause only contraction (Yanagisawa *et al.*, 1988; Fukuroda *et al.*, 1992) or relaxation at low concentrations (Fukuroda *et al.*, 1992; Ushio-Fukai *et al.*, 1992) in porcine coronary or pulmonary arteries. Intravenous bolus injections of ET-1 or ET-3 cause a transient, dose-dependent depressor response followed by a sustained pressor response in anaesthetized rats (Wright & Fozard, 1988; Gardiner *et al.*, 1989; Inoue *et al.*, 1989) and cats (Minkes *et al.*, 1989; Lippton *et al.*, 1988). ET-3 possesses greater vasodilator activity than ET-1 (Inoue *et al.*, 1989; Minkes *et al.*, 1989). The ET-induced vasodilatation has been considered to be mediated by endothelium-derived relaxing factor(s) (EDRF), since the depressor responses to ET are abolished by pretreatment with N^G-monomethyl-L-arginine (Fukuda *et al.*, 1990), a nitric oxide synthase inhibitor, or endothelium denudation (Warner *et al.*, 1989; Fukuda *et al.*, 1991; Ushio-Fukai *et al.*, 1992), and markedly inhibited by treatment with methylene blue or oxyhaemoglobin (Warner *et al.*, 1989) in rat isolated perfused mesenteric arteries. On the other hand, in *in vivo* experiments in dogs, N^G-nitro-L-arginine in concentrations sufficient to inhibit nitric oxide synthase partially inhibits but does not abolish the ET-induced depressor response, and the remaining response is abolished by additional treatment with cyclo-oxygenase inhibitors, suggesting the involvement of vasodilator prostaglandins (Yamashita *et al.*, 1991). An ability of endothelin to release prostacyclin (PGI_2) has been demonstrated in guinea-pig and rat isolated lungs (De Nucci *et al.*, 1988).

Recently, two different ET receptors have been cloned and termed ET_A and ET_B (Arai *et al.*, 1990; Sakurai *et al.*, 1990). BQ-123 has been introduced as a selective antagonist for the ET_A receptor subtype (Ihara *et al.*, 1992). The present study was, therefore, undertaken to examine the mechanism of ET-induced relaxations in dog isolated coronary arteries, with reference to endothelial function and receptor subtypes.

Methods

Mongrel dogs of either sex, weighing 8 to 15 kg, were anaesthetized with intravenous injections of sodium pentobarbitone (30 mg kg^{-1}) and killed by bleeding from the carotid arteries. The heart was rapidly removed. Descending and circumflex branches of the left coronary artery (0.6 to 0.8 mm outside diameter) were isolated and cut into helical strips approximately 20 mm long, with special care being taken to avoid endothelial damage. The tissue was fixed vertically between two hooks in a muscle bath containing modified Ringer-Locke solution, which was maintained at 37 ± 0.3°C and aerated with a mixture of 95% O_2 and 5% CO_2. The hook anchoring the upper end of the strip was connected to the level of a force-displacement transducer (Nihon-Kohden Kogyo Co., Tokyo, Japan). The resting tension was adjusted to 1.5 g, which is optimal for inducing the maximal contraction. The composition of the bathing medium was (mM): NaCl 120, KCl 5.4, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0 and dextrose 5.6. The pH of the solution was 7.35 to 7.42. The strips were allowed to equilibrate for 60 to 90 min in the bathing medium during which time the solution was replaced three times every 10 to 15 min.

Isometric contractions and relaxations were displayed on an ink-writing oscillograph (Nihon-Kohden Kogyo Co.). A contractile response to 30 mM K^+ was first obtained, and the artery strips repeatedly washed with the fresh medium and reequilibrated. The strips were partially contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-7} to 10^{-6} M), the contraction being in the range of 20 and 35% of the contraction induced by 30 mM K^+. Concentration-response curves for endothelin-3 (ET-3), BQ-123 and beraprost, a PGI_2 analogue (Toda, 1988), were obtained by adding the agents directly to the bathing medium in cumulative concentrations. The artery strips were treated for approximately 20 min with antagonists, before the concentration-response curves for agonists

[1] Author for correspondence.

were obtained. Because of a lack of reproducibility, the first concentration-response curves in the absence (control) and presence of pharmacological antagonists were compared in 2–4 strips obtained from the same dogs. At the end of each series of experiments, papaverine (10^{-4} M) was applied to attain the maximal relaxation. Contractions and relaxations induced by the test drugs are presented as percentage values relative to those caused by 30 mM K^+ and 10^{-4} M papaverine, respectively, unless otherwise mentioned. The endothelium of artery strips was removed by gently rubbing the intimal surface with a cotton pellet; unrubbed strips from the same dogs were used for comparison. Successful removal of the endothelium was determined by abolition of relaxations induced by 10^{-7} M acetylcholine and was confirmed histologically by the silver staining procedure.

The results shown in the text and figures are expressed as the mean ± s.e.mean. Statistical analyses were made using Student's paired and unpaired *t* test and Tukey's method after one-way analysis of variance. Drugs used were acetylcholine chloride (Daiichi Seiyaku Co., Tokyo, Japan), tranylcypropime, indomethacin (Sigma, St. Louis, MO, U.S.A.), prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical Co., Osaka, Japan), endothelin (ET)-1 and 3, N^G-nitro-L-arginine (Peptide Institute Inc., Minoh, Japan), diphloretin phosphate (Leo, Helsingborg, Sweden), beraprost (sodium (±)-4[1R,2R, 3aS,8bS) -1,2,3a,8b -tetrahydro-2-hydroxy-1-[(3S,4RS)-3-hydroxy-4-methyl-oct-6-yne-(E)-1-enyl] -5cylcopenta [b] benzofuranyl] butyrate; Toray industries, Inc., Tokyo, Japan) and papaverine hydrochloride (Dainippon Pharmaceutical Co., Osaka, Japan). BQ-123 (cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-) sodium) was synthesized in the Tsukuba Research Institute of the Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan).

Results

In dog coronary arterial strips partially contracted with $PGF_{2\alpha}$, the addition of ET-3 in concentrations of 3×10^{-9} and 10^{-8} M produced a concentration-dependent relaxation. The relaxations at 3×10^{-9} M were inconsistent; slight relaxations were observed in three out of six strips but no relaxations in the remaining three, whereas those at 10^{-8} M were consistent. Therefore, the mechanism underlying relaxations was analysed at 10^{-8} M ET-3. Relaxations at 10^{-8} M slowly reversed to contraction to the level that was lower or higher than that prior to the addition of ET-3. The strips responded to the peptide in concentrations higher than 3×10^{-8} M with only contraction. After stabilization of the response to 3×10^{-8} M ET-3, indomethacin caused a marked contraction (Figure 1a). Removal of the endothelium did not influence these responses induced by ET-3 and indomethacin (Figure 1b). Concentration-response curves to ET-3 in the arterial strips with and without the endothelium are shown in Figure 2, in which the maximal level of relaxations and contractions is presented. The apparent median effective concentration (EC_{50}) of ET-3 for the relaxation in the endothelium-intact strips was $[4.2 \pm 0.7] \times 10^{-9}$ M ($n = 11$), and the maximal relaxation attained at 10^{-8} M was 25.1 ± 5.5% ($n = 11$) relative to that induced by 10^{-4} M papaverine.

The responses caused by ET-3 were not reproducible on repeated administration, even though the strips were repeatedly rinsed after the application of ET-3. Therefore, modification by antagonists of the ET-3-induced relaxation was tested on the first responses in different strips obtained from the same dogs; one was used as a control and the others were for treatment with antagonists. In endothelium-intact strips, treatment with N^G-nitro-L-arginine (L-NNA, 10^{-5} M), sufficient to suppress the EDRF-mediated relaxant response (Toda *et al.*, 1990), did not inhibit the ET-3-induced relaxation. The relaxant responses to 10^{-8} M ET-3 in the control and L-NNA-treated strips were 23.7 ± 7.6% and 21.8 ± 5.3% ($n = 6$). In the endothelium-denuded strips, treatment with indomethacin (10^{-6} M) reversed the relaxation

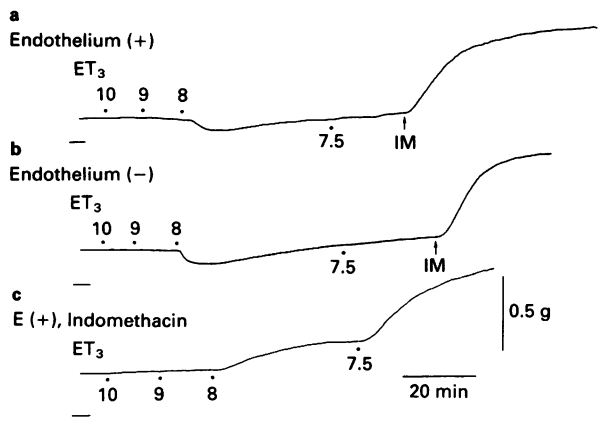

Figure 1 Modification by indomethacin (IM, 10^{-6} M) of the responses to endothelin-3 (ET-3) in dog coronary artery strips precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) with (a and c) and without (b) the endothelium. Concentrations of ET-3 from 10 to 7.5 = 10^{-10}, 10^{-9}, 10^{-8} and 3×10^{-8} M, respectively. Horizontal lines just left of each tracing represent the level before addition of $PGF_{2\alpha}$ (10^{-7} to 10^{-6} M).

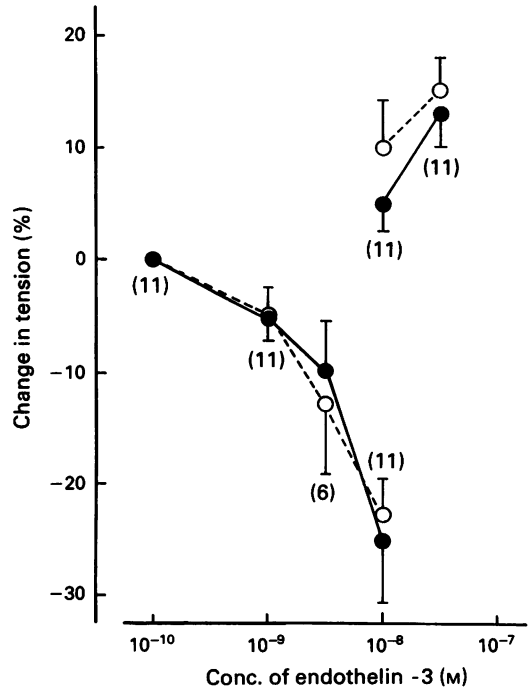

Figure 2 Concentration-response curves for endothelin-3 (ET-3) in dog coronary artery strips with (●) and without (○) the endothelium. The strips were partially contracted with prostaglandin $F_{2\alpha}$ (10^{-7} M to 10^{-6} M). Since ET-3 at 10^{-8} M caused a relaxation followed by a contraction, both repsonses to the peptide are plotted. Relaxations induced by 10^{-4} M papaverine and contractions by 30 mM K^+ were taken as 100% for relaxation and contraction, respectively; mean absolute values for the relaxation in the arteries with and without the endothelium were 270 ± 34 mg and 279 ± 39 mg ($n = 11$), respectively, and those for the contraction were 1722 ± 209 mg and 1752 ± 233 mg ($n = 11$), respectively. Points indicate means and bars indicate s.e.mean. Numbers in parentheses indicate the number of strips from separate dogs.

induced by 10^{-8} M ET-3 to a contraction. Typical tracings from strips with and without the endothelium are shown in Figures 1c and 3c, respectively. The ET-3-induced relaxation was markedly suppressed by tranylcypromine (3×10^{-4} M) or

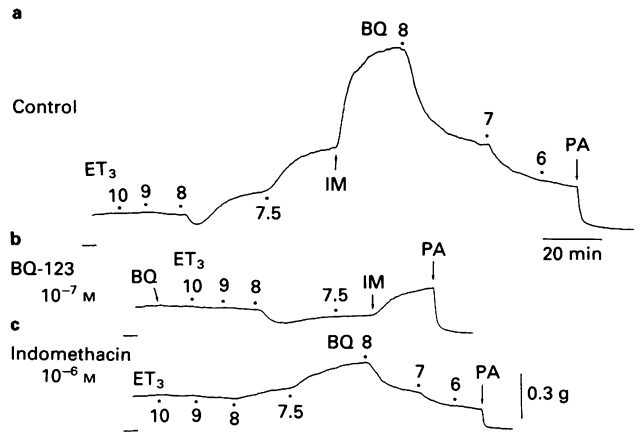

Figure 3 Responses to endothelin-3 (ET-3), indomethacin (IM, 10^{-6} M) and BQ-123 (BQ, 10^{-7} M) in dog coronary arteries without endothelium precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Concentrations of ET-3 from 10 to 7.5 = 10^{-10}, 10^{-9}, 10^{-8} and 3×10^{-8} M, respectively; concentrations of BQ-123 from 8 to 6 = 10^{-8}, 10^{-7} and 10^{-6} M, respectively; PA = 10^{-4} M papaverine. Horizontal lines just left of each tracing represent the level before addition of $PGF_{2\alpha}$ (10^{-7} to 10^{-6} M).

diphloretin phosphate (10^{-5} M). The quantitative data are summarized in Figure 4. Relaxations induced by beraprost were not significantly altered by treatment with tranylcypromine. The relaxant responses in the control and tranylcypromine-treated strips were 19.8 ± 6.8% and 31.3 ± 4.4%, respectively, at 10^{-8} M beraprost, and 55.5 ± 11.8% and 79.1 ± 2.4% ($n = 5$), respectively, at 10^{-7} M; the differences were not statistically significant.

ET-1 (10^{-9} to 10^{-8} M) elicited only concentration-dependent contractions in endothelium-intact and -denuded strips precontracted with $PGF_{2\alpha}$. The ET-1-induced contractions at 10^{-9} and 3×10^{-9} M were 22.5 ± 10.1% and 51.0 ± 17.6% relative to K^+ (30 mM)-induced contraction, respectively, in the endothelium-intact strips, and 27.3 ± 7.4% and 54.0 ± 11.2%, respectively, in the endothelium-denuded strips.

The addition of BQ-123 (10^{-8} to 10^{-6} M) produced a dose-dependent relaxation in the denuded strips contracted by ET-3 in the absence and presence of indomethacin (Figure 3 a and c). Mean values of the relaxations at 10^{-8}, 10^{-7} and 10^{-6} M BQ-123 were 69.0 ± 6.9%, 97.7 ± 1.6% and 99.4 ± 0.6% ($n = 7$), respectively, when the maximal contraction caused by 3×10^{-8} M ET-3 was taken as 100%. BQ-123 in these concentrations did not alter the tone of $PGF_{2\alpha}$-contracted strips. Pretreatment with BQ-123 of the denuded strips suppressed the contractions caused by ET-3 (Figure 3b, Figure 5), but prolonged (Figure 3b) and potentiated the relaxations by the peptide (Figure 5).

Discussion

ET-3 elicited relaxation at low concentrations (up to 10^{-8} M) and contraction at higher concentrations in dog isolated coronary arteries precontracted with $PGF_{2\alpha}$. Both responses were not endothelium-dependent, suggesting that endothelium-derived vasoactive substances such as EDRF (Furchgott & Zawadzki, 1980) or EDCF (De Mey & Vanhoutte, 1982; Toda *et al.*, 1988) are not involved. BQ-123 (Ihara *et al.*, 1992), a selective antagonist for ET_A receptors, significantly

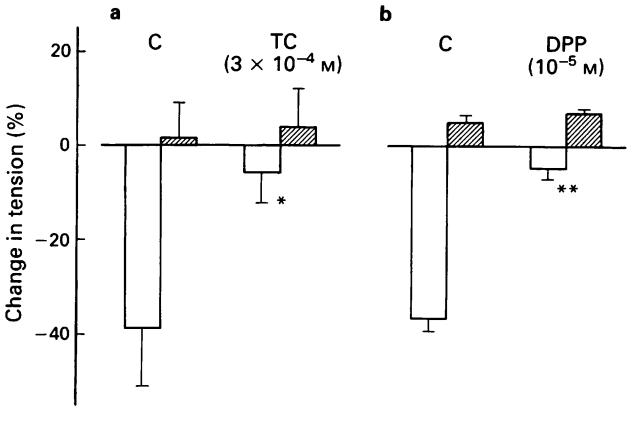

Figure 4 Modification by tranylcypromine (TC, 3×10^{-4} M; a) and diphloretin phosphate (DPP, 10^{-5} M; b) of responses to endothelin-3 (ET-3, 10^{-8} M) in dog coronary artery strips without the endothelium. The strips were partially contracted with prostaglandin $F_{2\alpha}$ (10^{-7} to 10^{-6} M). Contractions induced by 30 mM K^+ were taken as 100%; mean absolute values in control and TC-treated strips were 2242 ± 153 mg and 1895 ± 167 mg ($n = 5$), respectively, and those in control and DPP-treated strips were 1213 ± 187 mg and 1293 ± 354 mg ($n = 3$), respectively. Relaxations induced by 10^{-4} M papaverine were taken as 100%; mean absolute values in control and TC-treated strips were 220 ± 22 mg and 316 ± 77 mg ($n = 5$), respectively, and those in control and DPP-treated strips were 248 ± 29 and 223 ± 43 mg ($n = 3$), respectively. Significantly different from control (C), $*P < 0.01$; $**P < 0.001$. Columns indicate means and bars represent s.e.mean.

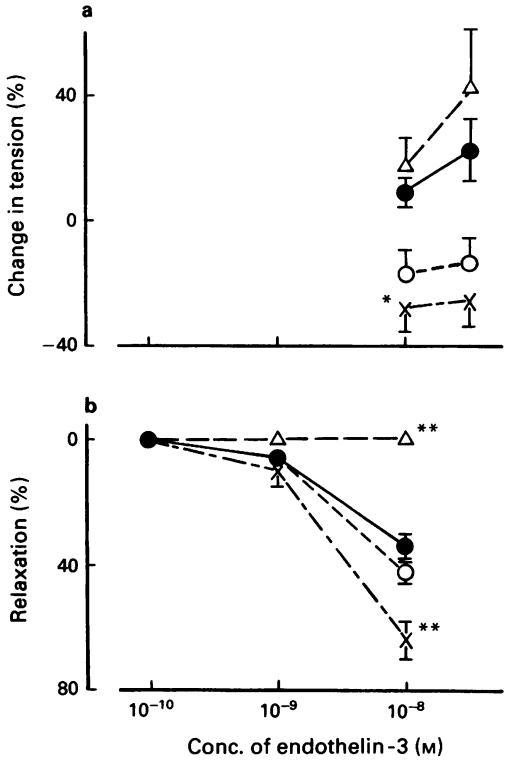

Figure 5 Modification by BQ-123 (BQ) and indomethacin (IM) of contractile (a) and relaxant (b) responses to endothelin-3 (ET-3) in dog coronary arteries without the endothelium. The strips were partially contracted with prostaglandin $F_{2\alpha}$ (10^{-7}–10^{-6} M). Since ET-3 at 10^{-8} M caused a relaxation followed by a contraction, both responses to the peptide are plotted. Contractions induced by 30 mM $K^+$and relaxations by 10^{-4} M papaverine were taken as 100% for contraction and relaxation, respectively; mean absolute values for the contraction in control (●), BQ (10^{-8} M)-treated (○), BQ (10^{-7} M)-treated (×) and IM (10^{-6} M)-treated (△) strips were 2344 ± 323 mg, 2190 ± 498 mg, 2230 ± 307 mg and 1924 ± 698 mg ($n = 5$), respectively; and those for the relaxation were 268 ± 49 mg, 294 ± 47 mg, 201 ± 12 mg and 308 ± 24 mg ($n = 5$), respectively. Points indicate means and bars represent s.e.mean. Significantly different from control, $*P < 0.05$; $**P < 0.01$ (Tukey's method).

inhibited the ET-3-induced contractions in control and indomethacin-treated arteries and potentiated the relaxations in a concentration-dependent manner, whereas the $PGF_{2\alpha}$-induced contraction was unaffected. Therefore, it appears that the contractions induced by ET-3 in dog coronary arteries are associated with ET_A receptors located in the smooth muscle as previously reported in porcine blood vessels (Fukuroda *et al.*, 1992), whereas the receptor subtype responsible for the relaxation induced by the peptide is distinct from ET_A. N^G-nitro-L-arginine failed to suppress the relaxations induced by ET-3 in endothelium-intact strips providing further evidence that the peptide-induced relaxation in dog coronary artery is unlikely to be mediated by endothelium-derived nitric oxide. On the other hand, indomethacin abolished the ET-3-induced relaxation in endothelium-intact and -denuded strips. Tranylcypromine, a PGI_2 synthetase inhibitor (Gryglewski *et al.*, 1976), markedly suppressed the relaxation caused by ET-3, but did not influence the response to beraprost, a PGI_2 analogue. Further, the peptide-induced relaxation was abolished by diphloretin phosphate, a prostaglandin receptor antagonist (Eakins, 1971), as is the response to PGI_2 (Akiba *et al.*, 1986). These results suggest that the relaxations elicited by ET-3 are mediated via liberation of PGI_2 by activation of non-ET_A receptors located in subendothelial tissues, possibly smooth muscle cells. Involvement of endogenous PGI_2 has been postulated in the relaxation induced by other peptides such as angiotensin II (Okamura *et al.*, 1990) and bradykinin (Toda *et al.*, 1987).

It is assumed that the ET receptor(s) responsible for vasoconstriction is (are) distinct from those mediating vasorelaxation (Vane, 1990). In the present study, ET-1 did not produce significant relaxation. The difference between ET-1 and ET-3 may be explained by their different affinities for ET_A and the receptor(s) mediating relaxation. Potent vasoconstriction by ET-1 may mask the weak relaxation by the peptide. Recently, contraction-mediated by ET_B receptors has been demonstrated in porcine isolated arteries (Fukuroda *et al.*, 1992); however, the present results suggest that this does not occur in dog coronary arteries. Although numerous studies have demonstrated that ET-3-induced relaxations are endothelium-dependent (Warner *et al.*, 1989; Fukuda *et al.*, 1990; 1991), the present study has revealed an endothelium-independent, PGI_2-mediated mechanism for the ET-induced relaxation.

Endothelial cells synthesize and release vasoactive substances spontaneously or in response to physical and chemical stimuli and thus play an important role in the regulation of vascular tone. Although ET was introduced as a potent vasoconstrictor (Yanagisawa *et al.*, 1988), low concentrations of ET-3 elicited hypotension *in vivo* and vasorelaxation in certain isolated blood vessels. Chronic infusion of ET did not cause hypertension (Tanabe *et al.*, 1992). Therefore, ET in physiological concentrations may not produce vasoconstriction but rather vasodilatation. The present study suggests that ETs might produce vasodilatation mediated via ET receptors distinct from ET_A even when endothelial functions are damaged by vascular disorders such as atherosclerosis or severe hypertension.

References

AKIBA, T., MIYAZAKI, M. & TODA, N. (1986). Vasodilator actions of TRK-100, a new prostaglandin I_2 analogue. *Br. J. Pharmacol.*, **89**, 703–711.

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding endothelin receptor. *Nature*, **348**, 730–732.

DE MEY, J.G. & VANHOUTTE, P.M. (1982). Heterogenous behavior of the canine arterial and venous wall-importance of the endothelium. *Circ. Res.*, **51**, 439–447.

DE NUCCI, G., THOMAS, R., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9797–9800.

EAKINS, K.E. (1971). Prostaglandin antagonism by polymeric phosphates of phloretin and related compounds. *Ann. N.Y. Acad. Sci.*, **180**, 386–395.

FUKUDA, N., IZUMI, Y., SOMA, M., WATANABE, Y., WATANABE, M., HATANO, M., SAKUMA, I. & YASUDA, H. (1990). L-N^G-monomethyl arginine inhibits the vasodilating effects of low dose of endothelin-3 on rat mesenteric arteries. *Biochem. Biophys. Res. Commun.*, **167**, 739–745.

FUKUDA, N., SOMA, M., IZUMI, Y., MINATO, M., WATANABE, Y., WATANABE, M. & HATANO, M. (1991). Low doses of endothelin-3 elicit endothelium dependent vasodilatation which accompanies with elevation of cyclic GMP. *Jpn. Circ. J.*, **55**, 617–622.

FUKURODA, T., NISHIKIBE, M., OHTA, Y., IHARA, M., YANO, M., ISHIKAWA, K., FUKAMI, T. & IKEMOTO, F. (1992). Analysis of responses to endothelins in isolated porcine blood vessels by using a novel endothelin antagonist, BQ-153. *Life Sci.*, **50**, 107–112.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1989). N^G-monomethyl-L-arginine does not inhibit the hindquarters vasodilator action of endothelin-1 in conscious rats. *Eur. J. Pharmacol.*, **171**, 237–240.

GRYGLEWSKI, R.J., BUNTING, W., MONCADA, S., FLOWER, R.J. & VANE, J.R. (1976). Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxides. *Prostaglandins*, **12**, 685–713.

INOUE, A., TANAGISAWA, M., KIMURA, S., KASUYA, Y., MIYAUCHI, T., GOTO, K. & MASAKI, T. (1989). The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 2863–2867.

IHARA, M., NOGUCHI, K., SAEKI, T., FUKURODA, T., TSUCHIDA, S., KIMURA, S., FUKAMI, T., ISHIKAWA, K., NISHIKIBE, M. & YANO, M. (1992). Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.*, **50**, 247–255.

LIPPTON, H., GOFF, J. & HYMAN, A. (1988). Effects of endothelin in the systemic and renal vascular beds in vivo. *Eur. J. Pharmacol.*, **155**, 197–199.

MINKES, R.K., MACMILLAN, L.A., BELLAN, J.A., KERSTEIN, M.D., MCNAMARA, D.B. & KADOWITZ, P.J. (1989). Analysis of regional responses to endothelin in hindquarters vascular bed of cats. *Am. J. Physiol.*, **256**, H598–H602.

OKAMURA, T., OKUNISHI, H., AYAJIKI, K. & TODA, N. (1990). Conversion of angiotensin I to angiotensin II in dog isolated renal artery: role of two different angiotensin II-generating enzymes. *J. Cardiovasc. Pharmacol.*, **15**, 353–359.

SAKURAI, T., YANAGISAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348**, 732–735.

TANABE, A., NARUSE, M., ZENG, Z.P., NARUSE, K., YOSHIHARA, I., HASE, M. & DEMURA, H. (1992). Is chronic elevation of plasma endothelin a cause of hypertension? *J. Hypertension*, **10** (Suppl. 4), S97.

TODA, N. (1988). Beraprost sodium. *Cardiovasc. Drug Rev.*, **6**, 222–238.

TODA, N., BIAN, K., AKIBA, T. & OKAMURA, T. (1987). Heterogeniety in mechanisms of bradykinin action in canine isolated blood vessels. *Eur. J. Pharmacol.*, **135**, 321–329.

TODA, N., INOUE, S., BIAN, K. & OKAMURA, T. (1988). Endothelium-dependent and -independent responses to prostaglandin H_2 and arachidonic acid in isolated dog cerebral arteries. *J. Pharmacol. Exp. Ther.*, **244**, 297–302.

TODA, N., MINAMI, Y. & OKAMURA, T. (1990). Inhibitory effects of L-N^G-nitro-arginine on the synthesis of EDRF and the cerebroarterial response to vasodilator nerve stimulation. *Life Sci.*, **47**, 345–351.

USHIO-FUKAI, M., NISHIMURA, J., AOKI, H., KOBAYASHI, S. & KANAIDE, H. (1992). Endothelin-1 inhibits and enhances contraction of porcine coronary arterial strips with an intact endothelium. *Biochem. Biophys. Res. Commun.*, **184**, 518–524.

VANE, J.R. (1990). Endothelins come home to roost. *Nature*, **348**, 673.

WARNER, T.D., DE NUCCI, G. & VANE, J.R. (1989). Rat endothelin is a vasodilator in the isolated perfused mesentery of the rat. *Eur. J. Pharmacol.*, **159**, 325–326.

WRIGHT, C.E. & FOZARD, J.R. (1988). Regional vasodilation is a prominent feature of the haemodynamic response to endothelin in anaesthetized, spontaneously hypertensive rats. *Eur. J. Pharmacol.*, **155**, 201–203.

YAMASHITA, Y., YUKIMURA, T., MIURA, K., OKUMURA, M. & YAMAMOTO, K. (1991). Effects of endothelin-3 on renal functions. *J. Pharmacol. Exp. Ther.*, **259**, 1256–1260.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

(Received April 10, 1992
Revised July 20, 1992
Accepted August 3, 1992)

Br. J. Pharmacol. (1992), **107**, 1042–1047

Pre-clinical pharmacology of ICI D2138, a potent orally-active non-redox inhibitor of 5-lipoxygenase

[1]R.M. McMillan, K.E. Spruce, G.C. Crawley, E.R.H. Walker & S.J. Foster

ICI Pharmaceuticals, Bioscience 1, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG

1 This paper describes the pre-clinical pharmacology of ICI D2138, a potent orally-active non-redox inhibitor of 5-lipoxygenase which is undergoing clinical evaluation.

2 ICI D2138 potently inhibited leukotriene synthesis in murine peritoneal macrophages (IC_{50} = 3 nM) and human blood (IC_{50} = 20 nM). In human and dog blood, ICI D2138 did not inhibit thromboxane B_2 synthesis at a concentration of 500 μM, thus the selectivity ratio (cyclo-oxygenase: 5-lipoxygenase) was greater than 20,000. In contrast, zileuton (a 5-lipoxygenase inhibitor also undergoing clinical evaluation) exhibited a selectivity ratio of 15–100.

3 ICI D2138 potently and dose-dependently inhibited *ex vivo* leukotriene B_4 (LTB_4) synthesis by rat blood with ED_{50} values of 0.9, 4.0 and 80.0 mg kg^{-1} p.o. at 3, 10 and 20 h respectively after dosing. Similar activity was observed for inhibition of LTB_4 production in a zymosan-inflamed rat air pouch model. Zileuton produced ED_{50} values of 5 and 20 mg kg^{-1} at 3 and 10 h respectively.

4 Oral administration of 1, 3 or 10 mg kg^{-1} ICI D2138 to dogs produced maximal inhibition of *ex vivo* LTB_4 synthesis by blood for 5, 9 and 31 h respectively. A dose of 5 mg kg^{-1} p.o. of zileuton caused maximal inhibition of LTB_4 for 24 h.

5 Oral administration of 10 mg kg^{-1} ICI D2138 caused total inhibition of LTB_4 production in zymosan-inflamed rabbit knee joint.

6 Topical administration of ICI D2138 to rabbit skin caused a dose-related inhibition of arachidonic acid-induced plasma extravasation with an ID_{30} of 1.08 nmol per site. Zileuton was approximately 40 times less potent.

7 Oral anti-inflammatory activity was assessed in an arachidonic acid-induced mouse ear oedema model in animals treated with indomethacin to block pro-inflammatory prostanoids. ICI D2138, given orally, caused dose-dependent inhibition of oedema with an approximate ID_{50} of 1.8 mg kg^{-1}. Zileuton was approximately 10 times less potent.

8 ICI D2138 caused a dose-dependent inhibition of antigen-induced broncho-constriction in guinea-pigs with an approximate ID_{50} of 0.1 mg kg^{-1}, i.v. Zileuton was approximately 10 times less potent.

9 In view of the pharmacological profile described here, ICI D2138 has the potential to provide improved clinical efficacy compared to existing lipoxygenase inhibitors such as zileuton.

Keywords: 5-Lipoxygenase inhibitor; leukotrienes; ICI D2138; zileuton; inflammation; anti-inflammatory; allergic bronchospasm; anti-asthmatic; inflammatory diseases

Introduction

Leukotrienes are a group of pro-inflammatory lipids that are derived from the metabolism of arachidonic acid by 5-lipoxygenase. Inhibitors of 5-lipoxygenase have therapeutic potential in a range of inflammatory diseases in which leukotrienes have been proposed to have a pathological role (for review see Salmon & Garland, 1991). These diseases include asthma, allergic rhinitis, rheumatoid arthritis, psoriasis and ulcerative colitis.

The mechanism of 5-lipoxygenase is thought to involve an iron-catalysed redox cycle and, of the large number of reported lipoxygenase inhibitors, the majority have the potential to ligand to iron or to participate in redox reactions. Use of such agents has provided support for the anti-inflammatory actions of lipoxygenase inhibitors in animal models (Foster *et al.*, 1990; Carter *et al.*, 1991) but drugs suitable for clinical evaluation have not been available. Recently, BW A4C, an acetohydroxamate which has the potential to chelate iron and possesses relatively weak redox properties, was shown to inhibit *ex vivo* leukotriene synthesis in man (Nicholls & Posner, 1991). A structurally related compound, zileuton, an N-hydroxy urea, inhibits *ex vivo* leukotriene synthesis and has been shown to produce clinical benefit in initial trials in ulcerative colitis, rheumatoid arthritis and pulmonary challenge studies (Collawn *et al.*, 1989; Israel *et al.*, 1990; Knapp, 1990; Weinblatt *et al.*, 1990).

Redox-based inhibitors and iron ligands have relatively low selectivity for 5-lipoxygenase compared to cyclo-oxygenase and evidence is lacking for a specific interaction of such agents with 5-lipoxygenase. For example, no difference in lipoxygenase inhibitor potency has been observed between enantiomers of optically-active acetohydroxamates (Salmon *et al.*, 1989).

We have described a novel series of lipoxygenase inhibitors, methoxyalkyl thiazoles, which have neither iron-liganding nor redox properties and exhibit enantioselective inhibition of 5-lipoxygenase (McMillan *et al.*, 1990). Further development of this series has produced the compound, ICI D2138 (6-([3-fluoro-5-(4-methoxy-3,4,5,6-tetrahydro-2*H*-pyran-4-yl)phenoxy]methyl)-methyl-2-quinolone) (Crawley *et al.*, 1992) which is undergoing clinical evaluation. This paper describes the pre-clinical pharmacology of ICI D2138.

Methods

Leukotriene synthesis in vitro *and* ex vivo

Leukotriene synthesis by mouse peritoneal macrophages and human blood *in vitro* and by rat blood *in vitro* and *ex vivo*

[1] Author for correspondence.

was measured as previously described (Foster *et al.*, 1990; McMillan *et al.*, 1990). For measurement of leukotriene synthesis in dog, a blood sample was withdrawn from a vein in the foreleg and collected into heparin (5 iu ml^{-1}). The animals were then given a gelatin capsule containing either micronised ICI D2138 or zileuton mixed with lactose (50:50 w:w). Blood samples were collected at various times after dosing and challenged with A23187 as described for human blood (Foster *et al.*, 1990). All studies were on the same colony of 6 male beagle dogs (12–16 kg).

Leukotriene synthesis in inflamed rat air pouch

Male Alderley Park rats (180–220 g) were anaesthetized with Halothane and an air pouch was formed by injecting sterile air (20 ml) into the subcutaneous tissue of the back of each animal by use of a 0.22 μM millipore filter attached to a syringe. Three days later the air pouches were reinflated with a second injection of sterile air (10 ml). After a further 3 days, groups of 15 animals were dosed with vehicle and 5 were dosed orally with compound formulated by dissolving the compound in 0.3 ml dimethyl sulphoxide and mixing the solution with 15 ml of 0.5% hydroxypropyl methyl cellulose containing 0.1% polysorbate 80 (HPMC). At the same time a 1% suspension of zymosan in physiological saline (PS) (1 ml) was injected directly into each air pouch. Before administration the zymosan suspension was boiled for 30 min in PS, washed three times by centrifugation in PS at 2000 *g* for 5 min, resuspended in PS to 1% then autoclaved. The rats were killed at various times after zymosan injection using a rising concentration of carbon dioxide and the air pouches were lavaged with PS (1 ml) containing 20 iu of heparin. Lavage fluids were immediately placed on ice, centrifuged in an Eppendorf bench centrifuge and the supernatants analysed for leukotriene B_4 (LTB_4) by radioimmunoassay. The effect of compounds on LTB_4 production was expressed as percentage inhibition of the control values.

Inflamed rabbit knee

Groups of 12 female New Zealand White rabbits (2.5–3.5 kg: Ranch Rabbits, Crawley Down, Sussex) were dosed orally with 10 mg kg^{-1} of ICI D2138 polytroned in HPMC (dose volume 1 ml kg^{-1}) or vehicle alone. Immediately after dosing the fur from both knees was removed with electric clippers and the exposed skin was swabbed with 70% ethanol. This was followed immediately by intra-articular injections of PS (1 ml) into the left knee joint space and zymosan (1 ml of a 1% suspension in PS prepared as indicated above) into the right knee joint space. Animals were given a lethal dose of sodium pentobarbitone (Euthatal) 4 h later and the knee joints were lavaged with 2 ml of PS containing 20 iu ml^{-1} heparin. Following centrifugation the lavage supernatants were stored frozen at −20°C for subsequent analysis of LTB_4 levels by radioimmunoassay.

Arachidonic acid-induced inflammation

Arachidonic acid-induced inflammation in rabbit skin was measured as previously described (McMillan *et al.*, 1990).

Leukotriene-dependent inflammation in mouse ear was measured by a modification of the procedure of Opas *et al.* (1985). Groups of 10–20 male or female AP mice (25–30 g) were dosed orally with indomethacin (10 mg kg^{-1} in HPMC) and with ICI D2138 or zileuton in HPMC or HPMC alone 1 h before the application of arachidonic acid (1 mg in 10 μl of Analar acetone) to the inside of the right ear. The contralateral ear did not receive acetone alone since previous experiments have shown this to be unnecessary. Mice were killed 1 h later by cervical dislocation and 6 mm discs were punched from both ears.

The effect of the test agent on inflammatory oedema was assessed by determining the mean difference in weight of the discs from the arachidonic acid-treated and untreated ears from both control and drug-treated groups of mice. Inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Control} - \text{Drug-treated} \times 100}{\text{Control}}$$

Allergic bronchospasm in guinea-pig

Leukotriene-dependent bronchospasm in guinea-pigs was measured by a modification of the procedure of Anderson *et al.* (1983). Male Dunkin Hartley guinea-pigs, weighing 250–300 g on delivery were housed in groups of 5 and allowed food and water *ad libitum*. After 4 days acclimatatization, animals were sensitized by an i.p. injection of 1 mg of ovalbumin 5×10^9 *Bordetella pertussis* organisms in 0.5 ml pyrogen-free saline on days 1, 4 and 8. Guinea-pigs were used for testing between days 28 and 35. On the day of the test guinea-pigs were anaesthetized with 1.0 g kg^{-1} urethane (ethyl carbamate, 0.25 g ml^{-1}) and 20 mg kg^{-1} Sagatal (sodium pentobarbitone, 60 mg ml^{-1} solution) given i.p. and left approximately 20 min before surgery. To measure ventilatory pressure, a tracheotomy tube was surgically implanted in the trachea using polythene tubing. This was connected to a 'T' piece on one side to a small animal respirator set at stroke rate volume^{-1} of 37 min^{-1} and 1.0 cc 100 g^{-1} body weight and on the other to a recording device consisting of a pressure transducer, an amplifier and a flat bed recorder. The recorder was calibrated with a mercury manometer to give full scale deflection at approximately 180 mmHg pressure.

Guinea-pigs were pretreated with indomethacin (10 mg kg^{-1}, i.v.), succinylcholine (1.2 mg kg^{-1}, i.v.), pyrilamine (1 mg kg^{-1}, i.v.) and propranolol (0.1 mg kg^{-1}) before induction of bronchoconstriction with antigen (5 mg ovalbumin kg^{-1}); under these conditions the bronchospasm is primarily mediated by leukotrienes (Anderson *et al.*, 1983). All agents were administered via an i.v. cannula inserted in the jugular vein.

Statistics

Statistical significance was assessed by Student's paired *t* test, analysis of variance or Dunnett's test, as indicated in the appropriate figure legends, with $P<0.05$ regarded as significant.

Materials

Materials used were as previously described (Foster *et al.*, 1990 and references therein). Rev 5901 (α-pentyl-3-(2-quinolinylmethoxy)-benzene methanol), A64077 (zileuton) (N-(1-(benzo(b)thien-2-yl)ethyl)-N-hydroxy urea) and ICI D2138 were synthesized in Chemistry Department I, ICI Pharmaceuticals. WY-50295 (S-α-methyl-6-(2-quinolinylmethoxy)-2-napthalene acetic acid, tromethamine salt) was kindly supplied by Dr B.M. Weichmann of Wyeth-Ayerst Research, Princeton, NJ, U.S.A. Succinylcholine chloride, pyrilamine maleate, and ovalbumin were purchased from Sigma (Poole, U.K.) and propranolol was from ICI Pharma-ceuticals. Urethane (ethyl carbamate) and hydroxyethyl-cellulose were purchased from Fluka, Glossop, U.K. *Bordetella pertussis* organisms (Per Vac) as adjuvant was purchased from Wellcome, U.K.

Results

ICI D2138 potently inhibited leukotriene C_4 synthesis in a plasma-free preparation of murine peritoneal macrophages with an IC_{50} of 0.003 μM, Figure 1). A reduction in the potency of ICI D2138 was observed when leukotriene B_4 synthesis by blood was measured (IC_{50} in human blood =

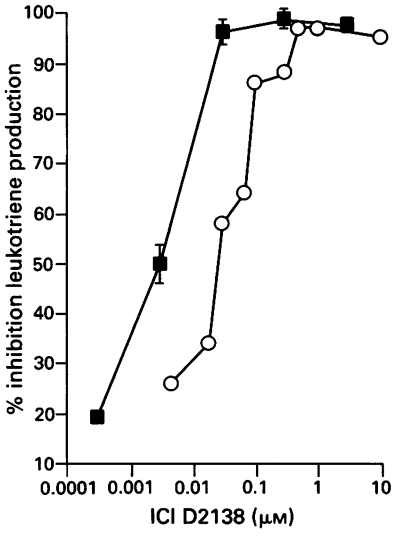

Figure 1 Inhibition of leukotriene production by ICI D2138. The calcium ionophore A23187 was used to stimulate leukotriene B_4 (LTB_4) production by human blood (○) and zymosan to stimulate LTC_4 production by mouse peritoneal macrophages (■) as previously described (Foster *et al.*, 1990). Values are the means of two experiments for human blood and the mean of 2 experiments (no error bars shown) or mean ± s.e.mean (error bars) of 3-5 experiments for mouse peritoneal macrophages.

0.02 μM, Figure 1).

Comparative data for inhibition of leukotriene synthesis in blood by ICI D2138 and 3 other lipoxygenase inhibitors are shown in Table 1. ICI D2138 was 25–100 times more potent than zileuton, depending on the species and also exhibited a higher level of selectivity. In dog and human blood, no inhibition of thromboxane B_2 (TxB_2) synthesis was observed with ICI D2138 at the highest concentration tested (500 μM) and the selectivity ratio (IC_{50} cyclo-oxygenase:IC_{50} 5-lipoxygenase) for ICI D2138 was greater than 20,000. In rat blood, significant inhibition of TxB_2 synthesis was apparent at 500 μM and the selectivity ratio was therefore reduced to 4000. In contrast, zileuton inhibited formation of TxB_2 synthesis at concentrations of 15–100 times those that inhibited LTB_4 synthesis.

Also shown in Table 1 are the effects of two previously described non-redox inhibitors, Rev 5901 and WY 50,295. Both compounds selectively inhibited LTB_4 synthesis in rat blood without inhibiting TxB_2 synthesis at concentrations up to at least 30 μM for Rev 5901 and 100 μM for WY 50,295. Rev 5901 and WY 50,295 were respectively 150 and 900 times less potent than ICI D2138 at inhibiting LTB_4 synthesis in rat blood and failed to inhibit LTB_4 synthesis in human blood at concentrations up to 40 and 100 μM respectively.

Figure 2a shows the effect of ICI D2138 on *ex vivo* LTB_4 synthesis by rat blood. Dose-dependent inhibition was observed at 3, 10 and 20 h with ID_{50} values of 0.9, 4.0 and 80.0 mg kg^{-1} p.o. respectively. In comparative studies (data

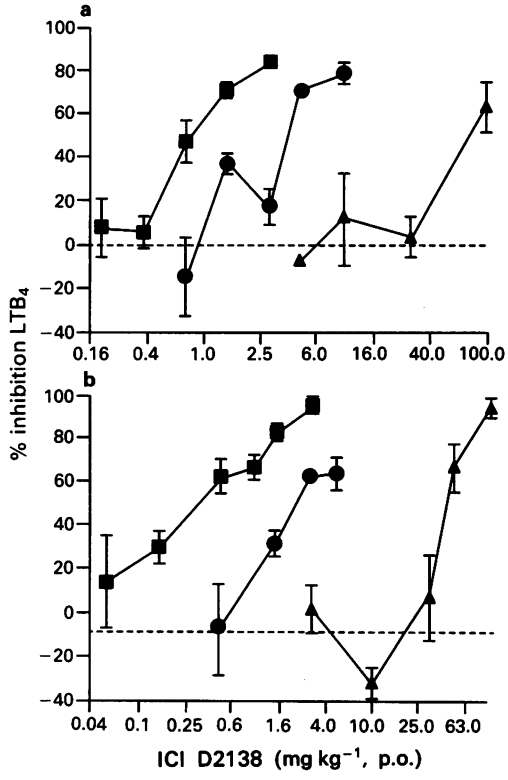

Figure 2 Dose-dependent inhibition of ICI D2138 on *ex vivo* leukotriene B_4 (LTB_4) synthesis by A23187-stimulated rat blood (a) as described in Foster *et al.* (1990) and LTB_4 synthesis in zymosan-inflamed rat air pouch (b) at 3 h (■), 10 h (●) and 20 h (▲) following oral administration. Data are the mean of two experiments (no error bars shown) or mean ± s.e.mean (error bars, 3 or 4 experiments).

Table 1 Inhibition of eicosanoid generation in blood

	Human		*Rat*		*Dog*	
	LTB_4	TxB_2	LTB_4	TxB_2	LTB_4	TxB_2
ICI D2138	0.024 (0.012-0.030) $n=4$	>500 $n=2$	0.033 (0.02-0.04) $n=3$	156 (150-162) $n=2$	0.020 $n=2$	>500 $n=2$
Zileuton	2.60 (2.4-2.8) $n=2$	40 – $n=1$	2.30 $n=1$	>100 $n=1$	0.56 (0.46–0.66) $n=2$	51 (27–75) $n=2$
Rev 5901	>40 ($n=2$)	>40 ($n=2$)	3.0 (2–5) ($n=5$)	>30[1] ($n=6$)	ND	ND
WY 50295	>100 ($n=2$)	>100 ($n=2$)	30 ($n=2$)	>100 ($n=2$)	ND	ND

Results are mean IC_{50} (μM) values with the range of individual values shown in parentheses.
[1]PGE_2 synthesis measured instead of TxB_2. The percentage inhibition caused by a concentration of 30 μM ranged from – 14 to 30 in 6 experiments.

not shown), zileuton produced ID_{50} values at 3 and 10 h of 5 mg kg^{-1} (mean of 2 experiments) and 20 mg kg^{-1} (mean of 3 experiments) respectively. Synthesis of LTB_4 in zymosan-inflamed rat air pouch was inhibited by comparable doses of ICI D2138: ID_{50} values of 0.3, 2.0 and 40.0 mg kg^{-1} p.o. were obtained at 3, 10 and 20 h after dosing (Figure 2b).

Oral administration of 1 mg kg^{-1} ICI D2138 to dogs produced transient inhibition of LTB_4 synthesis with maximal effects at 3–5 h. At 3 or 10 mg kg^{-1}, maximal inhibition was evident at the earliest time point studied (1 and 5 h respectively). Maximal inhibition was maintained for 9 h and at least 31 h following single oral doses of 3 and 10 mg kg^{-1} respectively (Figure 3). Zileuton also produced prolonged inhibition of LTB_4 synthesis in dog: inhibition following a dose of 5 mg kg^{-1} p.o. was maintained for 24 h (data not shown).

Figure 4 shows the effect of oral administration of ICI D2138 on LTB_4 synthesis in zymosan-inflamed rabbit knee joint. Intra-articular injection of zymosan stimulated LTB_4 concentrations in lavage fluid by approximately 6 fold to 3.0 ± 0.55 ng ml^{-1}. Prior administration of ICI D2138 reduced LTB_4 concentrations in lavage fluid from zymosan-inflamed knee to the baseline levels detected in fluid from control, saline-injected knee joint.

Topical administration of ICI D2138 to rabbit skin caused dose-related inhibition of plasma extravasation induced by arachidonic acid with an ID_{30} of 1.08 ± 0.33 nmol per site (mean ± s.d.; $n = 6$). Zileuton was approximately 40 times less potent with an ID_{30} of 42 nmol per site (mean of 2 experiments).

Oral anti-inflammatory activity was measured using arachidonic acid-induced mouse ear oedema. In the presence of indomethacin to block synthesis of pro-inflammatory prostanoids, ICI D2138 produced dose-dependent inhibition of oedema (Figure 5). In this model ICI D2138 was approximately 10 times more potent than zileuton: respective ID_{50} values 1.8 mg kg^{-1} p.o. and 18 mg kg^{-1} p.o. were obtained.

Figure 6 shows the effects of ICI D2138 and zileuton on allergic bronchospasm in the guinea-pig. In the presence of indomethacin, pyrillamine and propranolol, intravenous administration of ICI D2138 caused a dose-dependent inhibition of the antigen-induced increase in ventilatory pressure. In this system zileuton (ID_{50} approximately 1.0 mg kg^{-1}) was approximately 10 times less potent than ICI D2138 (ID_{50} approximately 0.1 mg kg^{-1}) (Figure 6).

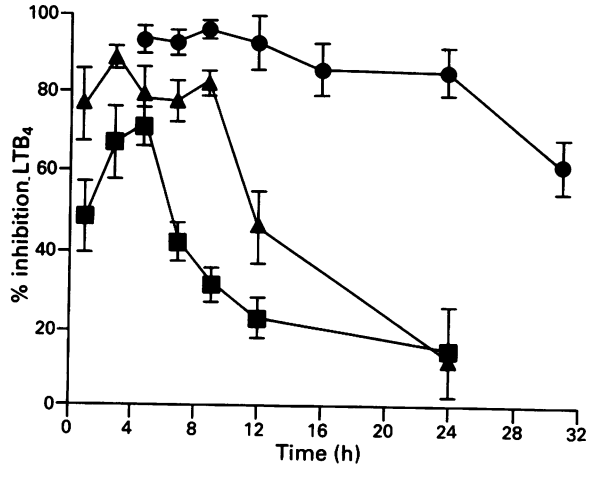

Figure 3 Time-dependent effect of ICI D2138 on *ex vivo* leukotriene B_4 (LTB_4) synthesis by A23187-stimulated dog blood as previously described in Foster *et al.* (1990) for human blood. Values are the means ± s.e.mean (vertical bars) % inhibition of LTB_4 production in 6 dogs treated with 1 mg kg^{-1} (■), 3 mg kg^{-1} (▲) or 10 mg kg^{-1} (●) of ICI D2138.

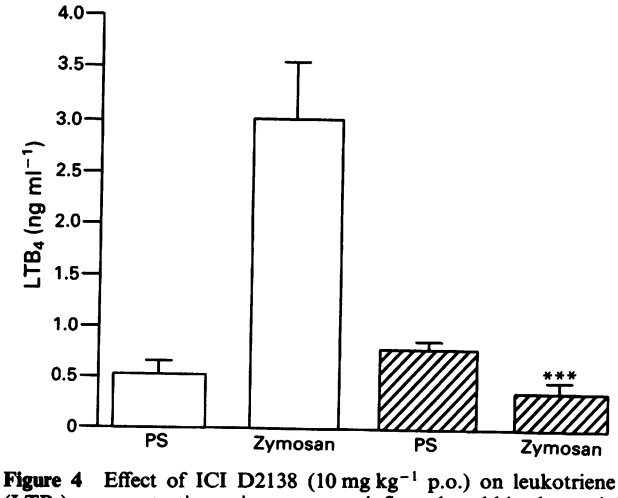

Figure 4 Effect of ICI D2138 (10 mg kg^{-1} p.o.) on leukotriene B_4 (LTB_4) concentrations in zymosan-inflamed rabbit knee joint. Zymosan suspension was injected into the right and PS into the left knee joint space respectively. Four hours later the animals were killed and the joints were lavaged with PS. Data are the means ± s.e.mean (vertical bars) ng LTB_4 ml^{-1} of lavage fluid from 11 vehicle-dosed (open columns) and 12 ICI D2138-dosed (cross-hatched columns) animals. ***Indicates $P < 0.001$ with respect to the zymosan-inflamed knee joint in the vehicle-dosed group calculated by Student's t test.

Discussion

The studies described here demonstrate that ICI D2138 is a potent, selective and orally-active inhibitor of 5-lipoxygenase. The compound has certain structural features in common with a series of methoxyalkyl thiazoles, which have been shown previously to produce enantioselective inhibition of 5-lipoxygenase (McMillan *et al.*, 1990).

The majority of previously reported 5-lipoxygenase inhibitors have the potential to participate in redox reactions or to ligand to iron. Such compounds usually exhibit only limited selectivity for 5-lipoxygenase compared to the related

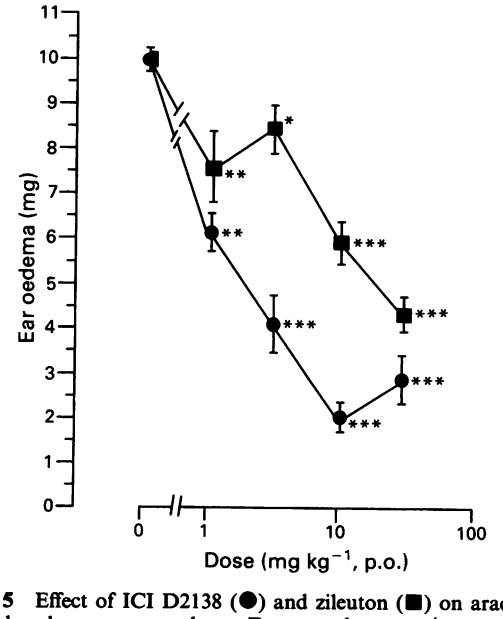

Figure 5 Effect of ICI D2138 (●) and zileuton (■) on arachidonic acid-induced mouse ear oedema. Data are the mean ± s.e.mean (vertical bars) mg oedema calculated from 8 separate experiments where the total number of control animals was 159 and the drug-treated group sizes were either 20 or 40. Statistical significance was calculated by analysis of variance. *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ with respect to controls.

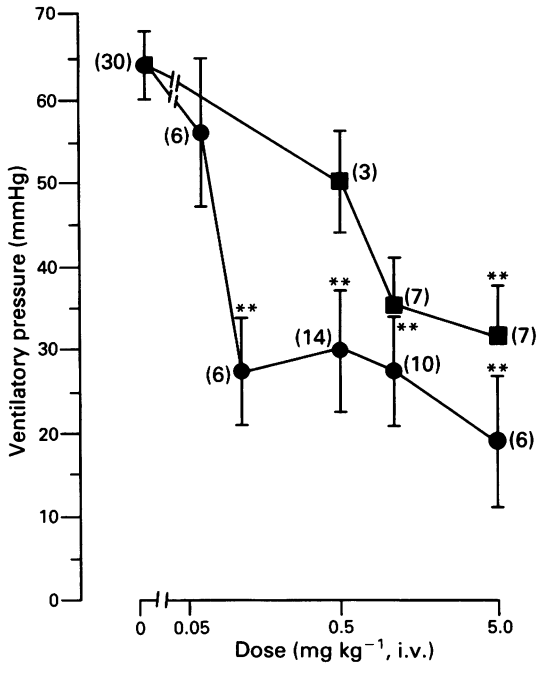

Figure 6 Effect of ICI D2138 (●) and zileuton (■) on antigen-induced bronchoconstriction in guinea-pigs. Values are the mean ± s.e.mean (vertical bars) ventilatory pressure (mmHg) with number of animals for each group shown in parentheses. Statistical significance was calculated by Dunnett's test to compare each dose group to control. $^{**}P<0.01$.

enzyme cyclo-oxygenase. For example, in these studies, zileuton, an N-hydroxy urea with iron liganding and weak redox properties, exhibits 15–100 fold selectivity depending on the *in vitro* assay employed. In contrast, ICI D2138 produced selectivity ratios (cyclo-oxygenase:5-lipoxygenase) of up to 25,000 fold. The effects of ICI D2138 on arachidonic acid metabolism are essentially the mirror image of those of potent non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin or flurbiprofen. In view of their potency and selectivity, ICI D2138 and related compounds can be considered to be the NSAIDs of the 5-lipoxygenase pathway.

Two other non-redox compounds, Rev 5901 and WY 50,295 also show high selectivity for inhibition of leukotriene synthesis. However, several features distinguish ICI D2138 from those compounds. First, WY 50,295 does not produce enantioselective inhibition of leukotriene synthesis (Musser & Kreft, 1990) whilst no evidence for enantioselective inhibition has been reported for Rev 5901. Second, ICI D2138 is considerably more potent than the other compounds: in rat blood it was 150 and 900 times more potent than Rev 5901 and WY 50,295 respectively. Finally, ICI D2138 exhibited comparable potency in rat, dog and man whereas the other compounds inhibited leukotriene synthesis in rat blood but failed to inhibit in human blood. The reduced potency of Rev 5901 is probably a consequence of insensitivity of human leucocytes to the compound (Coutts *et al.*, 1985). In the case of WY 50,295, the discrepancy between man and rat is at least partly due to differences in binding of the compound to human and rat plasma proteins (Carlsson, R.P., Personal communication).

A problem with previous lipoxygenase inhibitors lacking redox or iron liganding properties, including Rev 5901 and methoxyalkyl thiazoles, was that oral activity was either weak or absent (McMillan *et al.*, 1991). In contrast, ICI D2138 has potent oral activity in rat and dog with comparable ED_{50} values as inhibitors of leukotriene synthesis in blood of approximately 1 mg kg^{-1} 3 h after dosing. However there was a marked difference in duration of action in the two species. In dog, inhibition of leukotriene synthesis following a single oral dose of 10 mg kg^{-1} persisted for at least 32 h. In rat, inhibition by oral doses up to 30 mg kg^{-1} had reversed by 24 h. These data are consistent with differences in the half life of ICI D2138 in rat and dog of 2.0 and 6.0 h respectively (E. Pywell and M. Hutchinson, unpublished). ICI D2138 also inhibited LTB_4 synthesis at an inflammatory site in the rat with potency comparable to that demonstrated in blood *ex vivo*. Inhibition of LTB_4 synthesis in an inflamed rabbit knee joint was also demonstrated. Thus, ICI D2138 exhibits biochemical efficacy in both peripheral blood and inflammatory exudates.

Anti-inflammatory activity of ICI D2138 has been demonstrated by use of arachidonic acid-induced skin oedema. Inhibition of arachidonic acid-induced inflammation in rabbit skin has been shown to be related to 5-lipoxygenase inhibitor potency (Foster *et al.*, 1990) and topical administration of ICI D2138 in this model produced dose-related anti-inflammatory activity. In order to investigate oral anti-inflammatory activity, a smaller species was desirable and therefore arachidonic acid-induced oedema in mouse ear was adopted. In our experience, there is a variable contribution of pro-inflammatory prostaglandins in this model and this complicates evaluation of lipoxygenase inhibitors which also show variations in potency. To overcome this problem, anti-inflammatory activity of lipoxygenase inhibitors was evaluated in animals treated with indomethacin to block prostanoid synthesis. Under these conditions, the model was predominantly dependent on leukotrienes and ICI D2138 produced consistent anti-inflammatory activity. In both rabbit and mouse models ICI D2138 was at least 10 times more potent than zileuton.

The pulmonary actions of ICI D2138 were studied on a model of allergic bronchospasm in the guinea-pig. In animals pretreated with indomethacin, propranolol and pyrillamine, the bronchoconstriction is mediated primarily by leukotrienes (Anderson *et al.*, 1983). Under these conditions, ICI D2138 produced potent inhibition of the antigen-induced increase in ventilatory pressure and was approximately 10 times more potent than zileuton. A detailed evaluation of the effect of ICI D2138 on pulmonary mechanics changes in this model will be given elsewhere (Buckner and Kusner, unpublished).

Based on the biological profile described here, ICI D2138 was selected for clinical development. The compound was well tolerated in human volunteers and inhibited *ex vivo* leukotriene synthesis (Yates *et al.*, 1992). The beneficial clinical effects observed with zileuton in ulcerative colitis and rheumatoid arthritis and in challenge models of asthma and allergic rhinitis (Collawn *et al.*, 1989; Israel *et al.*, 1990; Knapp, 1990; Weinblatt *et al.*, 1990) support the therapeutic potential of lipoxygenase inhibitors in inflammatory diseases. In view of the pharmacological profile described here, ICI D2138 has the potential to provide improved clinical efficacy compared to existing lipoxygenase inhibitors such as zileuton.

The authors wish to thank Christine Potts, Margaret McCormick and Steve Moores for carrying out the experimental work and Mavis Bloor for secretarial assistance.

References

ANDERSON, W.H., O'DONNELL, M., SIMKO, B.A. & WELTON, A.F. (1983). An *in vivo* model for measuring antigen-induced SRS-A-mediated bronchoconstriction and plasma levels in the guinea pig. *Br. J. Pharmacol.*, **78**, 67–74.

CARTER, G.W., YOUNG, P.R., ALBERT, D.H., BOUSKA, J., DYER, R., BELL, R.L., SUMMERS, J.B. & BROOKS, D.W. (1991). 5-Lipoxygenase inhibitory activity of zileuton. *J. Pharmacol. Exp. Ther.*, **256**, 929–937.

COLLAWN, C., RUBIN, P., PEREZ, H., REYES, E., BOBADILLA, J., CABRERA, G., MORAN, M.A. & KERSHENOBICH, D. (1989). Experimental use of a 5-lipoxygenase inhibitor (Abbott-64077) in human ulcerative colitis (UC). *Am. J. Gastroenterol.*, **84**, 1178.

COUTTS, S.M., KHANDWALA, A., VAN INWEGAN, R., CHAKRABORTY, U., MUSSER, J., BRUENS, J., JARIWALA, N., DALLY-MEADE, V., INGRAM, R., PRUSS, T., JONES, H., NEISS, E. & WEINRYB, I. (1985). Arylmethyl phenyl ethers: a new class of specific inhibitors of 5-lipoxygenase. In *Prostaglandins, Leukotrienes and Lipoxins*. ed. Baily, M.J. pp. 627–637. New York: Plenum Press.

CRAWLEY, G.C., DOWELL, R.I., EDWARDS, P.N., FOSTER, S.J., MCMILLAN, R.M., WALKER, E.R.H. & WATERSON, D. (1992). Methoxytetrahydropyrans: A new series of selective and orally potent 5-lipoxygenase inhibitors. *J. Med. Chem.*, (in press).

FOSTER, S.J., BRUNEAU, P., WALKER, E.R.H. & MCMILLAN, R.M. (1990). 2-Substituted indazolinones: orally active and selective 5-lipoxygenase inhibitors with anti-inflammatory activity. *Br. J. Pharmacol.*, **99**, 113–118.

ISRAEL, E., DERMARKARIAN, R.M., ROSENBERG, M.A., SPERLING, R., TAYLOR, G., RUBIN, P. & DRAZEN, J.M. (1990). The effects of a 5-lipoxygenase inhibitor on asthma induced by cold, dry air. *New Engl. J. Med.*, **323**, 1740–1744.

KNAPP, H.R. (1990). Reduced allergen-induced nasal congestion and leukotriene synthesis with an orally active 5-lipoxygenase inhibitor. *N. Engl. J. Med.*, **323**, 1745–1748.

MCMILLAN, R.M., BIRD, T.G.C., CRAWLEY, G.C., EDWARDS, M.P., GIRODEAU, J-M., KINGSTON, J.F. & FOSTER, S.J. (1991). Methoxyalkyl thiazoles: A novel series of potent, orally active and enantioselective inhibitors of 5-lipoxygenase. *Agents Action*, **34**, 110–112.

MCMILLAN, R.M., GIRODEAU, J-M. & FOSTER, S.J. (1990). Selective chiral inhibitors of 5-lipoxygenase with anti-inflammatory activity. *Br. J. Pharmacol.*, **101**, 501–503.

MUSSER, J.H. & KREFT, A.F. (1990). Substituted-[2-quinolinyl-(bridged)aryl] compounds: modulators of eicosanoid biosynthesis and action. *Drugs of the Future*, **15**, 73–80.

NICHOLLS, A.J. & POSNER, J. (1991). Investigation of single doses of BWA4C, a selective 5-lipoxygenase inhibitor, in healthy volunteers. *Br. J. Clin. Pharmacol.*, **31**, 577P.

OPAS, E.E., BONNEY, R.J. & HUMES, J.L. (1985). Prostaglandin and leukotriene synthesis in mouse ears inflamed by arachidonic acid. *J. Invest. Dermatol.*, **84**, 253–256.

SALMON, J.A. & GARLAND, L.G. (1991). Leukotriene antagonists and inhibitors of leukotriene biosynthesis as potential therapeutic agents. *Prog. Drug Res.*, **37**, 9–90.

SALMON, J.A., JACKSON, W.P. & GARLAND, L.G. (1989). Development of 5-lipoxygenase inhibitors: potential drugs for asthma and inflammation. In *Therapeutic Approaches to Inflammatory Disease*. ed. Lewis, A.J., Doherty, N.S. & Ackerman, N.R. pp.137–146. New York: Elsevier.

WEINBLATT, M., KREMER, J., HELFGOTT, S., COBLYN, J., MAIER, A., SPERLING, R., PETRILLO, G., KESTERSAN, J., DUBE, L., HANSON, B., TEAH, H. & DUBIN, P. (1990). A 5-lipoxygenase inhibitor in rheumatoid arthritis (RA). *Arthritis Rheum.*, **33**, S152.

YATES, R.A., HUTCHINSON, M., SPRUCE, K., MCMILLAN, R.M., GILMORE, E. & WILLIAMS, A.J. (1992). Tolerability, pharmacokinetics and effects on blood 5-lipoxygenase of ICI D2138, a new lipoxygenase inhibitor. *Br. J. Clin. Pharmacol.*, (in press).

(Received April 24, 1992
Revised July 1, 1992
Accepted August 4, 1992)

Br. J. Pharmacol. (1992), **107**, 1048–1056

Cardiovascular selectivity of adenosine receptor agonists in anaesthetized dogs

R.Z. Gerencer, *B.A. Finegan & [1]A.S. Clanachan

Departments of Pharmacology and *Anaesthesia, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

1 In order to determine the relevance of adenosine (Ado) receptor classification obtained from *in vitro* methods to the cardiovascular actions of Ado agonists *in vivo*, the cardiovascular effects of adenosine 5'-monophosphate (AMP), N^6-cyclohexyladenosine (CHA, 400 fold A_1-selective), 5'-N-ethylcarboxamidoadenosine (NECA, $A_1 \approx A_2$) and 2-phenylaminoadenosine (PAA, 5 fold A_2-selective) were compared in open-chest, fentanyl-pentobarbitone anaesthetized dogs.

2 Graded doses of CHA (10 to 1000 $\mu g\,kg^{-1}$), NECA (0.5 to 100 $\mu g\,kg^{-1}$) or PAA (0.1 to 20 $\mu g\,kg^{-1}$) were administered intravenously and changes in haemodynamics and myocardial contractility were assessed 10 min following each dose. The effects of graded infusions of AMP (200 to 1000 $\mu g\,kg^{-1}\,min^{-1}$) were also evaluated.

3 AMP and each of the Ado analogues (NECA > PAA > CHA) increased the systemic vascular conductance index (SVCI) in a dose-dependent manner and reduced mean arterial pressure (MAP). At doses causing similar increases in SVCI, these agonists caused (i) similar reflex increases in heart rate (HR) and cardiac index (CI) and decreases in AV conduction interval (AV_i) and (ii) similar increases in coronary vascular conductance (CVC).

4 After cardiac autonomic blockade with atropine (0.2 $mg\,kg^{-1}$) and propranolol (1 $mg\,kg^{-1}$), AMP, CHA and PAA still increased SVCI and CVC and decreased MAP. CHA and PAA had no marked effects on HR, CI or AV_i. As in the absence of cardiac autonomic blockade, equieffective vasodilator doses of CHA and PAA had identical effects on CVC, CI and AV_i.

5 Myocardial contractility, as assessed by E_{max} measurements, was stimulated by AMP in control animals. Following cardiac autonomic blockade, PAA increased contractility while AMP and CHA had no significant effects.

6 Despite marked differences in receptor selectivity *in vitro*, no marked differences between the actions of these A_1- and A_2-selective Ado receptor agonists on the cardiovascular system *in vivo* were apparent. Difficulties therefore exist in the application of *in vitro* Ado receptor selectivity data to the prediction of the cardiovascular effects of Ado agonists *in vivo*.

Keywords: Adenosine receptors; haemodynamics; myocardial contractility; AV nodal conduction; adenosine 5'-monophosphate; N^6-cyclohexyladenosine; 5'-N-ethylcarboxamidoadenosine; 2-phenylaminoadenosine

Introduction

Adenosine (Ado) is an endogenous nucleoside that participates in a multitude of biochemical and physiological processes throughout the body. In the cardiovascular system, Ado affects both vascular conductance (see review by Collis, 1989) and cardiac function (see review by Belardinelli *et al.*, 1989). It is used clinically in the diagnosis and management of some forms of cardiac dysrhythmias (DiMarco *et al.*, 1983) and for the induction of deliberate hypotension during anaesthesia (Sollevi *et al.*, 1984). Ado effects are mediated by two main subtypes of cell-surface receptors classified as A_1 or A_2 based on the relative pharmacological potencies of a series of reference Ado agonists (Linden, 1991). Based mostly on studies *in vitro*, A_1-selective agonists are considered to generate negative inotropic, chronotropic and dromotropic responses (Evans *et al.*, 1982; DiMarco *et al.*, 1983; Romano *et al.*, 1991) that would tend to reduce the oxygen demand of the myocardium. A_2-mediated responses include coronary vasodilatation (Berne, 1980; King *et al.*, 1990) and inhibition of platelet aggregation (Quattrin *et al.*, 1988) and neutrophil function (Cronstein *et al.*, 1985) and would therefore tend to preserve or increase myocardial oxygen supply. Ado-induced changes in myocardial efficiency (Headrick & Willis, 1989) and energy substrate utilization (Finegan *et al.*, 1992) have also been demonstrated.

The marked systemic vasodilatation resulting from Ado receptor stimulation may be of therapeutic benefit in the management of acute, low output cardiac failure. We have shown previously (Finegan *et al.*, 1990), in normal anaesthetized dogs, that afterload reduction due to Ado receptor-mediated vasodilatation, increases cardiac output more than equieffective afterload reducing doses of sodium nitroprusside (SNP). A similar result was also observed in a canine model of acute left ventricular dysfunction (Finegan & Clanachan, 1991).

Potential advantages of Ado receptor agonists in this role include inhibition of atrial and ventricular automaticity (Szentmiklosi *et al.*, 1980; Wainwright & Parratt, 1988), anti-renin activity (Lagerkranser *et al.*, 1989) and protection of cardiac muscle from ischaemic damage (Olafsson *et al.*, 1987; Babbitt *et al.*, 1989; Pitarys *et al.*, 1991) in addition to their potent vascular smooth muscle relaxant properties (Collis, 1989).

It is not clear what balance of A_1 to A_2 activity would be ideal. A_2 receptor stimulation is required for vasodilatation and afterload reduction while A_1 stimulation, although apparently responsible for cardioprotection (Liu *et al.*, 1991) and attenuating reflex tachycardia, may, if excessive, cause depression of myocardial contractility or blockade of atrio-ventricular (AV) conduction.

Considerable data on the selectivity of Ado agonists in CNS tissues have been derived from receptor binding assays *in vitro*, but the relevance of these classification systems to the cardiovascular effects of Ado agonists *in vivo* is unclear. Indeed, some reports have indicated that Ado receptors involved in canine sino-atrial (SA) nodal function *in vivo*

[1] Author for correspondence.

have an unusual order of agonist potency that does not conform to the conventional A_1 receptor (Belloni *et al.*, 1989a) identified from tissue and binding studies *in vitro*.

This study was designed to examine in detail the effects of selective Ado receptor agonists on haemodynamics and myocardial contractility *in vivo* and to determine if their classification based on *in vitro* methods is applicable to the prediction of their cardiovascular properties *in vivo*.

Methods

This study was approved by the Health Sciences Animal Care Committee of the University of Alberta. Experiments were performed on healthy mongel dogs ($n = 28$) of either sex weighing between 24 and 28 kg (mean 26.2 kg). Animals were anaesthetized with pentobarbitone (30 mg kg^{-1}) and ventilated with an O_2-enriched air mixture to maintain arterial O_2 tension greater than 120 mmHg. Anaesthesia was maintained by a constant infusion (4 ml kg^{-1} h^{-1}) of fentanyl (20 μg kg^{-1} h^{-1}), pentobarbitone (3 mg kg^{-1} h^{-1}) and pancuronium (60 μg kg^{-1} h^{-1}) in 0.9% NaCl following loading doses of fentanyl (40 μg kg^{-1}) and pancuronium (80 μg kg^{-1}).

The right external jugular vein was cannulated for passage of a pulmonary artery thermodilution catheter (Swan-Ganz) for determination of cardiac output (average of 3 individual measurements), pulmonary artery pressure (PAP) and central venous pressure (CVP) and for fluid loading (10 ml kg^{-1} of 6% Dextran 70 in 0.9% NaCl) and anaesthetic infusion. A triple lumen catheter was inserted in the left external jugular vein for infusion of test drugs. A catheter was placed in the aortic arch via the left femoral artery to measure aortic pressure and allow blood sampling at regular intervals for determination of blood gasses, pH and electrolytes. Normal CO_2 tension was maintained by continuous monitoring of end-tidal CO_2. Acidosis and hypokalaemia were corrected, when required, by infusion of sodium bicarbonate and potassium chloride, respectively. The right femoral artery was cannulated for insertion of a precalibrated, high fidelity (Millar) pressure transducer tipped catheter into the left ventricle (LV) for measurement of LV pressure.

Following a left lateral thoracotomy, two piezoelectric crystals (5 MHz, 1.5–2.0 mm diameter) were inserted into the LV subendocardium along its long axis to allow continuous measurement of myocardial segment length (SL) and an electromagnetic flow probe was positioned on the left anterior descending coronary artery (LAD) proximal to the first diagonal branch for the continuous measurement of LAD blood flow. Positive end-expiratory pressure of 5 cm H_2O was applied to the airway to prevent atelectasis.

Data Collection

Heart rate (HR), ECG, systolic, diastolic and mean (MAP) arterial pressure, LAD blood flow, LV pressure, and SL were continuously recorded. For the assessment of AV nodal conduction intervals (AV_i), stimulus artifact-R intervals were averaged from 5 consecutive cardiac cycles during 5 s periods of atrial pacing at 2.5 Hz (a bipolar pacing electrode was placed in the right atrium via the left femoral vein). Myocardial contractility was assessed by E_{max}, which was determined from the end-systolic pressure segment length (ESPSL) relationship (Nozawa *et al.*, 1988). E_{max} was determined at appropriate times of the protocol when cardiac preload was altered by inflation of a 40 ml 22F balloon catheter that was placed in the inferior vena cava. Inflation was maintained for only 4 to 6 cardiac cycles to minimize potential baroreceptor-mediated changes in the inotropic state of the LV. Stroke index (SI), cardiac index (CI), coronary vascular conductance (CVC) and pulmonary (PVCI) and systemic vascular conductance (SVCI) indices were calculated from standard formulae (Keefer & Barash, 1985).

Drug administration protocol

Separate groups of dogs were used to evaluate the cardiovascular effects of N^6-cyclohexyladenosine (CHA), 2-phenyl-aminoadenosine (PAA) and 5'-N-ethylcarboxamidoadenosine (NECA). Each animal within a drug treatment group received all doses of a particular agonist. The effects of adenosine 5'-monophosphate (AMP) were also studied, but because of its extremely short plasma half-life, it was administered as continuous infusions of 200, 400 and 1000 μg kg^{-1} min^{-1} for 10 min per dose level.

Initial baseline values were recorded 30 min following completion of instrumentation and repeated every 10 min for 30 min to assess the stability of the experimental preparation. Haemodynamic variables were then recorded in the steady state after 10 min of infusion of AMP or 10 min after the slow (1 min) intravenous administration of each dose level of each Ado analogue.

Cardiac autonomic blockade

In separate groups of animals, the effects of AMP, CHA and PAA were determined following cardiac autonomic blockade. After baseline measurements, propranolol (1 mg kg^{-1}) and atropine (0.2 mg kg^{-1}) were administered intravenously and the blockade was maintained by constant infusion of propranolol (0.5 mg kg^{-1} h^{-1}) and atropine (0.05 mg kg^{-1} h^{-1}). Completeness of blockade was confirmed by the absence of a cardiac response to isoprenaline and acetylcholine. AMP, CHA and PAA were then administered as described above.

Statistical analysis

Cardiovascular data in the text are means ± s.e. and drug dosages are expressed as geometric means with 95% confidence limits. Statistical analysis was performed by means of analysis of variance for repeated measures and between group comparisons were made by the Mann-Whitney U test. Differences were judged to be statistically significant when $P < 0.05$.

Drugs

AMP and atropine were dissolved in 0.9% saline. Stock solutions of propranolol, PAA and NECA were prepared with saline containing 10% dimethylsulphoxide (DMSO). CHA was dissolved initially in 95% ethanol and then diluted in saline. Ado analogues were purchased from Research Biochemicals, Inc. and all other agents were obtained from Sigma Chemical Co.

Results

Baseline cardiovascular parameters in control animals are shown in Table 1. It should be noted that the addition of fentanyl to the anaesthetic regimen resulted in values for HR that approximated those of normal conscious animals (90 beats min^{-1}) and are unlike those reported during anaesthesia with pentobarbitone alone (150 beats min^{-1}).

Haemodynamic effects of AMP in control animals

Graded infusions of AMP (Table 1) produced rapid, stepwise reductions in MAP that were due to systemic vasodilatation as evidenced by marked increases in SVCI. HR and CI increased; the greater proportional increase in CI indicated that SI also increased. As expected, AMP elevated coronary blood flow by up to 4 fold. CVC, a parameter that is independent of mean arterial pressure, and hence coronary perfusion pressure, was also significantly increased by up to 5.5 fold. Despite the increase in CI, there was only a slight, but significant increase in PAP due to a concomitant increase

Table 1 Cardiovascular effects of intravenous infusions of AMP in fentanylpentobarbitone anaesthetized dogs

	Baseline	*AMP infusion rate* ($\mu g\ kg^{-1}\ min^{-1}$) *200*	*400*	*1000*	P
MAP	112 ± 3	102 ± 2	98 ± 2	90 ± 3	<0.0001
SVCI	360 ± 20	560 ± 50	720 ± 50	940 ± 60	<0.0001
HR	89 ± 3	111 ± 5	143 ± 9	163 ± 8	<0.0001
CI	3.0 ± 0.1	4.2 ± 0.3	5.2 ± 0.3	6.2 ± 0.3	<0.0001
SI	34 ± 1	38 ± 2	37 ± 2	39 ± 2	<0.05
CBF	35 ± 2	58 ± 17	115 ± 17	146 ± 14	<0.0001
CVC	5.0 ± 0.5	9.2 ± 2.8	19.3 ± 3.1	27.5 ± 3.2	<0.0001
LVEDP	10 ± 1	9 ± 1	8 ± 1	7 ± 1	<0.05
CVP	5.6 ± 0.3	5.3 ± 0.3	5.3 ± 0.3	5.3 ± 0.3	<0.05
PAP	17 ± 1	19 ± 1	20 ± 1	22 ± 1	<0.0001
PRVI	220 ± 20	200 ± 20	190 ± 10	170 ± 10	<0.0001
LVSWI	48.1 ± 2.9	48.3 ± 2.5	44.8 ± 2.6	42.0 ± 1.5	<0.01
RVSWI	5.2 ± 0.2	6.8 ± 0.4	7.6 ± 0.5	8.5 ± 0.5	<0.0001
RPP	11.7 ± 0.3	13.8 ± 0.6	17.5 ± 1.0	19.1 ± 1.1	<0.0001
AV_i	151 ± 7	121 ± 7	108 ± 8	101 ± 7	<0.0001

Values are means ± s.e., $n = 15$. Data are shown for baseline conditions and during three graded infusions of AMP on MAP, mean arterial pressure (mmHg); SVCI, systemic vascular conductance index (dyne s cm^{-5} $m^2)^{-1}$; HR, heart rate (beats min^{-1}); CI, cardiac index ($l\ min^{-1}\ m^{-2}$); SI, stroke index ($ml\ m^{-2}$); CBF, coronary blood flow ($ml\ min^{-1}$); CVC, coronary vascular conductance (dyne s cm^{-5} $m^2)^{-1}$; LVEDP, left ventricular end diastolic pressure (mmHg); CVP, central venous pressure (mmHg); PAP, mean pulmonary artery pressure (mmHg); PVRI, pulmonary vascular resistance index (dyne s cm^{-5} m^2); LVSWI, left ventricular stroke work index (g-m m^{-2} $beat^{-1}$); RVSWI, right ventricular stroke work index (g-m m^{-2} $beat^{-1}$); RPP, rate pressure product (mmHg beats min^{-1}); AV_i, atrio-ventricular nodal conduction (PR) interval (ms). *P* values were determined by analysis of variance for repeated measures.

in PVCI. CVP was unchanged. Calculation of indices of myocardial work (Table 1) showed that rate-pressure product (RPP) and right ventricular stroke work (RVSWI) were elevated by AMP whereas left ventricular stroke work (LVSWI) was reduced by AMP.

Haemodynamic effects of AMP after cardiac autonomic blockade

Relative to baseline values (Table 1), cardiac autonomic blockade with propranolol and atropine significantly increased HR (to 154 ± 9, $P<0.001$) and altered the other cardiovascular parameters that are HR-dependent; namely, it increased CI (to 3.6 ± 0.4, $P<0.01$) and RPP (to 21.5 ± 1.3, $P<0.001$) and depressed AV_i (to 115 ± 7, $P<0.01$) and SI (to 23 ± 3, $P<0.0001$). There were no alterations in the other baseline values. The graded doses of AMP, administered during cardiac autonomic blockade, reduced MAP ($P<0.0001$) and increased SVCI ($P<0.0001$). Although HR was slightly depressed ($P<0.05$) by AMP, CI was increased ($P<0.001$) due to a significant increase in SI of about 60% ($P<0.0001$). While coronary blood flow was elevated ($P<0.05$) by up to about 2 fold, CVC was increased ($P<0.0001$) by up to 4.5 fold to a value similar to that in unblocked dogs. Myocardial work, as assessed by RPP was reduced ($P<0.001$) by AMP, while RVSWI was increased ($P<0.001$) and LVSWI remained unchanged ($P<0.05$).

Haemodynamic effects of Ado analogues in control animals

Each of the Ado analogues, CHA, PAA and NECA decreased MAP (Figure 1). As an index of their relative hypotensive potency, doses that caused a 20 mmHg decrease in MAP (ED_{-20}) were calculated. Geometric mean (with 95% confidence limits) were as follows ($nmol\ kg^{-1}$); CHA, 270 (90 to 810), NECA, 7.7 (1.9 to 31); PAA, 6.6 (1.3 to 33) indicating an order of hypotensive effectiveness of PAA = NECA 35× > CHA. All agonists increased SVCI (Figure 1); mean ED_{+300} doses were CHA, 630 (380 to 1030), NECA, 7.1 (4.1 to 12) and PAA, 22 (11 to 42) which indicates an order of effectiveness as systemic vasodilators of NECA 3.1× > PAA 29× > CHA. HR (Figure 2) was elevated by each of the agonists with an order of potency that was similar to that for the changes in MAP; ED_{+50} doses ($nmol\ kg^{-1}$) were CHA, 770 (545 to 1080), NECA, 5.1 (2.3 to 11) and PAA 11 (6.2 to 20). CI (Figure 2) was also increased but SI was unchanged.

Coronary blood flow was increased by each of the Ado analogues (Figure 3) due to increases in CVC. Doses ($nmol\ kg^{-1}$) causing increases in CVC of 10 units (ED_{+10}) were as follows; CHA 290 (160 to 540), NECA 4.2 (2.7 to 6.4), PAA 18 (7.6 to 42) yielding an order of effectiveness of NECA 4× > PAA 22× > CHA. NECA had a marked biphasic effect on coronary blood flow with higher doses tending to cause smaller alterations. This was probably due to marked reductions in MAP, and hence coronary perfusion pressure, with the higher doses; no biphasic responses were observed in the changes in CVC (Figure 3). CHA, NECA and PAA also produced dose-dependent increases in AV nodal excitation, as indicated by reductions in AV_i (Figure 4).

Haemodynamic effects of Ado analogues after cardiac autonomic blockade

After cardiac autonomic blockade, the Ado analogues, CHA and PAA still caused marked decreases in MAP. ED_{-20} values ($nmol\ kg^{-1}$) for CHA and PAA were 79 (46 to 140) and 2.4 (0.5 to 10), respectively. The hypotensive potency ratio of PAA and CHA (33 fold) was similar to that in the unblocked condition (Figure 1). Also, their potency ratio (21 fold) for increases in SVCI (ED_{+300} values for CHA and PAA were 500 (300 to 820) and 23 (12 to 46) $nmol\ kg^{-1}$, respectively) was similar to that in the unblocked condition (Figure 1). The increases in HR and CI, that accompanied drug-induced hypotension in control animals, were absent following cardiac autonomic blockade (Figure 2). At the highest dose of CHA studied, maximal increases in SVCI were demonstrable that were accompanied by only small decreases in HR. Both CHA and PAA increased coronary blood flow (Figure 3) and the changes in CVC (ED_{+10} values of 234 (208 to 263) and 5.7 (4.3 to 7.5) $nmol\ kg^{-1}$, respectively) were similar to those in control animals (Figure 3). No

changes in AV_i, were demonstrable with the doses of CHA or PAA used in this study (Figure 4).

Comparison of the effects of Ado analogues at equi-effective vasodilator doses

As a further probe of the selectivity of these Ado analogues *in vivo*, changes in HR and AV_i (presumed A_1-mediated events) were compared at equieffective vasodilator (a presumed A_2-mediated event) dosages (Figure 5). Reflex tachycardia, that was observed with each of the analogues in control animals, was greatest with PAA. After cardiac autonomic blockade, no consistent alterations in HR occurred. No differences between the effects of CHA and PAA on AV_i were seen; with both agents (reflex) reductions in AV_i were observed in control animals, but following cardiac autonomic blockade AV_i remained constant.

The relative selectivity between effects on the coronary and

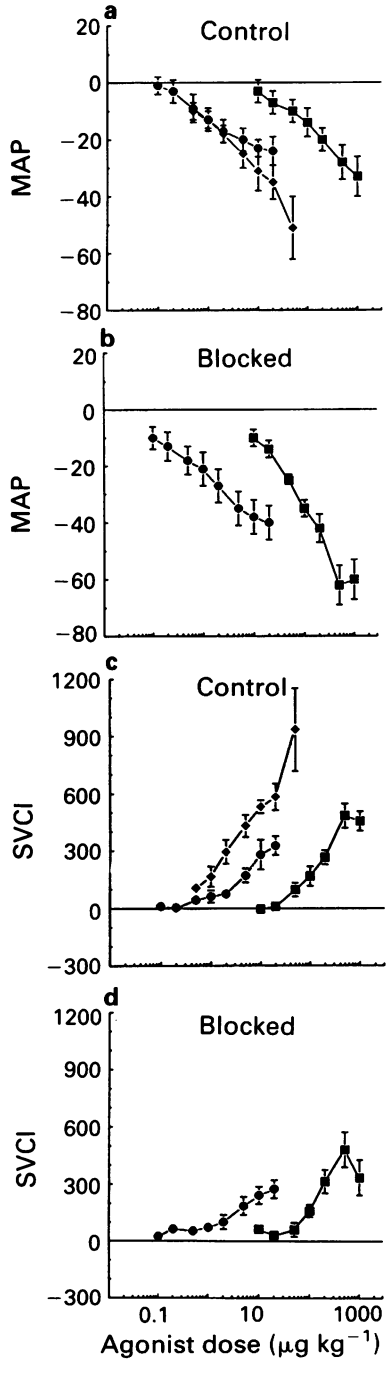

Figure 1 Dose-response relationships for the effects of adenosine (Ado) receptor agonists on mean arterial pressure (mmHg, a,b) and systemic vascular conductance index (conductance units, CU, (dyne s cm^{-5} $m^{-2})^{-1}$, c,d) in control animals (a,c) or following cardiac autonomic blockade (b,d). Graded doses (ordinates; μg kg^{-1}, log scale) of N^6-cyclohexyladenosine (■), 2-phenylaminoadenosine (●) and 5'N-ethylcarboxamidoadenosine (◆) were administered intravenously and responses recorded after 10 min. Values are mean changes from baseline ± s.e.(vertical bars), $n > 5$ observations.

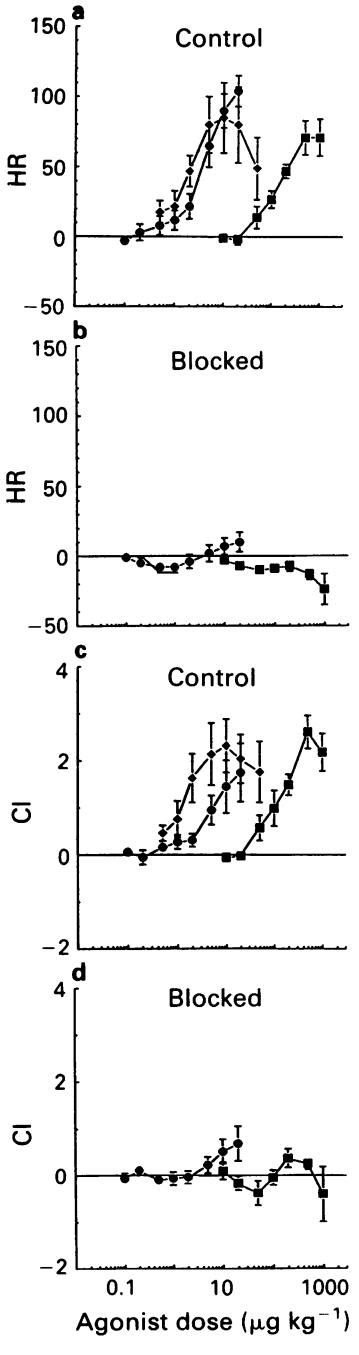

Figure 2 Dose-response relationships for the effects of adenosine (Ado) receptor agonists on heart rate (beats, min^{-1}, a,b) and cardiac index (1 min^{-1} m^{-2}, c,d) in control animals (a,c) or following cardiac autonomic blockade (b,d). Graded doses (ordinates: μg kg^{-1}, log scale) of N^6-cyclohexyladenosine (■), 2-phenylaminoadenosine (●) and 5'N-ethylcarboxamidoadenosine (◆) were administered intravenously and responses recorded after 10 min. Values are mean changes from baseline ± s.e.(vertical bars), $n > 5$ observations.

systemic vasculature were assessed by plotting agonist-induced increases in CVC against increases in SVCI (Figure 6). The resulting relationships were identical for CHA, NECA and PAA in control animals and for CHA and PAA in animals following cardiac autonomic blockade. Consequently, preferential coronary, relative to systemic, vasodilator activity of either of these Ado receptor agonists was not apparent.

Effects of AMP and Ado analogues on myocardial contractility (E_{max})

In order to assess the direct effects of AMP and the Ado receptor agonists on myocardial contractility, independent of changes in HR and cardiac loading, E_{max} values were estimated from LV pressure-dimension loops (Table 2) following equieffective vasodilator doses. AMP significantly in-

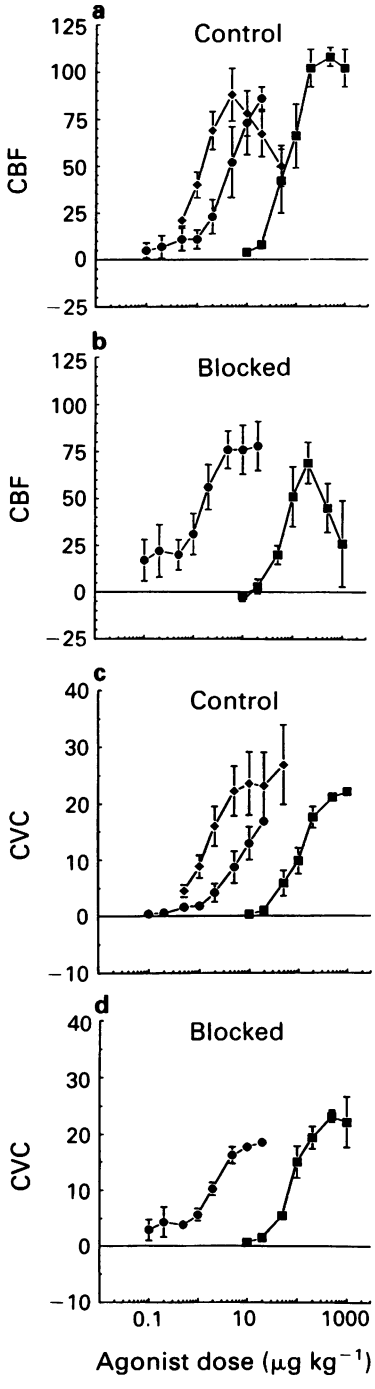

Figure 3 Dose-response relationships for the effects of adenosine (Ado) receptor agonists on coronary artery blood flow (ml min^{-1}, a,b) and coronary vascular conductance (conductance units, CU, (dyne s cm^{-5} m^{-2})$^{-1}$, c,d) in control animals (a,c) or following cardiac autonomic blockade (b,d). Graded doses (ordinates: μg kg^{-1}, log scale) of N^6-cyclohexyladenosine (■), 2-phenylaminoadenosine (●) and 5'N-ethylcarboxamidoadenosine (◆) were administered intravenously and responses recorded after 10 min. Values are mean changes from baseline ± s.e.(vertical bars), $n > 4$ observations.

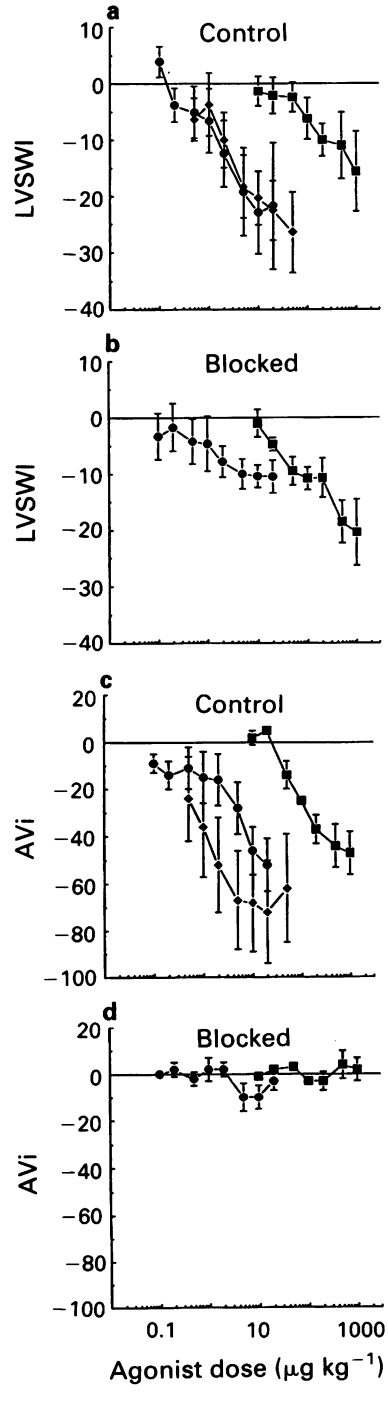

Figure 4 Dose-response relationships for the effects of adenosine (Ado) receptor agonists on LV stroke work index (g-m beat^{-1}, a,b) and AV_i (paced PR interval, ms, c,d) in control animals (a,c) or following cardiac autonomic blockade (b,d). Graded doses (ordinates: μg kg^{-1}, log scale) of N^6-cyclohexyladenosine (■), 2-phenylaminoadenosine (●) and 5'N-ethylcarboxamidoadenosine (◆) were administered intravenously and responses recorded after 10 min. Values are mean changes from baseline ± s.e.(vertical lines), $n > 5$ observations.

creased E_{max}, but no significant changes were demonstrable with the low numbers of animals included in the CHA, NECA and PAA groups. Following cardiac autonomic blockade, PAA significantly increased this index of myocardial contractility whereas the other agents had no effect.

Discussion

We found that the predominant action of AMP and the three Ado receptor agonists (CHA, PAA, and NECA) was systemic and coronary vasodilatation. The reduction in MAP elicited reflex increases in HR, CI and AV nodal conduction velocity. Even after cardiac autonomic blockade with atropine and propranolol, and when reflex activation of the heart was eliminated, still only minor differences between CHA and PAA were apparent. CHA is an effective vasodilator, but less potent that PAA or NECA, and each agonist elicited similar cardiac effects at equieffective vasodilator dosages. N^6-[(R)-1-methyl-2-phenylethyl]adenosine (R-PIA) elicited responses that were similar to CHA ($n = 3$, data not shown). Thus, despite marked selectivity of these agonists in *in vitro* assays, no obvious differences in their cardiovascular selectivity were demonstrable *in vivo*.

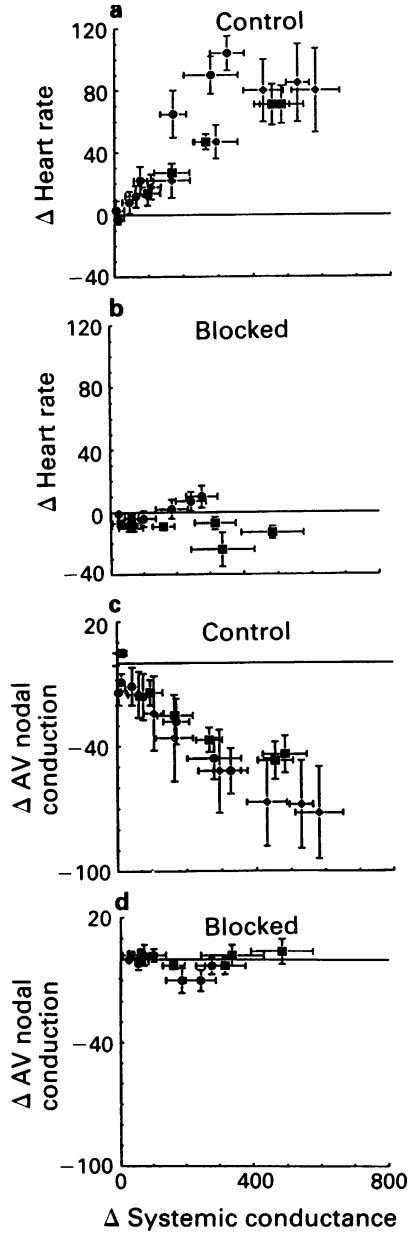

Figure 5 Relationships between drug-induced changes in heart rate (beats min^{-1}, a,b) and AV_i (paced PR interval, ms, c,d) and changes in systemic vascular conductance index (conductance units, CU, $(dyne\ s\ cm^{-5}\ m^{-2})^{-1}$) in control animals (a,c) or following cardiac autonomic blockade (b,d). Graded doses of N^6-cyclohexyladenosine (■), 2-phenylaminoadenosine (●) and 5'N-ethylcarboxamidoadenosine (◆) were administered intravenously and responses recorded after 10 min. Values are mean ± s.e., $n = 5$ subjects.

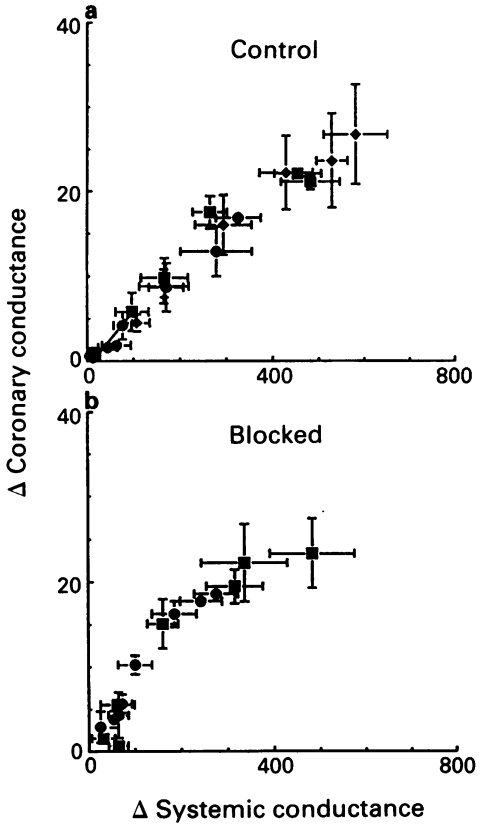

Figure 6 Relationships between drug-induced changes in coronary vascular conductance and changes in systemic vascular conductance index (conductance units, CU, $(dyne\ s\ cm^{-5}\ m^{-2})^{-1}$) in control animals (a) or following cardiac autonomic blockade (b). Graded doses of N^6-cyclohexyladenosine (■), 2-phenylaminoadenosine (●) and 5'N-ethylcarboxamidoadenosine (◆) were administered intravenously and responses recorded after 10 min. Values are mean ± s.e., $n = 5$ subjects.

Table 2 Effect of equieffective vasodilator doses of adenosine 5'-monophosphate (AMP) and adenosine (Ado) analogues on myocardial contractility (E_{max}, $mmHg\ mm^{-1}$) in anaesthetized dogs in the absence (control) or presence of cardiac autonomic blockade

	Control	*Blocked*
Baseline	32 ± 3 (15)	45 ± 6 (8)
AMP	*50 ± 5 (15)	49 ± 8 (6)
CHA	49 ± (2)	39 ± 6 (4)
NECA	39 ± 8 (3)	ND
PAA	65 ± 15 (3)	*95 ± 14 (4)

Values are shown for equieffective systemic vasodilating doses of AMP ($400\ \mu g\ kg^{-1}\ min^{-1}$), N^6-cyclohexyladenosine (CHA, $200\ \mu g\ kg^{-1}$), 5'-N-ethylcarboxamideadenosine (NECA, $2\ \mu g\ kg^{-1}$) and 2-phenylaminoadenosine (PAA, $10\ \mu g\ kg^{-1}$) and are means ± s.e. (*n* observations). Significant differences from baseline are indicated by *. ND indicates not done.

Table 3 Relative potencies of N^6-cyclohexyladenosine (CHA), 5'-N-ethylcarboxamidoadenosine (NECA) and 2-phenylaminoadenosine (PAA) *in vitro* and *in vivo*

	CHA	*NECA*	*PAA*
In vitro (nM) data from rat striatal membranes (Bruns *et al.*,1986)			
A_1 affinity	1.3 (1)	6.3 (0.21)	560 (0.002)
A_2 affinity	510 (1)	10 (51)	120 (4.3)
A_2/A_1 selectivity	390	1.6	0.21
In vivo (nmol kg^{-1})		*Control*	
MAP (ED_{-20})	270 (1)	7.7 (35)	6.6 (41)
SVCI (ED_{+300})	625 (1)	7.1 (88)	22 (28)
CVC (ED_{+10})	293 (1)	4.2 (70)	18 (16)
In vivo (nmol kg^{-1})		*Cardiac Autonomic Blockade*	
MAP (ED_{-20})	80 (1)	—	2.4 (34)
SVCI (ED_{+300})	500 (1)	—	23 (21)
CVC (ED_{+10})	234 (1)	—	5.7 (41)

For *in vivo* data, doses of agonist (nmol kg^{-1}) that elicited decreases in MAP of 20 mmHg (ED_{-20}) and increases in SVCI of 300 units (ED_{+300}) and CVC of 10 units (ED_{+10}) were determined from individual dose-response curves for each animal. Values in Table represent geometric means from 5 experiments. Values in parentheses indicate potency relative to CHA. For abbreviations, see footnote to Table 1.

Definitive classification of Ado receptors in cardiovascular tissues has not yet been accomplished due to the relatively small population of receptors in the various parts of the heart and the lack of high affinity and selective Ado receptor antagonists. Furthermore, determination of the functional effects of these receptor subtypes has been difficult due to the often contrasting results obtained with the use of different experimental preparations. Nevertheless, current attempts revolve around the use of the presently available selective A_1 and A_2 agonists. Studies using receptor binding methodology (e.g., Bruns *et al.*, 1986) have shown that many of the N^6-substituted Ado analogues are A_1 selective, such as CHA (A_1/A_2 potency ratio: 390) which we used to determine the functional effects of A_1 receptor agonists. To study the effects of A_2 receptor activation, we used the A_2 selective compound, PAA (CV-1808, A_1/A_2 potency ratio: 0.2), which is of particular interest since there is some evidence that it possesses coronary selectivity (Kawazoe *et al.*, 1980). The potent, but non-selective 5'-substituted analogue NECA (Bruns *et al.*, 1986) was used to elucidate the cardiovascular effects arising from simultaneous A_1 and A_2 receptor activation and to compare its effects to those of its parent compound Ado. Ado was administered as the soluble prodrug, AMP, which is rapidly dephosphorylated to Ado by 5'-nucleotidase located on the plasma membrane of many cells, including vascular endothelial cells (Pelleg *et al.*, 1987; Coade & Pearson, 1989).

Numerous studies have shown Ado-induced reductions in both the rate and force of cardiac contractions (Evans *et al.*, 1982), depression of A-V nodal conduction velocity (DiMarco *et al.*, 1983) and inhibition of atrial and ventricular automaticity (Szentmiklosi *et al.*, 1980; Wainwright & Parratt, 1988). These effects are mediated by the A_1 subclass of Ado receptors and are easily demonstrable with A_1-selective agonists such as CHA (Haleen & Evans, 1985; Hamilton *et al.*, 1987; Oei *et al.*, 1988) and R-PIA (Caparrotta *et al.*, 1987; Jahnel & Nawrath, 1989; Shryock *et al.*, 1989). A_1-mediated responses, including negative chronotropic and dromotropic actions as well as cardioprotection, are also apparent in canine hearts, although some evidence suggests that dogs possess an unusual form of A_1 receptor. Consequently it was surprising that in this study, an effect of each of theses agonists was an apparent cardiac excitation. This was mediated by baroreceptor reflex activation in response to drug-induced hypotension, for in the presence of cardiac autonomic blockade, cardiac excitation was absent. Nevertheless, even under conditions of autonomic blockade, marked cardiac depression was not observed, even with the supposedly A_1 selective agonist, CHA, at doses that caused maximal systemic vasodilatation. This absence of A_1 receptor responses may be due to a lower A_1 receptor density and/or less efficient transduction mechanisms relative to A_2 receptor systems.

A-V nodal blockade can be easily demonstrated in dogs (Belhassen & Pelleg, 1984) and man (DiMarco *et al.*, 1983) with bolus dosing protocols (high cardiac and lower systemic concentrations of agonist). In this study, the slower drug administration and the measurement of responses under equilibrium conditions 10 min after drug administration, probably contributed to the observed lack of significant cardiac depression. The drug evaluation procedure used here, although subject to potential differences in the tissue partitioning of each agonist, is more appropriate than bolus dosing for the assessment of receptor selectivity among different cardiovascular effector systems.

A second factor that may have contributed to the observed lack of cardiac depression was the drug combination used for anaesthesia. Our animals were anaesthetized with fentanyl and pentobarbitone and had a basal HR of 89 beats min^{-1}, a value close to that in conscious dogs. Most of the *in vivo* studies that have demonstrated Ado receptor-mediated depression of cardiac rate and/or contractility have usually been in animals anaesthetized with pentobarbitone alone. In such canine preparations, basal HR is approximately 150 beats min^{-1}, indicating an autonomic imbalance (consisting of a relative sympathetic excitation) relative to the conscious state (Belloni *et al.*, 1989b). Indeed, in conscious man (Biaggioni *et al.*, 1987) and rabbits (Chen & Clanachan, unpublished) intravenous Ado causes tachycardia. The involvement of an intact baroreceptor reflex response may even give the appearance of a selective Ado-induced tachycardia (Fuller *et al.*, 1987) because increased efferent sympathetic activity, that attempts to increase HR, systemic vascular resistance and cardiac output, may be able to compensate adequately for concomitant Ado-induced systemic vasodilatation and hypotension.

In this study, AMP-induced hypotension elicited reflex increases in myocardial contractility in normal anaesthetized animals. After autonomic blockade, E_{max} was unaltered by AMP and by CHA. PAA, however, appeared to stimulate myocardial contractility as it significantly increased E_{max}. The

mechanism for this positive inotropic action was not investigated in this study but may have been related to 'Greggs phenomenon', where increased coronary blood flow enhances LV contractile function (Goto *et al.*, 1991). If this were the case, CHA and AMP, that also caused similar increases in coronary blood flow, would also be expected to increase E_{max}. The lack of any such increase in E_{max} with these agonists is consistent with a mild direct negative inotropic action that was compensated for by the Gregg effect. An alternate explanation for the PAA-induced increase in E_{max} is that it arose from stimulation of A_2 receptors that have been shown to be present on myocytes (Behnke *et al.*, 1990) and that may be capable of eliciting small positive inotropic responses (Bruckner *et al.*, 1985).

Comparison of the relative potencies of the agonists used in this study (Table 3) shows that, for A_2-mediated responses *in vivo*, e.g., vasodilatation, NECA had a potency ratio to CHA (ranging from 38 fold for CVC to 88 fold for SVRI) that was similar to that observed in *in vitro* binding assays (approximately 51 fold). However, for PAA, significantly greater potency ratios relative to CHA were found *in vivo* (ranging from 13 to 29 for CVC and SVCI, respectively) than that reported previously based on *in vitro* assays (4 to 5). This apparent greater A_2-selectivity *in vivo* may be related to additional activities of this substance such as nucleoside transport (NT) inhibition (Chiba & Watanabe, 1983). Other studies have demonstrated that PAA has an affinity for NT inhibitory sites in endothelial cells (K_i value of about 200 nM) that is comparable to its affinity for A_2 receptors (Clanachan, unpublished).

An equivalent comparison of relative potencies of these agonists for A_1 receptors *in vivo* and *in vitro* cannot be made because no clear A_1 responses were demonstrable *in vivo*.

Some Ado agonists, e.g., PAA, have been reported to exert coronary-selective vasodilatation (Kawazoe *et al.*, 1980). Comparison of changes in CVC with changes in SVCI showed these relationships to be identical for PAA, CHA and NECA, either before, or after cardiac autonomic blockade. Thus, using experimental conditions that avoid pharmacokinetic conditions favouring cardiac and coronary responses, no coronary selectivity was observed.

Our research shows that both systemic and coronary vasodilatation without A-V blockade and direct depression of LV contractility (E_{max}) is demonstrable when agonists are administered slowly and allowed to equilibrate among cardio-vascular effector tissues. These results are of importance in the design and evaluation of new Ado agonists for cardiovascular indications. Sufficient Ado receptor-mediated systemic vasodilatation and afterload reduction to reduce cardiac workload and O_2 consumption, in combination with direct A_1 receptor-mediated cardioprotection (Liu *et al.*, 1991), should be obtainable without adverse negative inotropic and dromotropic effects.

While classification of Ado receptor subtypes can be performed in isolated tissues or with binding affinities *in vitro*, functional selectivity of Ado agonists *in vivo* cannot be predicted easily because of several interfering mechanisms, including compensation by autonomic reflexes, relative density of receptors, drug inactivation rates and efficiency of transduction mechanisms.

Supported by M.R.C. (Canada). R.Z.G. thanks the Pharmaceutical Manufacturers Association of Canada for a Summer Studentship.

References

BABBITT, D.G., VIRMANI, R. & FORMAN, M.B. (1989). Intracoronary adenosine administered after reperfusion limits vascular injury after prolonged ischemia in the canine model. *Circulation*, **80**, 1388–1399.

BEHNKE, N., MULLER, W., NEUMANN, J., SCHMITZ, W., SCHOLZ, H. & STEIN, B. (1990). Differential antagonism by 1,3-dipropylxanthine-8-cyclopentylxanthine and 9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo(1,5-c)quinazoli n-5-imine of the effects of adenosine derivatives in the presence of isoprenaline on contractile response and cyclic AMP content in cardiomyocytes. Evidence for the coexistence of A1- and A2-adenosine receptors on cardiomyocytes. *J. Pharmacol. Exp. Ther.*, **254**, 1017–1023.

BELARDINELLI, L., LINDEN, J. & BERNE, R.M. (1989). The cardiac effects of adenosine. *Prog. Cardiovasc. Dis.*, **32**, 73–97.

BELHASSEN, B. & PELLEG, A. (1984). Electrophysiologic effects of adenosine triphosphate and adenosine on the mammalian heart: clinical and experimental aspects. *J. Am. Coll. Cardiol.*, **4**, 414–424.

BELLONI, F.L., BELARDINELLI, L., HALPERIN, C. & HINTZE, T.H. (1989a). An unusual receptor mediates adenosine-induced SA nodal bradycardia in dogs. *Am. J. Physiol.*, **256**, H1553-H1564.

BELLONI, F.L., BROWN, I. & HINTZE, T.H. (1989b). Mechanism of the apparent parasympathetic inhibition of adenosine induced heart rate slowing in the dog. *Cardiovasc. Res.*, **23**, 239–248.

BERNE, R.M. (1980). The role of adenosine in the regulation of coronary blood flow. *Circ. Res.*, **47**, 807–813.

BIAGGIONI, I., OLAFSSON, B., ROBERTSON, R.M., HOLLISTER, A.S. & ROBERTSON, D. (1987). Cardiovascular and respiratory effects of adenosine in conscious man. Evidence for chemoreceptor activation. *Circ. Res.*, **61**, 779–786.

BRUCKNER, R., FENNER, A., MEYER, W., NOBIS, T.M., SCHMITZ, W. & SCHOLZ, H. (1985). Cardiac effects of adenosine and adenosine analogs in guinea-pig atrial and ventricular preparations: evidence against a role of cyclic AMP and cyclic GMP. *J. Pharmacol. Exp. Ther.*, **234**, 766–774.

BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1986). Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.*, **29**, 331–346.

CAPARROTTA, L., FASSINA, G., FROLDI, G. & POJA, R. (1987). Antagonism between (−)-N^6-phenylisopropyladenosine and the calcium channel facilitator Bay K 8644, on guinea-pig isolated atria. *Br. J. Pharmacol.*, **90**, 23–30.

CHIBA, S. & WATANABE, H. (1983). Potentiation of the negative chronotropic and inotropic effects of adenosine by 2-phenylaminoadenosine. *Clin. Exp. Pharmacol. Physiol.*, **10**, 1–5.

COADE, S.B. & PEARSON, J.D. (1989). Metabolism of adenine nucleotides in human blood. *Circ. Res.*, **65**, 531–537.

COLLIS, M.G. (1989). The vasodilator role of adenosine. *Pharmacol. Ther.*, **41**, 143–162.

CRONSTEIN, B.N., ROSENSTEIN, E.D., KRAMER, S.B., WEISSMANN, G. & HIRSCHHORN, R. (1985). Adenosine; a physiologic modulator of superoxide anion generation by human neutrophils. Adenosine acts via an A2 receptor on human neutrophils. *J. Immunol.*, **135**, 1366–1371.

DIMARCO, J.P., SELLERS, T.D., BERNE, R.M., WEST, G.A. & BELARDINELLI, L. (1983). Adenosine: electrophysiologic effects and therapeutic use for terminating paroxysmal supraventricular tachycardia. *Circulation*, **68**, 1254–1263.

EVANS, D.B., SCHENDEN, J.A. & BRISTOL, J.A. (1982). Adenosine receptors mediating cardiac depression. *Life Sci.*, **31**, 2425–2432.

FINEGAN, B.A., CHEN, H.J., SINGH, Y.N. & CLANACHAN, A.S. (1990). Comparison of hemodynamic changes induced by adenosine monophosphate and sodium nitroprusside alone and during dopamine infusion in the anesthetized dog. *Anesth. Analg.*, **70**, 44–52.

FINEGAN, B.A. & CLANACHAN, A.S. (1991). Comparison of the haemodynamic effects of adenosine monophosphate with sodium nitroprusside in a canine model of acute left ventricular dysfunction. *Br. J. Pharmacol.*, **103**, 1691–1696.

FINEGAN, B.A., CLANACHAN, A.S., COULSON, C.S. & LOPASCHUK, G.D. (1992). Adenosine modification of energy substrate use in isolated hearts perfused with fatty acids. *Am. J. Physiol.* (in press).

FULLER, R.W., MAXWELL, D.L., CONRADSON, T.B., DIXON, C.M. & BARNES, P.J. (1987). Circulatory and respiratory effects of infused adenosine in conscious man. *Br. J. Clin. Pharmacol.*, **24**, 306–317.

GOTO, Y., SLINKER, B.K. & LEWINTER, M.M. (1991). Effect of coronary hyperemia on Emax and oxygen consumption in blood-perfused rabbit hearts. Energetic consequences of Gregg's phenomenon. *Circ. Res.*, **68**, 482–492.

HALEEN, S.J. & EVANS, D.B. (1985). Selective effects of adenosine receptor agonists upon coronary resistance and heart rate in isolated working rabbit hearts. *Life Sci.*, **36**, 127–137.

HAMILTON, H.W., TAYLOR, M.D., STEFFEN, R.P., HALEEN, S.J. & BRUNS, R.F. (1987). Correlation of adenosine receptor affinities and cardiovascular activity. *Life Sci.*, **41**, 2295–2302.

HEADRICK, J.P. & WILLIS, R.J. (1989). Endogenous adenosine improves work rate to oxygen consumption ratio in catecholamine stimulated isovolumic rat heart. *Pflügers Arch.*, **413**, 354–358.

JAHNEL, U. & NAWRATH, H. (1989). Characterization of adenosine receptors in guinea-pig isolated left atria. *Br. J. Pharmacol.*, **97**, 1182–1190.

KAWAZOE, K., MATSUMOTO, N., TANABE, M., FUJIWARA, S., YANAGIMOTO, M., HIRATA, M. & KIKUCHI, K. (1980). Coronary and cardiohemodynamic effects of 2-phenylamino-adenosine (CV-1808) in anesthetized dogs and cats. *Arzneimittelforschung*, **30**, 1083–1087.

KEEFER, J.R. & BARASH, P.G. (1985). Pulmonary artery catheterization. In *Monitoring in Anesthesia and Critical Care Medicine*. ed. Blitt, C.D. pp. 177–228. New York: Churchill Livingstone.

KING, A.D., MILAVEC-KRIZMAN, M. & MULLER-SCHWEINITZER, E. (1990). Characterization of the adenosine receptor in porcine coronary arteries. *Br. J. Pharmacol.*, **100**, 483–486.

LAGERKRANSER, M., BERGSTRAND, G., GORDON, E., IRESTEDT, L., LINDQUIST, C., STANGE, K. & SOLLEVI, A. (1989). Cerebral blood flow and metabolism during adenosine-induced hypotension in patients undergoing cerebral aneurysm surgery. *Acta Anaesthesiol. Scand.*, **33**, 15–20.

LINDEN, J. (1991). Structure and function of A_1 adenosine receptors. *FASEB J.*, **5**, 2668–2676.

LIU, G.S., THORNTON, J., VAN WINKLE, D.M., STANLEY, A.W., OLSSON, R.A. & DOWNEY, J.M. (1991). Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation*, **84**, 350–356.

NOZAWA, T., YASUMURA, Y., FUTAKI, S., TANAKA, N., UENISHI, M. & SUGA, H. (1988). Efficiency of energy transfer from pressure-volume area to external mechanical work increases with contractile state and decreases with afterload in the left ventricle of the anesthetized closed-chest dog. *Circulation*, **77**, 1116–1124.

OEI, H.H., GHAI, G.R., ZOGANAS, H.C., STONE, G.A., ZIMMERMAN, M.B., FIELD, F.P. & WILLIAMS, M. (1988). Correlation between binding affinities for brain A1 and A2 receptors of adenosine agonists and antagonists and their effects on heart rate and coronary vascular tone. *J. Pharmacol. Exp. Ther.*, **247**, 882–888.

OLAFSSON, B., FORMAN, M.B., PUETT, D.W., POU, A., CATES, C.U., FRIESINGER, G.C. & VIRMANI, R. (1987). Reduction of reperfusion injury in the canine preparation by intracoronary adenosine: importance of the endothelium and the no-reflow phenomenon. *Circulation*, **76**, 1135–1145.

PELLEG, M., MITSUOKA, T., MICHELSON, E.L. & MENDUKE, H. (1987). Adenosine mediates the negative chronotropic action of adenosine 5'-triphosphate in the canine sinus node. *J. Pharmacol. Exp. Ther.*, **242**, 791–795.

PITARYS, C.J., VIRMANI, R., VILDIBILL, H.D., Jr., JACKSON, E.K. & FORMAN, M.B. (1991). Reduction of myocardial reperfusion injury by intravenous adenosine administered during the early reperfusion period. *Circulation*, **83**, 237–247.

QUATTRIN, S., GENOVESE, A., CIRILLO, R., FORMISANO, S. & MARONE, G. (1988). Functional and biochemical evidence of a specific adenosine A2/Ra receptor on human platelets. *Ric. Clin. Lab.*, **18**, 105–118.

ROMANO, F.D., NAIMI, T.S. & DOBSON, J.G.J. (1991). Adenosine attenuation of catecholamine-enhanced contractility of rat heart in vivo. *Am. J. Physiol.*, **260**, H1635–H1639.

SHRYOCK, J., PATEL, A., BELARDINELLI, L. & LINDEN, J. (1989). Downregulation and desensitization of A1-adenosine receptors in embryonic chicken heart. *Am. J. Physiol.*, **256**, H321-H327.

SOLLEVI, A., LAGERKRANSER, M., IRESTEDT, L., GORDON, E. & LINDQUIST, C. (1984). Controlled hypotension with adenosine in cerebral aneurysm surgery. *Anesthesiology*, **61**, 400–405.

SZENTMIKLOSI, A.J., NEMETH, M., SZEGI, J., PAPP, J.G. & SZEKERES, L. (1980). Effect of adenosine on sinoatrial and ventricular automaticity of the guinea pig. *Naunyn Schmiedebergs Arch. Pharmacol.*, **311**, 147–149.

WAINWRIGHT, C.L. & PARRATT, J.R. (1988). An antiarrhythmic effect of adenosine during myocardial ischaemia and reperfusion. *Eur. J. Pharmacol.*, **145**, 183–194.

(Received February 4, 1992
Revised June 27, 1992
Accepted August 5, 1992)

Br. J. Pharmacol. (1992), **107**, 1057–1060

Stereospecific antiarrhythmic effects of naloxone against myocardial ischaemia and reperfusion in the dog

Andrew Ying-Siu Lee

Heart Research Laboratory, Department of Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan

1 The effects of both the (−)- and (+)-stereoisomers of naloxone in anaesthetized dogs with arrhythmias induced by acute coronary artery occlusion followed by reperfusion were investigated.

2 Following coronary artery occlusion and reperfusion, all dogs in the control group developed ischaemia- and reperfusion-induced cardiac arrhythmias, bradycardia and hypotension.

3 The opiate antagonist (−)-naloxone prevented the arrhythmias, bradycardia and hypotension due to myocardial ischaemia and reperfusion.

4 The (+)-stereoisomer of naloxone, which is inactive as an opiate antagonist, was without beneficial effects.

5 These results indicate a possible involvement of endogenous opioid peptides in the cardiac effects due to myocardial ischaemia and reperfusion, mediated by opiate receptors through opiate antagonism.

Keywords: Endogenous opioid peptide; naloxone; stereoisomer; cardiac arrhythmias; myocardial ischaemia and reperfusion

Introduction

It has been shown that endogenous opioid peptides (EOP) are involved in cardiac arrhythmogenesis (for a review, see Lee, 1990) and that the opiate antagonists naturally possess antiarrhythmic activity (for a review, see Lee, 1989). The pure opiate antagonist, naloxone, has been found to inhibit cardiac arrhythmias resulting from coronary artery occlusion in rats (Fagbemi *et al.*, 1982; Lee *et al.*, 1992) and dogs (Huang *et al.*, 1986), suggesting that EOP may be released from the heart upon myocardial ischaemia thus causing arrhythmias, and naloxone, by virtue of its antagonistic action against opiates, rectifies this irregular cardiac rhythm. Further studies demonstrated that the antiarrhythmic effects of opiate antagonists (Mr 1452 and WIN 44,441-3) against myocardial ischaemia were stereospecific and thus mediated by opiate receptors since their (+)-isomers, which possess no opiate antagonistic properties, were less effective (Parratt & Sitsapesan, 1986). On the other hand, it has also been reported that both (−)- and (+)-stereoisomers of naloxone were antiarrhythmic in rats subjected to intracarotid administrations of adrenaline, suggesting that the antiarrhythmic action of naloxone was probably not mediated by opiate receptors (Sarne *et al.*, 1988). To clarify the discrepancy between the results obtained from the above two experimental models (ischaemia- or adrenaline-induced arrhythmias), and to provide more compelling evidence that EOP are indeed involved in the pathophysiology of myocardial ischaemia and reperfusion, the effects of both (−)- and (+)-stereoisomers of naloxone were investigated in anaesthetized dogs with arrhythmias induced by acute coronary artery ligation followed by reperfusion.

Methods

Mongrel dogs of either sex weighing between 10 to 20 kg were used. All experiments were conducted according to the guidelines for animal experiments at Taichung Veterans General Hospital Medical Research Centre. The animals were anaesthetized with pentobarbitone sodium (25 mg kg^{-1}) administered intravenously into the lateral saphenous vein. They were intubated and artificially ventilated. Respiratory rate was synchronized with that of the dog (16–18 strokes min^{-1}; 300 ml kg^{-1} min^{-1}). The left femoral artery and vein were cannulated for the measurement of blood pressure (BP) and heart rate (HR) with a Statham pressure transducer and a Biotechnometer (Gould), and for the administration of drugs, respectively. Electrocardiograms (ECG) were recorded from lead II limb leads, using the Lifepak ECG Monitor (Physio-Control., USA).

A similar procedure for coronary artery ligation in the dog to that described by Benfey *et al.* (1984) was adopted. Median thoracotomy was performed. The heart was exposed by cutting open the pericardium. The left anterior descending coronary artery (LAD) was isolated for ligation. A silk suture with a short polyethylene tube threaded around it was placed under the LAD. The dog was then allowed to equilibrate for 20 min. Afterwards, (−)-naloxone (with opiate antagonistic properties), (+)-naloxone (without opiate antagonistic properties), or 0.9% NaCl solution (as control) were infused into the femoral vein over a period of 10 min. At 2 min after the start of infusion, the LAD was occluded by applying tension on the suture and clamping immediately above the polyethylene tubing surrounding the artery. Occlusion was maintained for 20 min followed by reperfusion by simply releasing the clamp for 30 min.

Doses of (−)-naloxone used were 0.92 and 2.75 μmol kg^{-1}, and that of (+)-naloxone was 2.75 μmol kg^{-1}. They were dissolved in 5 ml of 0.9% NaCl solution. ECG, BP and HR were continuously monitored throughout the experiment. Arrhythmias were assessed by recording the incidence and onset of ventricular premature contraction (VPC), ventricular tachycardia (VT) and ventricular fibrillation (VF). A Chi-squared test was used to analyse the difference in the incidence of arrhythmias between control and drug-treated groups. Student's *t* test was used to test the difference in the onset of arrhythmias between control and drug-treated groups. Analysis of variance was used to compare the difference in time course changes in mean arterial pressure and heart rate between control and treated groups. A P value of less than 0.05 was considered as statistically significant.

Results

Effects of stereoisomers of naloxone on cardiac arrhythmias

Table 1 summarizes the effects of the (−)- and (+)-stereoisomers of naloxone on cardiac rhythm following coronary artery occlusion and reperfusion in the dog. Myocar-

Table 1 Effects of stereoisomers of naloxone on cardiac arrhythmias during coronary occlusion and reperfusion

	Occlusion (20 min)							*Reperfusion* (30 min)						
			VPC onset		*VT* onset		*VF* onset		*VPC* onset		*VT* onset		*VF* onset	
	N	*n*	(min)	*n*	(min)	*n*	(min)	*n*	(min)	*n*	(min)	*n*	(min)
Control	8	8	1.69 ± 0.42	4	6.50 ± 3.84	3	9.00 ± 5.00	8	1.69 ± 0.53	4	1.13 ± 0.31	5	1.20 ± 0.46
(−)-Naloxone (0.92 μmol kg^{-1})	8	7	3.57 ± 0.72*	1	2.00	1	4.00	5	2.60 ± 0.81	1	1.00	2	3.00 ± 1.00
(−)-Naloxone (2.75 μmol kg^{-1})	8	7	7.00 ± 2.43	0*		0		6	2.50 ± 0.56	0*		0*	
(+)-Naloxone (2.75 μmol kg^{-1})	8	8	1.13 ± 0.21	4	4.25 ± 0.85	1	9.00	8	1.31 ± 0.33	3	1.33 ± 0.33	3	1.88 ± 1.05

N and *n* represent the number of animals; VPC – ventricular permature contraction; VT – ventricular tachycardia; VF – ventricular fibrillation.
Statistical difference from the corresponding control values at the levels of $*P<0.05$ by chi-squared test, and $*P<0.05$ by Student's *t* test.

dial ischaemia and reperfusion invariably caused malignant ventricular arrhythmias including VPC, VT and VF. In agreement with previous findings (Penny & Sheridan, 1983; Zhan *et al.*, 1986), arrhythmias were more frequent and severe during the reperfusion period. Of 8 dogs in the control group, 8 showed VPC, 4 VT and 3 VF during ischaemia and 8 showed VPC, 4 VT and 5 VF during the reperfusion period. The onset of arrhythmias for VPC, VT and VF were 1.69, 6.50 and 9.00 min during ischaemia and 1.69, 1.13 and 1.20 min during the reperfusion periods, respectively. Pretreatment with (−)-naloxone significantly reduced the incidence and delayed the onset of arrhythmias in both the ischaemia and reperfusion periods in a dose-related manner. Of 8 dogs receiving 0.92 μmol kg^{-1} (−)-naloxone, 7 showed VPC, 1 VT and 1 VF during ischaemia and 5 showed VPC, 1 VT and 2 VF during the reperfusion period. The onset of arrhythmias for VPC, VT and VF were 3.57, 2.00 and 4.00 min during ischaemia and 2.60, 1.00 and 3.00 min during the reperfusion periods, respectively. Moreover, of 8 dogs receiving 2.75 μmol kg^{-1} (−)-naloxone, 7 showed VPC during ischaemia and 6 showed VPC during the reperfusion periods whilst no dog developed VT or VF. The onset of arrhythmias for VPC were 7.00 min during ischaemia and 2.5 min during reperfusion, respectively. Pretreatment with (+)-naloxone (2.75 μmol kg^{-1}), however, was without beneficial effects in preventing the ischaemia- or reperfusion-induced arrhythmias. Of 8 dogs, 8 showed VPC, 4 VT and 1 VF during ischaemia and 8 showed VPC, 3 VT and 3 VF during the reperfusion periods. The onset of arrhythmias for VPC, VT and VF were 1.13, 4.25 and 9.00 min during ischaemia and 1.31, 1.33 and 1.88 min during reperfusion, respectively. These differences were not statistically significant compared to the control group.

Effects of stereoisomers of naloxone on blood pressure and heart rate

In the doses used in this study, both stereoisomers of naloxone had no significant effects on BP or HR. The BPs in the control group before and after injection of saline were 99 ± 7 and 98 ± 8 mmHg respectively. while the corresponding values in the groups treated with 2.7 μmol kg^{-1} of (−)-naloxone were 93 ± 9 and 98 ± 9 mmHg, and with (+)-naloxone were 99 ± 7 and 102 ± 6 mmHg. Similarly the HRs in the control group before and after administration of saline were 189 ± 12 and 188 ± 13 beats min^{-1} respectively, whereas the corresponding values in the groups treated with 2.75 μmol kg^{-1} (−)-naloxone were 189 ± 2 and 193 ± 9 beats min^{-1}, and with (+)-naloxone were 186 ± 9 and 185 ± 8 beats min^{-1}.

The effects of the (−)- and (+)-stereoisomers of naloxone on the BP and HR following coronary artery occlusion and reperfusion in the dog are shown in Figures 1 and 2. Myocardial ischaemia and reperfusion invariably caused a marked decrease in both BP and HR. Pretreatment with (−)-naloxone significantly prevented the reduction in BP and HR during both the ischaemia and reperfusion periods in a dose-related manner. Pretreatment with (+)-naloxone, however, was without beneficial effects in reversing the ischaemia- or reperfusion-induced hypotension and bradycardia.

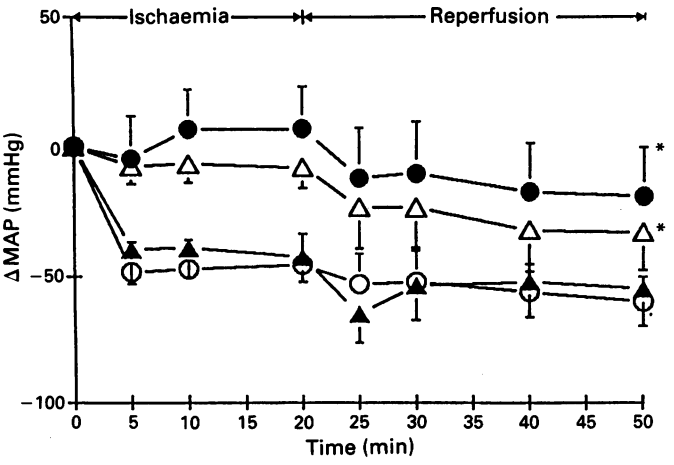

Figure 1 Effects of the stereoisomers of naloxone on the change in mean arterial pressure (Δ MAP) following coronary artery occlusion and reperfusion in the dog: (○) saline; (△) (−)-naloxone 0.92 μmol kg^{-1}; (●) (−)-naloxone 2.75 μmol kg^{-1}; (▲) (+)-naloxone 2.75 μmol kg^{-1}. Values are means and s.e.mean (vertical bars) of eight animals. $*P<0.05$ vs control by analysis of variance.

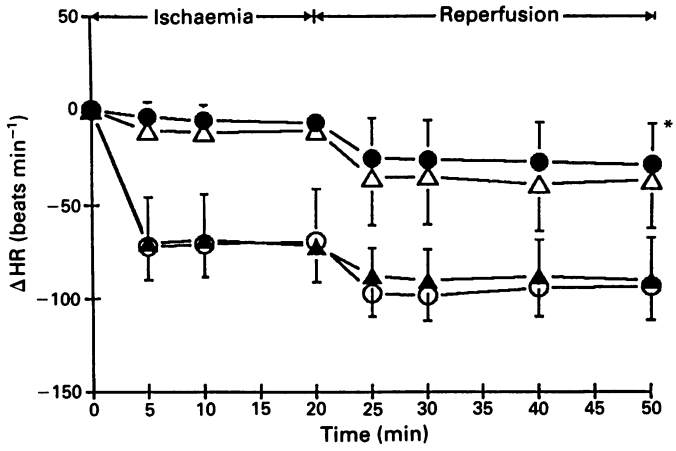

Figure 2 Effects of the stereoisomers of naloxone on the change in heart rate (Δ HR) in beats min^{-1} following coronary artery occlusion and reperfusion in the dog. For key to symbols see legend to Figure 1.

Discussion

It is well-known that coronary artery occlusion and reperfusion can lead to cardiogenic shock, bradycardia and arrhythmias, all of which may be fatal complications secondary to acute myocardial infarction. Similar observations were made in the present study in the dog in which coronary artery occlusion and reperfusion soon led to a marked reduction in arterial blood pressure, bradycardia and malignant ventricular arrhythmias. Sinus bradycardia commonly occurs during the early phases of acute myocardial infarction, secondary to bradyarrhythmias or because of extensive damage to the heart with destruction of the conduction pathway. The post-occlusion decreases in arterial blood pressure in control and (+)-naloxone-treated animals were very marked. This may be due to extensive ischaemic injury of the heart, as blood pressure and heart rate (rather high) are within normal ranges (Bolton, 1975) in the pre-occlusion period. Moreover, adequate anaesthesia was maintained during the entire experimental period, using the same dose of pentobarbitone sodium (25 mg kg^{-1}, i.v.) as used in previous studies (Huang *et al.*, 1986; Sakamoto *et al.*, 1989). In addition, the absence of purposeful movement and no tachycardia or pressor responses to leg pinch or pinprick indicated that adequate anaesthesia was maintained throughout the experiment.

In this study, (−)-naloxone prevented the hypotension, bradycardia, ischaemic and reperfusion arrhythmias due to coronary artery occlusion and reperfusion in the dog. These results are in agreement with those obtained in the rat (Fagbemi *et al.*, 1982; Lee *et al.*, 1992) and by Huang *et al.* (1986) in the dog, subjected to myocardial ischaemia and reperfusion. It is of interest to note that the doses of naloxone that produced antiarrhythmic effects in rats and dogs were of a similar order of magnitude. We have previously shown that both β-endorphin (Lee *et al.*, 1984) and dynorphin (Lee & Wong, 1987) are arrhythmogenic in the rat isolated heart, which is the first piece of evidence suggesting that EOP may be involved in cardiac arrhythmogenesis. The second piece of evidence in support of this suggestion is the demonstration of an antiarrhythmic effect of naloxone both *in vivo* (Fagbemi *et al.*, 1982; Lee *et al.*, 1992) and *in vitro* (Zhan *et al.*, 1986; Sarne *et al.*, 1988). However, the possibility that naloxone exerts its antiarrhythmic action via its membrane stabilizing effect rather than by opiate antagonism cannot be excluded. The finding in the present study that (−)-naloxone is antiarrhythmic whilst the isomer lacking opiate antagonistic actions is not, provides more compelling evidence that EOP are indeed involved in the pathophysiology of myocardial ischaemia and reperfusion and that the opiate antagonism is responsible for the antiarrhythmic effect.

The antiarrhythmic effect of naloxone may result from the influence of the central nervous system on the opioid receptors, vagal reflex, prevention of post-occlusion hypotension with a consequent improvement in coronary blood flow, as well as from the receptors located in the heart (Ehrenpresis, 1976; Bergey & Beil, 1983; Lin *et al.*, 1992). Brasch (1986) has demonstrated that naloxone increased both the cardiac action potential duration and the functional refractory period, thus rendering the heart less vulnerable to cardiac arrhythmias. He suggested that naloxone exerted a negative chronotropic effect due to the inhibition of the time-dependent membrane potassium outward current. Others reported prolongation of the conductance in the frog node of Ranvier (Carratu & Mitolo-Chieppa, 1982), and rat heart (Sarne *et al.*, 1988), which suggested inhibition of the inward sodium or calcium currents. In addition, naloxone has a significant influence on the electrophysiological properties of the proximal part of the heart conduction system. It has been reported to lengthen the sinoatrial, intra-atrial and atrioventricular node conduction times, and to prolong the atrial and atrioventricular node effective refractory periods (Markiewicz *et al.*, 1991).

The present results can be explained on the basis that blockade of opioid receptors, perhaps in the myocardium itself, inhibits ischaemia- and reperfusion-induced arrhythmias by reducing the effects of EOP released as a consequence of the stress of myocardial ischaemia and reperfusion. This is in agreement with the recent finding by Parratt & Sitsapesan (1986) who found that two opiate antagonists, (−)-Mr1452 and (−)-WIN 44,441-3 are antiarrhythmic while their isomers, without opiate antagonistic properties, are not. They are not compatible, however, with the finding of Sarne *et al.* (1988) that both stereoisomers of naloxone (with and without opiate antagonistic properties) reduced the incidence and severity of cardiac arrhythmias induced in rats by intracarotid administration of adrenaline. This discrepancy may suggest that different mechanisms may be involved in these two events (ischaemia- and adrenaline-induced arrhythmogenesis). Further studies are needed to define the extent of involvement of EOP in cardiac arrhythmogenesis and to elucidate both the mechanisms of action of EOP in the pathophysiology of myocardial ischaemia and reperfusion, and the electrophysiological effects of opioid receptor activation and blockade in cardiac muscle.

I would like to thank Miss Chun-Jou Lin for her excellent technical assistance. (+)-Naloxone was kindly supplied by National Institute on Drug Abuse, U.S.A. The study was supported by the National Science Council and the National Institute of Health, Taiwan.

References

BENFEY, B.G., ELFELLAH, M.S., OGILVIE, R.I. & VARMA, D.R. (1984). Antiarrhythmic effects of prazosin and propranolol during coronary artery occlusion and reperfusion in dogs and pigs. *Br. J. Pharmacol.*, **82**, 717–725.

BERGEY, J.L. & BEIL, M.E. (1983). Antiarrhythmic evaluation of naloxone against acute coronary occlusion-induced arrhythmias in pigs. *Eur. J. Pharmacol.*, **90**, 427–431.

BOLTON, G.R. (1975). *Handbook of Canine Electrocardiology*. Philadelphia: Saunders.

BRASCH, H. (1986). Influence of the optical isomers (−)- and (+)-naloxone on beating frequency, contractile force and action potentials of guinea-pig isolated cardiac preparation. *Br. J. Pharmacol.*, **88**, 733–740.

CARRATU, M.R. & MITOLO-CHIEPPA, D. (1982). Inhibition of ionic currents in frog node of Ranvier treated with naloxone. *Br. J. Pharmacol.*, **77**, 115–124.

EHRENPRESIS, S. (1976). Actions of opiates and their antagonist on cholinergic transmission in the guinea pig ileum. In *Advances in General and Cellular Pharmacology*, Vol. I, ed. Narahashi, T. & Bianchi, C.P. p. 67. New York: Plenum Press.

FAGBEMI, O., LEPRAN, I., PARRATT, J.R. & SZEKERES, L. (1982). Naloxone inhibits early arrhythmias resulting from acute coronary ligation. *Br. J. Pharmacol.*, **76**, 504–506.

HUANG, X.D., LEE, A.Y.S., WONG, T.M., ZHAN, C.Y. & ZHAO, Y.Y. (1986). Naloxone inhibits arrhythmias induced by coronary artery occlusion and reperfusion in anaesthetized dogs. *Br. J. Pharmacol.*, **87**, 475–477.

LEE, A.Y.S. (1989). Naloxone as an antiarrhythmic agent. *Acta Cardiol. Sin.*, **5**, 301–306.

LEE, A.Y.S. (1990). Endogenous opioid peptides and cardiac arrhythmias. *Int. J. Cardiol.*, **27**, 145–151.

LEE, A.Y.S., CHEN, Y.T., KAN, M.N., PENG, F.K., CHAI, C.Y. & KUO, J.S. (1992). Consequences of opiate agonist and antagonist in myocardial ischaemia suggest a role of endogenous opioid peptides in ischaemic heart disease. *Cardiovasc. Res.*, **26**, 392–395.

LEE, A.Y.S. & WONG, T.M. (1987). Effects of dynorphin 1-13 on cardiac rhythm and cyclic adenosine monophosphate (cAMP) levels in the isolated perfused rat heart. *Neurosci. Lett.*, **80**, 289–292.

LEE, A.Y.S., ZHAN, C.Y. & WONG, T.M. (1984). Effects of B-endorphin on the contraction and electrical activity of the isolated perfused rat heart. *Int. J. Pept. Prot. Res.*, **24**, 525–528.

LIN, C.J., CHEN, Y.T., KUO, J.S. & LEE, A.Y.S. (1992). Antiarrhythmic action of naloxone: suppression of picrotoxin-induced cardiac arrhythmias in the rat. *Jpn Heart J.*, **33**, 365–372.

MARKIEWICZ, K., KUS, W., CHOLEWA, M. & BUBINSKI, R. (1991). Electrophysiologic effects of blocking and stimulating the opioid system in patients with unexplained heart palpitations. *Clin. Cardiol.*, **14**, 813–820.

PARRATT, J.R. & SITSAPESAN, R. (1986). Stereospecific antiarrhythmic effect of opioid receptor antagonists in myocardial ischaemia. *Br. J. Pharmacol.*, **87**, 621–622.

PENNY, W.J. & SHERIDAN, D.J. (1983). Arrhythmias and cellular electrophysiological changes during myocardial ischaemia and reperfusion. *Cardiovasc. Res.*, **17**, 363–372.

SARNE, Y., HOCHMAN, I., ESHED, M. & OPPENHEIMER, E. (1988). Antiarrhythmic action of naloxone: Direct, non-opiate effect on the rat heart. *Life Sci.*, **43**, 859–864.

SAKAMOTO, S., STONE, C.K., WOOLF, P.D. & LIANG, C.S. (1989). Opiate receptor antagonism in right-sided congestive heart failure: Naloxone exerts salutary hemodynamic effects through its action on the central nervous system. *Circ. Res.*, **65**, 103–114.

ZHAN, C.Y., LEE, A.Y.S. & WONG, T.M. (1986). Naloxone blocks the cardiac effects of myocardial ischaemia and reperfusion in the rat isolated heart. *Clin. Exp. Pharmacol. Physiol.*, **12**, 373–378.

(Received May 26, 1992
Revised June 27, 1992
Accepted August 5, 1992)

Br. J. Pharmacol. (1992), **107**, 1061–1067

Blockade by antiarrhythmic drugs of glibenclamide-sensitive K^+ channels in *Xenopus* oocytes

[1]Hidenari Sakuta, Koichi Okamoto & Yasuhiro Watanabe

Department of Pharmacology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359, Japan

1 The outward K^+ current induced by KRN2391 (K^+ channel opener) in *Xenopus* oocytes is blocked by glibenclamide. We have investigated the effects of various classes (I–IV) of antiarrhythmic drugs on this KRN2391-induced response.

2 All class I antiarrhythmic drugs (Na^+ channel blockers) tested concentration-dependently suppressed KRN2391-induced responses with the rank order of potency (IC_{50} in μM), disopyramide (17.8) > aprindine (29.5) > propafenone (63.1) > ajmaline (145) > quinidine (151). Flecainide, SUN1165, lignocaine, mexiletine and procainamide were much less potent (IC_{50}, 450–>1000 μM) than quinidine.

3 The class II antiarrhythmic drugs (β-blockers), timolol, (−)- and (±)- propranolol, and (+)-propranolol (a non-β-blocker) inhibited KRN2391-induced K^+ currents in a concentration-dependent manner with values for IC_{50} (μM) of 79, 131, 151 and 129, respectively, whilst butoxamine, oxprenolol, alprenolol, pindolol, nadolol, metoprolol and acebutolol were either weak (IC_{50}, 300 μM–600 μM) or virtually inactive (IC_{50}, >1000 μM).

4 The class III antiarrhythmic drugs, amiodarone and (+)-sotalol scarcely affected KRN2391 responses.

5 All class IV drugs (Ca^{2+} antagonists) tested suppressed KRN2391-induced responses in a concentration-dependent manner with an IC_{50} of 6.3 μM for bepridil, 38 μM for prenylamine, 85 μM for verapamil and 135 μM for diltiazem.

6 In conclusion, antiarrhythmic drugs of classes I, II and IV potently blocked glibenclamide-sensitive K^+ channels in *Xenopus* oocytes.

Keywords: K^+ channel; glibenclamide; *Xenopus* oocyte; KRN2391; antiarrhythmic drugs; W-7

Introduction

Adenosine 5'-triphosphate (ATP)-regulated K^+ channels, which were first demonstrated in cardiac muscle (Noma, 1983), are known to be present also in the pancreas (Ashcroft *et al.*, 1984; Cook & Hales, 1984), vascular smooth muscle (Quast & Cook, 1988), skeletal muscle (Spruce *et al.*, 1985) and brain (Ashford *et al.*, 1988). While the ATP-sensitive K^+ channel is inactivated by an increase in intracellular ATP, it is activated by K^+ channel openers such as cromakalim and pinacidil (Escande *et al.*, 1988) and inactivated by antidiabetic sulphonylureas such as glibenclamide and tolbutamide (Sturgess *et al.*, 1985).

The oocyte of *Xenopus laevis* possesses a K^+ channel which is activated by K^+ channel openers such as cromakalim and pinacidil and is blocked by glibenclamide (Honoré & Lazdunski, 1991a,b). This K^+ channel is insensitive to charybdotoxin (a Ca^{2+}-activated K^+ channel blocker) and to dendrotoxin (a voltage-dependent K^+ channel blocker) (Honoré & Lazdunski, 1991a,b). Although not yet proven by single channel studies, this oocyte K^+ channel is thought to be an ATP-sensitive K^+ channel (Honoré & Lazdunski, 1991a,b).

We have found that a glibenclamide-sensitive K^+ current is induced by KRN2391 in *Xenopus* oocytes. KRN2391 is a novel K^+ channel opener structurally related to nicorandil and has been reported to enhance ^{86}Rb efflux from the rat isolated aorta more potently than cromakalim or pinacidil (Kashiwabara *et al.*, 1991).

Since antiarrhythmic drugs such as quinidine and verapamil suppress the increase of K^+ conductance measured by the ^{86}Rb uptake induced by ATP deficiency in isolated myocardial cells (Haworth *et al.*, 1989), these drugs are thought to block ATP-sensitive K^+ channels in myocardial cells. However, the effects of antiarrhythmic drugs on the ATP-sensitive K^+ channel have not been investigated comprehensively. In the present study, we tentatively regarded the KRN2391-activated and glibenclamide-inhibited K^+ channel in *Xenopus* oocytes as an ATP-sensitive K^+ channel, and investigated in detail the effects of various antiarrhythmic drugs on the KRN2391-induced activation of the glibenclamide-sensitive K^+ channels in oocytes. As a result, it was found that glibenclamide-sensitive K^+ channels in oocytes are relatively potently blocked by the antiarrhythmic drugs which distribute in the class Ia (Na^+ channel blockers prolonging the action potential duration, APD), class Ib (Na^+ channel blockers shortening the APD), class Ic (Na^+ channel blockers not affecting the APD), class II (β-blockers) and class IV (Ca^{2+} antagonists) of Vaughan Williams's classification of antiarrhythmic drug actions (Vaughan Williams, 1984; 1989; Woosley, 1991).

Methods

Electrophysiological recording

Follicle-enclosed oocytes at stage V–VI were collected from female frogs (*Xenopus laevis*) anaesthetized in ice and were incubated in modified Barth's medium for one to four days for stabilization (Sakuta *et al.*, 1992). *Xenopus laevis* female frogs were purchased from Hamamatsu Biological Research Service, Inc. (Hamamatsu, Japan).

Each oocyte was placed in a recording well (about 0.2 ml in capacity) and superfused at a constant rate of 3 ml min^{-1} with frog Ringer solution consisting of (in mM): NaCl 120, KCl 2, $CaCl_2$ 1.8 and HEPES 5 (pH 7.4). The membrane potential of each oocyte was voltage-clamped routinely at −20 mV with two glass microelectrodes each filled with 3 M

[1] Author for correspondence.

KCl (1–2 Mohm) using the voltage-clamp amplifier, CEZ-1200 (Nihon Kohden, Tokyo, Japan), and current responses were recorded by the thermal array recorder, RTA-1100 (Nihon Kohden), as previously described (Sakuta *et al.*, 1992). A clamped potential of − 20 mV was chosen so as to minimize the Cl^- current and to maximize the K^+ current in oocytes (Honoré & Lazdunski, 1991a). All electrophysiological experiments were carried out at room temperature (19–21°C).

Application of drugs

KRN2391 was dissolved in frog Ringer solution to be 100 μM or to be 10–1000 μM for concentration-response studies, and applied to a voltage-clamped oocyte routinely for a duration of 20 s by constant flow superfusion (3 ml min^{-1}). When KRN2391 was applied repeatedly to the same oocyte, 6 min intervals were taken to provide a period for applying test drugs between KRN2391 applications.

All the drugs tested except for glibenclamide were dissolved in frog Ringer solution (pH was adjusted to 7.4, if necessary). When the effects of these drugs on KRN2391-induced K^+ currents were studied, each drug solution was applied to an oocyte for a constant period of 2 min by superfusion (3 ml min^{-1}), because most drugs tested required about 2 min to exert their maximum effects. Then, the oocyte was washed for 15 s by superfusion with frog Ringer solution before the application of KRN2391. This 15 s washing period was taken, because lignocaine and propranolol (which required mM concentrations to show maximum effects) induced by themselves small inward currents (< 10 nA) which disappeared within about 15 s. Separate experiments showed that the suppressive effects of all other drugs tested on KRN2391-induced currents were not reduced by this washing period.

Glibenclamide was dissolved in ethanol to a concentration of 2.5 mM, then diluted with frog Ringer solution to a final concentration of 5 nM–10 μM (pH 7.4) and applied to each oocyte by superfusion in the same manner as test drugs. The solution of 10 μM glibenclamide contained 0.4% ethanol which was confirmed not to affect current responses to KRN2391.

Drugs used

Acebutolol hydrochloride, ajmaline, (−)-alprenolol (+-tartrate, amiodarone hydrochloride, atenolol, bepridil hydrochloride, butoxamine hydrochloride, diltiazem hydrochloride, disopyramide phosphate, lignocaine hydrochloride, (±)-metoprolol (+)-tartrate, nadolol, oxyprenolol hydrochloride, pindolol, prenylamine lactate, procainamide hydrochloride, (+)-, (−)- and (±)-propranolol hydrochloride, quinidine sulphate, timolol maleate, (±)-verapamil hydrochloride, W-7 (N-6-aminohexyl)-5-chloro-1-naphthalenesulphonamide) and W-5 (N-(6-aminohexyl)-1-naphthalene sulphonamide) were purchased from Sigma Chemical Co. Ltd. (St. Louis, U.S.A.). KRN2391 (N-cyano-N'-(2-nitroxyethyl)-3-pyridine carboximidamide methane sulphonate) was a gift from Dr. Koji Nishikori, Kirin Brewery Co. Ltd. (Takasaki, Japan). (+)-Sotalol was presented by Bristol Myers Squibb (Aichi, Japan). Aprindine hydrochloride was obtained from Mitsui Pharmaceutical Co. Ltd. (Tokyo, Japan), mexiletine hydrochloride was from Tanabe Pharmaceutical Co. Ltd. (Osaka, Japan), and glibenclamide, charybdotoxin and dendrotoxin were from Funakoshi Co. Ltd. (Tokyo, Japan). Flecainide was a gift from Esai Pharmaceutical Co. Ltd. (Tokyo, Japan), SUN1165 (N-(2, 6-dimethylphenyl)-8-pyrrolizinylacetamide hydrochloride hemihydrate) from Suntory Co. Ltd. (Tokyo, Japan) and propafenone from Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). All other chemicals used were from Wako Pure Chemical Industry Co. Ltd. (Tokyo, Japan).

Estimation of IC_{50} and EC_{50} values

The IC_{50} value for the inhibtiion of KRN2391-induced currents by each test drug was estimated from the log concentration-inhibition curve drawn by linearly connecting observed mean values (cf. Figures 2–7). EC_{50} values for KRN2391 was also estimated from the log concentration-response curve in the same manner (cf. Figure 1).

Results

Properties of KRN2391-induced K^+ currents in Xenopus *oocytes*

KRN2391, a novel K^+ channel opener (Kashiwabara *et al.*, 1991), induced non-oscillating and slowly rising outward currents in a concentration-dependent manner over the range of 20–1000 μM with an EC_{50} of about 400 μM in the follicle-enclosed oocytes voltage-clamped at − 20 mV (Figure 1a). KRN2391-induced outward currents were carried by K^+ ions, because they were reversed at a membrane potential of about − 100 mV and were associated with an increase in membrane conductance (data not shown). KRN2391-induced outward current was concentration-dependently and reversibly suppressed by 5 nM–2 μM glibenclamide, a specific blocker of ATP-sensitive K^+ channels, with an IC_{50} of about 300 nM (Figure 1b–d). Neither 50 nM charybdotoxin (Ca^{2+}-activated K^+ channel blocker) nor 100 nM dendrotoxin (voltage-dependent K^+ channel blocker) affected KRN2391 responses (data not shown).

Effects of class I antiarrhythmic drugs on KRN2391-induced K^+ currents

As shown in Figure 2, class Ia antiarrhythmic drugs, disopyramide (a), quinidine (b), ajmaline (c) and procainamide (d) reversibly blocked KRN2391-induced K^+ currents in a concentration-dependent manner (e) with IC_{50} values of 17.8 μM, 145 μM, 151 μM and 2700 μM, respectively.

Of the class Ib antiarrhythmic drugs tested, aprindine was found to block KRN2391-induced K^+ currents most potently (IC_{50}, 29.5 μM) as shown in Figure 3a and d, whilst mexiletine and lignocaine showed very weak suppressive effects on KRN2391 responses as indicated by their IC_{50} values of

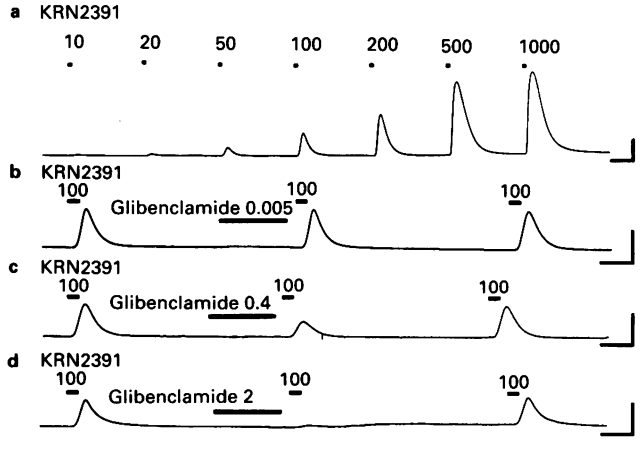

Figure 1 (a) The concentration-dependence of KRN2391-induced outward K^+ currents. (b–d) Concentration-dependent suppression of KRN2391 (100 μM) responses by glibenclamide. All numbers on bars are drug concentrations in μM, and KRN2391 was applied for a constant period of 20 s (short bars). Glibenclamide was applied for 2 min (long bars). Calibrations: 200 nA vertical and 2 min horizontal for (a), and 100 nA and 1 min for (b)–(d). Records (a)–(d) were from different oocytes voltage-clamped at − 20 mV.

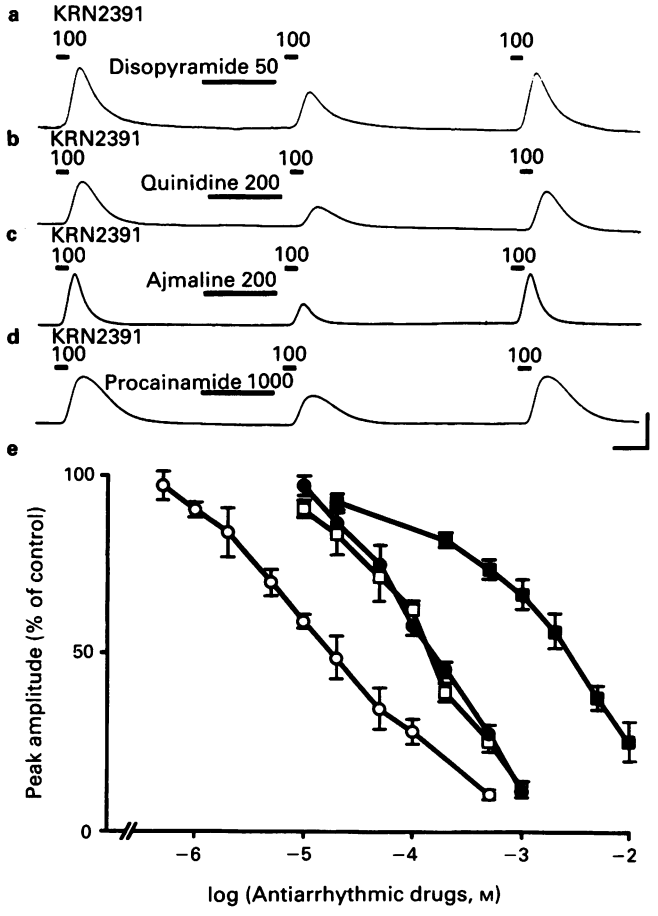

Figure 2 Effects of class Ia antiarrhythmic drugs on KRN2391 responses. In (a)–(d), KRN2391 (100 μM) was applied for 20 s (short bars), and test drugs were applied for 2 min (long bars) at concentrations shown in μM. Calibrations: 100 nA and 1 min. Records (a)–(d) were from different oocytes voltage-clamped at −20 mV. (e) Concentration-inhibition curves (mean ± s.d., $n = 5$) for the inhibition of KRN2391-induced currents by disopyramide (○), quinidine (●), ajmaline (□) and procainamide (■). The ordinate scale is the % peak amplitude of the KRN2391-induced current in the absence of an antiarrhythmic drug, and the abscissa scale is the log molar concentration of the antiarrhythmic drugs.

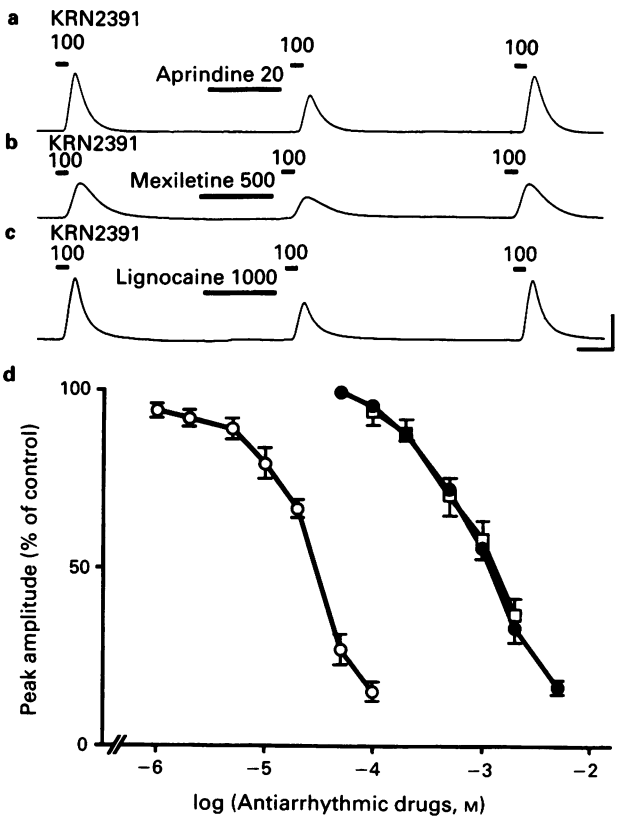

Figure 3 Effects of class Ib antiarrhythmic drugs on KRN2391 responses. In (a)–(c), KRN2391 (100 μM) was applied for 20 s (short bars), and test drugs were applied for 2 min (long bars) at concentrations shown in μM. Records (a)–(c) were from different oocytes voltage-clamped at −20 mV. Calibrations: 100 nA and 1 min. (d) Concentration-inhibition curves (mean ± s.d., $n = 5$) for the inhibition of KRN2391-induced currents by aprindine (○), lignocaine (●), and mexiletine (□). The ordinate scale shows the % peak amplitude of the KRN2391-induced current in the absence of an antiarrhythmic drug, and the abscissa scale shows the log molar concentration of the antiarrhythmic drugs.

1.3 mM for mexiletine and 1.2 mM for lignocaine (Figure 3b–d).

Similarly for the class Ic antiarrhythmic drugs tested, propafenone (IC_{50}, 63.1 μM) was most effective (Figure 4a and d), and flecainide (IC_{50}, 447 μM) and SUN1165 (IC_{50}, 832 μM) were much weaker than propafenone (Figure 4b–d), in blocking KRN2391 responses.

Effects of class II (β-blockers) antiarrhythmic drugs on KRN2391-induced K^+ currents

Among the β-blockers tested, timolol (a nonselective β-blocker) potently inhibited KRN2391 responses (IC_{50}, 79.4 μM) (Figure 5a and c), and more than 1 h was required for recovery from the timolol effect as shown in Figure 5a. The nonselective β-blocker, (−)- and (±)-propranolol, and the non-β-blocker, (+)-propanolol had a similar potency to quinidine in blocking KRN2391 responses, their IC_{50} values were 131, 151 and 129 μM, respectively. Except for propranolol and timolol, all other β-blockers tested showed either weak blocking effects or no effect on KRN2391 responses as exemplified by atenolol in Figure 5c. The IC_{50} values for these were as follows; 275 μM for butoxamine (β_2-blocker), 631 μM for oxprenolol (nonselective β-blocker), and >1000 μM for alprenolol, pindolol and nadolol (nonselective β-blockers) and for metoprolol, acebutolol and atenolol (β_1-blockers).

Effects of class III and IV antiarrhythmic drugs (Ca^{2+} antagonists) on KRN2391-induced K^+ currents

The class III antiarrhythmic drugs, amiodarone and (+)-sotalol scarcely affected KRN2391 responses (Figure 6e). The β-blockers, oxprenolol and nadolol also possess class III activity (Taggart *et al.*, 1984), but they showed very weak suppressive effects on KRN2391 responses as mentioned above.

The class IV antiarrhythmic drugs, bepridil and prenylamine are known also to block Na^+ channels i.e., exhibit class I activity (Fleckenstein, 1988; Singh *et al.*, 1985). Verapamil has a very weak class I activity (Woosley, 1991), whilst diltiazem is devoid of class I activity. All four of these Ca^{2+} antagonists concentration-dependently blocked KRN-2391 responses (Figure 6a–e) with the rank order of potency as follows (IC_{50} in μM); bepridil (6.3)>prenylamine (38)>verapamil (85)>diltiazem (135). As indicated by these IC_{50} values, bepridil was the most potent of all the antiarrhythmic drugs tested but was weaker than glibenclamide (IC_{50}, 0.3 μM). However, the IC_{50} values for verapamil and diltiazem to block KRN2391 responses are much higher than those reported to block Ca^{2+} channels, which are 0.1–1 μM (Fleckenstein, 1988).

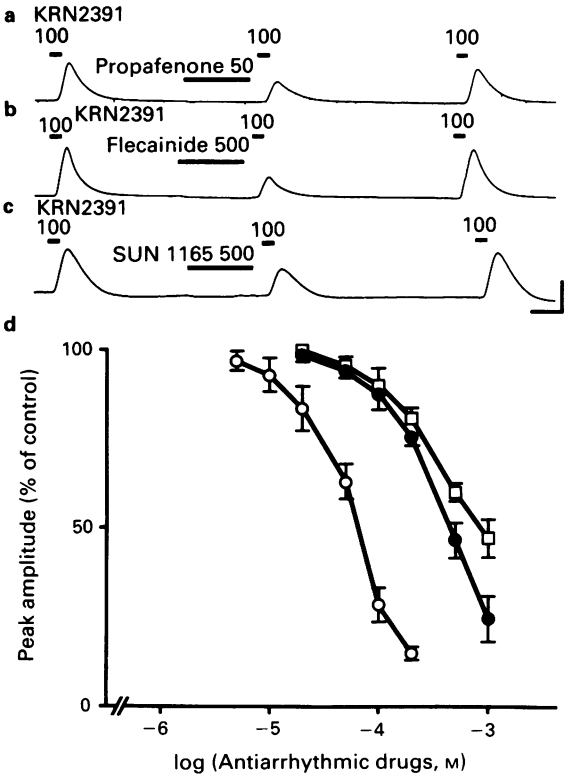

Figure 4 Effects of class Ic antiarrhythmic drugs on KRN2391 responses. In (a)–(c), KRN2391 (100 μM) was applied for 20 s (short bars), and test drugs were applied for 2 min (long bars) at concentrations shown in μM. Records (a)–(c) were from different oocytes voltage-clamped at − 20 mV. Calibrations: 100 nA and 1 min. (d) Concentration-inhibition curves (mean ± s.d., $n = 5$) for the inhibition of KRN2391-induced currents by propafenone (○), flecainide (●), and SUN1165 (□). The ordinate scale shows the % peak amplitude of the KRN2391-induced current in the absence of an antiarrhythmic drug, and the abscissa scale shows the log molar concentration of the antiarrhythmic drugs.

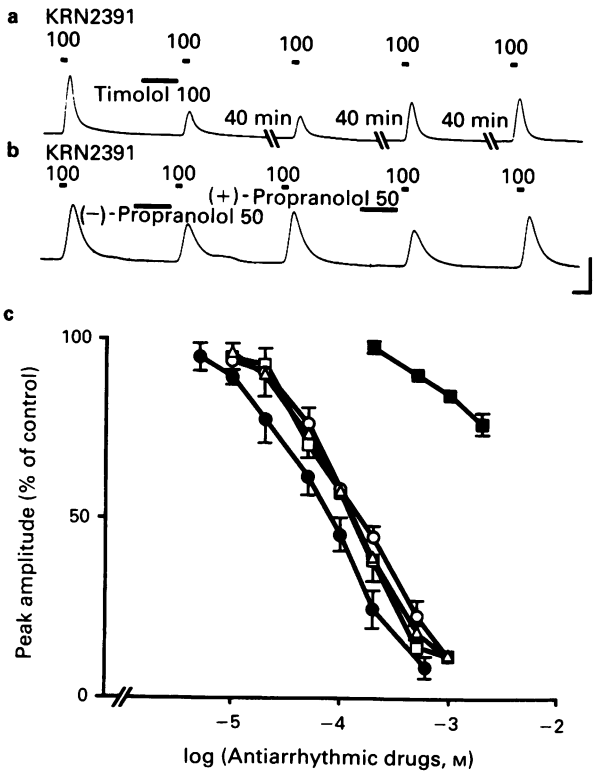

Figure 5 Effects of class II antiarrhythmic drugs on KRN2391 responses. In (a)–(d), KRN2391 (100 μM) was applied for 20 s (short bars), and test drugs were applied for 2 min (long bars) at concentrations shown in μM. Records (a) and (b) were from different oocytes voltage-clamped at − 20 mV. Calibrations: 100 nA and 1 min. Note 40 min interruptions in (a) indicating slow recovery from the effect of timolol. (c) Concentration-inhibition curves (mean ± s.d., $n = 5$) for the inhibition of KRN2391-induced currents by (±)-propranolol (○), (−)-propranolol (□), (+)-propranolol (△), timolol (●), and atenolol (■). The ordinate scale shows the % peak amplitude of the KRN2391-induced current in the absence of an antiarrhythmic drug, and the abscissa scale shows the log molar concentration of the antiarrhythmic drugs.

Effects of calmodulin antagonists on KRN2391-induced K^+ currents

Of all the antiarrhythmic drugs tested in this study, bepridil, prenylamine and aprindine, which showed relatively potent blocking effects on KRN2391 responses, are known to possess fairly strong antagonistic activities on calmodulin (Hidaka *et al.*, 1980; Itoh *et al.*, 1984; Zimmer & Hofmann, 1987). We have previously reported that cromakalim-induced glibenclamide-sensitive K^+ currents in oocytes are blocked by various calmodulin antagonists including W-7 (Sakuta *et al.*, 1992). KRN2391-induced K^+ currents in oocytes were also found to be reversibly and concentration-dependently blocked by W-7 with an IC_{50} of 26.9 μM (Figure 7a and b), and this value was comparable to the IC_{50} (26 μM) obtained for W-7 to inhibit Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase (Ca^{2+}-PDE) (Hidaka *et al.*, 1981). W-5, which is a dechlorinated derivative of W-7 and a weaker calmodulin antagonist (IC_{50}, 240 μM) than W-7 (Hidaka *et al.*, 1981), was found to be less potent (IC_{50}, 141 μM) than W-7 in blocking KRN2391 responses (Figure 7b).

Discussion

Seven drugs, namely, bepridil (IC_{50} in μM, 6.3), disopyramide (17.8), aprindine (29.5), prenylamine (38.0), propafenone (63.1), timolol (79.4) and verapamil (85.0), were found to induce a potent block of KRN2391-induced, glibenclamide-sensitive K^+ currents in oocytes. Except for bepridil, the IC_{50} values of these drugs are greater than their therapeutic concentrations. The concentrations of quinidine, verapamil and lignocaine required to inhibit ATP deficiency-induced increase in K^+ conductance measured by ^{86}Rb uptake by rat myocardial cells are more than 10 times higher than the concentrations required to block KRN2391 responses (Haworth *et al.*, 1989). Thus, if the ATP/glibenclamide-sensitive K^+ channel of myocardial cells is assumed to be 10 times more sensitive to K^+ channel blockers than the oocyte K^+ channel of this type, it may be inferred that not only bepridil but also disopyramide, aprindine, prenylamine and propafenone may block the myocardial ATP/glibenclamide-sensitive K^+ channels at their therapeutic concentrations.

Vaughan Williams' classification and the blockade of the KRN2391/glibenclamide-sensitive K^+ channel

Disopyramide, ajmaline and quinidine belong to Vaughan Williams' class Ia, aprindine to class Ib, propafenone to class Ic, timolol and propranolol to class II, and bepridil, prenylamine, verapamil and diltiazem to class IV (Vaughan Williams, 1984; 1989; Woosley, 1991). Thus, the drugs which can relatively potently block KRN2391 responses were found distributed in all Vaughan Williams' classes of antiarrhythmic actions excepting for class III.

The K^+ channel blocking action of class I antiarrhythmic

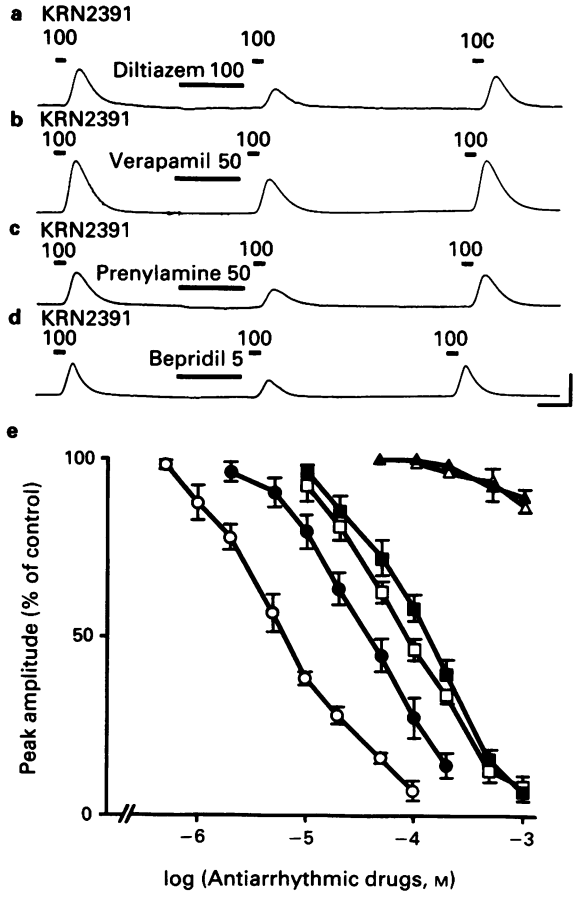

Figure 6 Effects of class III and IV antiarrhythmic drugs on KRN2391 responses. In (a)–(d), KRN2391 (100 μM) was applied for 20 s (short bars), and test drugs were applied for 2 min (long bars) at concentrations shown in μM. Records (a)–(d) were from different oocytes voltage-clamped at −20 mV. Calibrations: 100 nA and 1 min. (e) Concentration-inhibition curves (mean ± s.d., $n = 5$) for the inhibition of KRN2391-induced currents by bepridil (○), prenylamine (●), verapamil (□), diltiazem (■), (+)-sotalol (△) and amiodarone (▲). The ordinate scale shows the % peak amplitude of the KRN2391-induced current in the absence of an antiarrhythmic drug, and the abscissa scale shows the log molar concentration of the antiarrhythmic drugs.

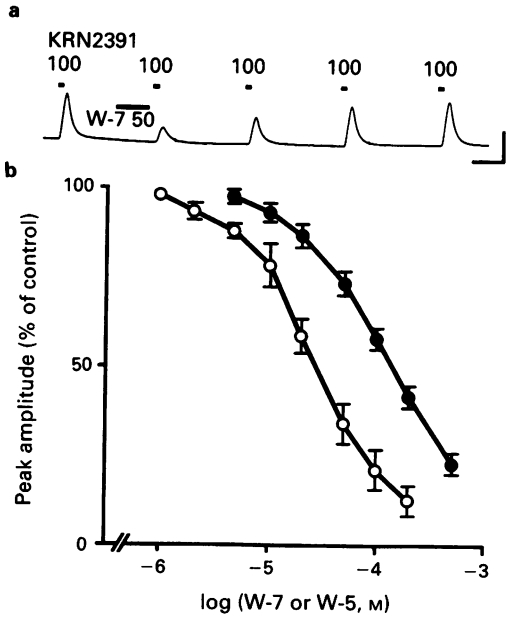

Figure 7 Effects of W-7 and W-5 on KRN2391 responses. (a) Suppression of KRN2391-induced currents by W-7. KRN2391 (100 μM) was applied for 20 s (short bars), and W-7 (50 μM) was applied for 1 min (long bar). Note slow recovery from inhibition by W-7. Calibrations: 100 nA and 2 min. (b) Concentration-inhibition curves (mean ± s.d., $n = 5$) for the inhibition of KRN2391-induced currents by W-7 (○) and W-5 (●). The ordinate scale shows the % peak amplitude of the KRN2391-induced current in the absence of an antiarrhythmic drug, and the abscissa scale shows the log molar concentration of the antiarrhythmic drugs.

drugs seemed to be independent of their blocking effect on Na^+ channels, because disopyramide (class Ia), aprindine (Ib) and propafenone (Ic) were more potent than quinidine, whilst other drugs tested in these classes were almost inactive (cf. Figures 2–4).

Despite the fact that (+)-propanolol is virtually devoid of β-blocking activity, (−)-propranolol and (+)-propranolol were equipotent in blocking KRN2391 responses, with an IC_{50} of about 130 μM for both (cf. Figure 5b and c). Moreover, pindolol, which is 5–10 times more effective than (±)-propranolol as a β-blocker, was nearly inactive (IC_{50} > 1 mM) in blocking KRN2391 responses. Thus, it is likely that the blocking effect of β-blockers on KRN2391 responses is not due to their property as β-adrenoceptor antagonists. The membrane stabilizing actions of β-blockers (Pruett *et al.*, 1980) may also not contribute to their blocking effect on KRN2391 responses, because, among all β-blockers tested, timolol, which has no membrane stabilizing activity, most potently (IC_{50}, 79.4 μM) blocked KRN2391 responses.

(+)-Sotalol and amiodarone, which are class III antiarrhythmic drugs with an APD-prolonging activity, scarcely inhibited KRN2391 responses (cf. Figure 6). Since amiodarone and (+)-sotalol are known to block delayed rectifier K^+ channels and to prolong the APD in non-ischaemic heart (Carmeliet, 1985; Sanguinetti & Jurkiewicz, 1990; Basler *et al.*, 1991), the APD prolonging effect of class III drugs in cardiac muscle may result from the inhibition of these or other (but not ATP/glibenclamide-sensitive) K^+ channels.

Non-selective and weak Ca^{2+} antagonists such as prenylamine and bepridil (Winslow *et al.*, 1986; Fleckenstein, 1988) suppressed KRN2391 responses more potently than the specific Ca^{2+} antagonists, verapamil and diltiazem (cf. Figure 6). It may be inferred, therefore, that Ca^{2+} antagonism may not directly contribute to the blockade of KRN2391 responses by these Ca^{2+} antagonists.

Thus, all the basic properties for Vaughan Williams' classification of antiarrhythmic drugs, namely, Na^+ channel blockade, β-adrenoceptor antagonism, APD-prolonging activity and Ca^{2+} antagonism, do not seem to contribute directly to the inhibition of KRN2391/glibenclamide-sensitive K^+ currents in oocytes.

Glibenclamide (Kantor *et al.*, 1990), tolbutamide (Wolleben *et al.*, 1988) and 5-hydroxydecanoate (Notsu *et al.*, 1992) exert their antiarrhythmic effects primarily by inhibiting ATP/glibenclamide-sensitive K^+ channels. In non-ischaemic myocardial cells, however, ATP-sensitive K^+ channels are thought to be closed. Therefore, drugs which inhibit myocardial ATP-sensitive K^+ channels would not prolong the APD. Thus, this antiarrhythmic action cannot be classified by the conventional Vaughan Williams' classification. The inhibition of ATP/glibenclamide-sensitive K^+ channels may be a novel mechanism required to be added to the conventional classification of antiarrhythmic actions. It is also conceivable that the inhibition of ATP/glibenclamide-sensitive K^+ channels may underlie some of the antiarrhythmic effects of conventional antiarrhythmic drugs.

Calmodulin antagonism as a possible mechanism of the blockade of glibenclamide-sensitive K^+ channel by some antiarrhythmic drugs

We have previously reported that calmodulin-dependent processes are involved in the activation of glibenclamide-

sensitive K^+ channels by K^+ channel openers in oocytes (Sakuta *et al.*, 1992), and W-7 was confirmed to block KRN2391 responses in the present study (cf. Figure 7). Several antiarrhythmic drugs possess calmodulin antagonizing activities. Bepridil, prenylamine and aprindine, which are relatively potent blockers of KRN2391 responses (cf. Figures 3a, 6a and 6b), are known as relatively strong calmodulin-antagonists with IC_{50} values for Ca^{2+}-PDE of 8 μM, 18 μM and 3 μM, respectively (Hidaka *et al.*, 1980; Itoh *et al.*, 1984; Zimmer & Hofmann, 1987). Both (+)- and (−)-propranolol blocked KRN2391 responses with similar potencies, and they are known to inhibit Ca^{2+}-PDE with an IC_{50} value of 180 μM for each (Volpi *et al.*, 1981). Diltiazem and verapamil also have calmodulin-antagonizing activities (Epstein *et al.*, 1982; Zimmer & Hofmann, 1987). Typical local anaesthetics such as lignocaine and procaine also possess very weak calmodulin-antagonizing activities (Muto *et al.*, 1983).

Thus, it is possible at least for some antiarrhythmic drugs that calmodulin antagonism underlies their inhibitory effects on glibenclamide-sensitive K^+ currents. Calmodulin antagonists such as W-7 and trifluoperazine have been reported to prevent reperfusion-induced ventricular arrhythmia as bepridil does (Barron *et al.*, 1986), though little has been known about the mechanism of this *in vivo* antiarrhythmic effect. Our present results suggest that the suppression of glibenclamide-sensitivie K^+ channels may underlie at least partly the mechanism of antiarrhythmic effects of calmodulin-antagonizing antiarrhythmic drugs. In this connection, it is noteworthy that bepridil, a potent calmodulin antagonist, is expected to be clinically effective for ventricular arrhythmia (Marshall *et al.*, 1983; Singh *et al.*, 1985).

Lipid solubilities of antiarrhythmic drugs may not contribute to their potencies to inhibit KRN2391 responses. For instance, in the case of class I antiarrhythmic drugs, the rank order of octanol/water partition coefficients is quinidine> lignocaine> disopyramide> mexiletine> flecainide (Vaughan Williams, 1989), which does not parallel that of their potencies in blocking KRN2391 responses, disopyramide> quinidine> flecainide> lignocaine = mexiletine. For class IV antiarrhythmic drugs, the octanol/Ringer solution partition coefficient of verapamil is about 3400 times greater than that of diltiazem (Pang & Sperelakis, 1984), but verapamil is only slightly more potent than diltiazem in blocking glibenclamide-sensitive K^+ currents (cf. Figure 6).

In conclusion, some antiarrhythmic drugs which do not belong to class III but are classifed as classes I, II, and IV blocked KRN2391/glibenclamide-sensitive K^+ currents in *Xenopus* oocytes more potently than quinidine. Calmodulin antagonism was suggested to underlie the mechanism of action of some of these antiarrhythmic drugs.

We would like to express our sincere thanks to Dr Koji Nishikori, Kirin Brewery Co., Ltd., Takasaki, Japan for the kind supply of KRN2391. We are also grateful to Esai Pharmaceutical Co., Ltd., Tokyo, Japan for the supply of flecainide, to Suntory Institute, Osaka, Japan for SUN1165, to Bristol Myers Squibb Co., Aichi, Japan for (+)-sotalol, and to Yamanouchi Pharmaceutical Co., Tokyo, Japan for propafenone.

References

ASHCROFT, F.M., HARRISON, D.E. & ASHCROFT, S.J.H. (1984). Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. *Nature*, **312**, 446–448.

ASHFORD, M.L.J., STURGESS, N.C., TROUT, N.J., GARDNER, N.J. & HALES, C.N. (1988). Adenosine-5'-triphosphate-sensitive ion channels in neonatal rat cultured central neurones. *Pflügers Arch.*, **412**, 297–304.

BARRON, E., MARSHALL, R.J., MARTORANA, M. & WINSLOW, E. (1986). Comparative antiarrhythmic and electrophysiological effects of drugs known to inhibit calmodulin (TFP, W7 and bepridil). *Br. J. Pharmacol.*, **89**, 603–612.

BASLER, J.R., BENETT, P.B., HONDEGHEM, L.C. & RODEN, D.M. (1991). Suppression of time-dependent outward current in guinea pig ventricular myocytes. Actions of quinidine and amiodarone. *Circ. Res.*, **69**, 519–529.

CARMELIET, E. (1985). Electrophysiologic and voltage clamp analysis of the effects of sotalol on isolated cardiac muscle and Purkinje fibers. *J. Pharmacol. Exp. Ther.*, **232**, 817–825.

COOK, D.L. & HALES, C.N. (1984). Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature*, **311**, 271–273.

EPSTEIN, P.M., FISS, K., HACHISU, R. & ANDRENYAK, D.M. (1982). Interaction of calcium antagonists with cyclic AMP phosphodiesterases and calmodulin. *Biochem. Biophys. Res. Commun.*, **105**, 1142–1149.

ESCANDE, D., THURINGER, D., LEGUERN, S. & CAVERO, I. (1988). The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent K^+ channels in isolated cardiac myocytes. *Biochem. Biophys. Res. Commun.*, **154**, 620–625.

FLECKENSTEIN, A. (1988). Historical overview: The calcium channel of the heart. *Ann. N.Y. Acad. Sci.*, **522**, 1–15.

HAWORTH, R.A., GOKNUR, A.B. & BERKOFF, H.A. (1989). Inhibition of ATP-sensitive potassium channels of adult rat heart cells by antiarrhythmic drugs. *Circ. Res.*, **65**, 1157–1160.

HIDAKA, H., ASANO, M. & TANAKA, T. (1981). Activity-structure relationship of calmodulin antagonists: Naphthalenesulfonamide derivatives. *Mol. Pharmacol.*, **20**, 571–578.

HIDAKA, H., YAMAKI, T., NAKA, M., TANAKA, T., HAYASHI, H. & KOBAYASHI, R. (1980). Calcium-regulated modulator protein interacting agents inhibit smooth muscle calcium-stimulated protein kinase and ATPase. *Mol. Pharmacol.*, **17**, 66–72.

HONORÉ, E. & LAZDUNSKI, M. (1991a). Hormone-regulated K^+ channels in follicle-enclosed oocytes are activated by vasorelaxing K^+ channel openers and blocked by antidiabetic sulfonylureas. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 5438–5442.

HONORÉ, E. & LAZDUNSKI, M. (1991b). Two different types of channels are targets for potassium channel openers in *Xenopus* oocytes. *FEBS Lett.*, **287**, 75–79.

ITOH, H., ISHIKAWA, T. & HIDAKA, H. (1984). Effects on calmodulin of bepridil, an antianginal agent. *J. Pharmacol. Exp. Ther.*, **230**, 737–741.

KANTOR, P.F., COETZEE, W.A., CARMELIET, E.E., DENNIS, S.C. & OPIE, L.H. (1990). Reduction of ischemic K^+ loss and arrhythmias in rat hearts. Effect of glibenclamide, a sulfonylurea. *Circ. Res.*, **66**, 478–485.

KASHIWABARA, T., NAKAJIMA, S., IZAWA, T., FUKUSHIMA, H. & NISHIKORI, K. (1991). Characteristics of KRN2391, a novel vasodilator, compared with those of cromakalim, pinacidil and nifedipine. *Eur. J. Pharmacol.*, **196**, 1–7.

MARSHALL, R.J., MUIR, A.W. & WINSLOW, E. (1983). Effects of antiarrhythmic drugs on ventricular fibrillation thresholds of normal and ischaemic myocardium in the anaesthetized rat. *Br. J. Pharmacol.*, **78**, 165–171.

MUTO, Y., KUDO, S. & NOZAWA, Y. (1983). Effects of local anesthetics on calmodulin-dependent guanylate cyclase in the plasma membrane of *Tetrahymena pyriformis*. *Biochem. Pharmacol.*, **32**, 3559–3563.

NOMA, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature*, **305**, 147–148.

NOTSU, T., TANAKA, I., TAKANO, M. & NOMA, A. (1992). Blockade of the ATP-sensitive K^+ channel by 5-hydroxydecanoate in guinea pig ventricular myocytes. *J. Pharmacol. Exp. Ther.*, **260**, 702–708.

PANG, D.C. & SPERELAKIS, N. (1984). Uptake of calcium antagonistic drugs into muscles as related to their lipid solubilities. *Biochem. Pharmacol.*, **33**, 821–826.

PRUETT, J.K., WALLE, T. & WALLE, U.K. (1980). Propranolol effects on membrane repolarization time in isolated canine Purkinje fibers: Threshold tissue content and the influence of exposure time. *J. Pharmacol. Exp. Ther.*, **215**, 539–543.

QUAST, U. & COOK, N.S. (1988). Potent inhibitors of the effect of the K^+ channel opener BRL34915 in vascular smooth muscle. *Br. J. Pharmacol.*, **93**, 204P.

SAKUTA, H., SEKIGUCHI, M., OKAMOTO, K. & SAKAI, Y. (1992). Inactivation of glibenclamide-sensitive K^+ channels in *Xenopus* oocytes by various calmodulin antagonists. *Eur. J. Pharmacol.*, **226**, 199–207.

SANGUINETTI, M.C. & JURKIEWICZ, N.K. (1990). Two components of cardiac delayed rectifier K^+ current. Differential sensitivity to block by class III antiarrhythmic agents. *J. Gen. Physiol.*, **96**, 195–215.

SINGH, B.N., BAKY, S. & NADEMANEE, K. (1985). Second-generation calcium antagonists: search for greater selectivity and versatility. *Am. J. Cardiol.*, **55**, 214B–221B.

SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1985). Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature*, **316**, 736–738.

STURGESS, N.C., ASHFORD, M.L.J., COOK, D.L. & HALES, C.N. (1985). The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet*, **ii**, 474–475.

TAGGART, P., DONALDSON, R., ABED, J. & NASHAT, F. (1984). Class III action of β-blocking agents. *Cardiovasc. Res.*, **18**, 683–689.

VAUGHAN WILLIAMS, E.M. (1984). A classification of antiarrhythmic actions reassessed after a decade of new drugs. *J. Clin. Pharmacol.*, **24**, 129–147.

VAUGHAN WILLIAMS, E.M. (1989). Classification of antiarrythmic actions. In *Antiarrhythmic Drugs*. ed. Vaughan Williams, E.M. & Campbell, T.J. pp. 45–67. Berlin & Heidelberg: Springer-Verlag.

VOLPI, M., SHA'AFI, R.I., EPSTEIN, P.M., ANDRENYAK, D.M. & FEINSTEIN, M.B. (1981). Local anesthetics, mepacrine, and propranolol are antagonists of calmodulin. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 795–799.

WINSLOW, E., FARMER, S., MARTORANA, M. & MARSHALL, R.J. (1986). The effects of bepridil compared with calcium antagonists on rat and rabbit aorta. *Eur. J. Pharmacol.*, **131**, 219–228.

WOLLEBEN, C.D., SANGUINETTI, M.C. & SIEGL, P.K.S. (1989). Influence of ATP-sensitive potassium channel modulators on ischemia-induced fibrillation in isolated rat hearts. *J. Mol. Cell. Cardiol.*, **21**, 783–788.

WOOSELY, R.L. (1991). Antiarrhythmic drugs. *Annu. Rev. Pharmacol. Toxicol.*, **31**, 427–455.

ZIMMER, M. & HOFMANN, F. (1987). Differentiation of the drug-binding sites of calmodulin. *Eur. J. Biochem.*, **164**, 411–420.

(Received June 1, 1992
Revised August 4, 1992
Accepted August 5, 1992)

Br. J. Pharmacol. (1992), **107**, 1068–1074

Lack of effect of potassium channel openers on ATP-modulated potassium channels recorded from rat ventromedial hypothalamic neurones

A.J. Sellers, *P.R. Boden & [1]M.L.J. Ashford

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ and *Park-Davis Research Unit, Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1QJ

1 Single neuronal cells were freshly isolated from the ventromedial hypothalamic nuclei (VMHN) of the rat brain. Currents through ATP-modulated and large conductance (160 and 250 pS) calcium-activated potassium channels were recorded by the cell-attached and excised inside-out patch techniques.

2 BRL38227 (lemakalim; 30–90 μM) applied to the superfusing medium produced no change in firing rate of isolated glucose-receptive VMHN neurones in cell-attached recordings.

3 BRL38227, at concentrations of between 30–100 μM applied to the intracellular (cytoplasmic) aspect of inside-out patches, had no effect on the activity of ATP-sensitive K^+ channels in the absence of ATP or in the presence of a sub-maximal inhibitory concentration (3 mM) of ATP. Cromakalim, pinacidil, minoxidil sulphate and diazoxide also produced no effect under these conditions.

4 The potassium channel openers (KCO's) were tested on ATP-activated potassium channels recorded from a further subpopulation of VMHN neurones. Application of BRL38227 (up to and including 100 μM) to this channel in inside-out patches either in the absence of ATP or when activated by 5 mM ATP had no effect on channel activity. Identical results were obtained with cromakalim and pinacidil.

5 BRL38227 had no effect on either of the large conductance (250 pS and 160 pS) calcium-activated potassium channels in VMHN neurones.

6 Intracellular recordings were made from glucose-receptive VMHN neurones in rat brain slices. Cromakalim (50 μM) or diazoxide (60 μM) did not alter the firing rate or passive membrane properties of these neurones demonstrated to be sensitive to tolbutamide (0.1 mM).

7 These results show that the KCO's tested in this study have no effect either on VMHN neurones contained in brain slices or on the activity of any of the ATP-modulated potassium channels under isolated patch conditions associated with these neurones.

Keywords: Potassium channel openers; BRL38227; ATP-sensitive K^+ channels; Ca^{2+}-activated K^+ channels; hypothalamic neurones

Introduction

The heterogeneous class of drugs known collectively as the potassium channel openers (KCO's), have been widely researched as powerful smooth muscle relaxing agents with both hypotensive and bronchodilator activity *in vivo* (for review see Robertson & Steinberg, 1990). Recently it has been suggested that these compounds may be of some use in diseases of the central nervous system (Miller, 1990; Aronson, 1992). Cromakalim (BRL 34915) is an isomeric benzopyran molecule with activity residing mainly in the 3S-4R configuration (BRL 38227, [lemakalim]). In the CNS, cromakalim has been shown to be effective in reducing seizure activity in two models of epilepsy (Gandolfo *et al.*, 1989a,b), and has also been shown to affect neuronal excitability (Alzheimer *et al.*, 1988; Politi & Rogawski, 1991). Furthermore, potassium channel openers have been shown to block neurosecretion (Schmid-Antomarchi *et al.*, 1990). The target channel for these agents has been identified in some peripheral tissues. For instance, in cardiac muscle cromakalim has been shown to activate a glibenclamide-sensitive potassium current in isolated ventricular myocytes (Escande *et al.*, 1988; Sanguinetti *et al.*, 1988), and adenosine 5'-triphosphate (ATP)-sensitive K^+ channels isolated in membrane patches from the same cells have also been shown to be activated by cromakalim (Escande *et al.*, 1988) and pinacidil (Fan *et al.*, 1990). In skeletal muscle cells, potassium channel openers including cromakalim, pinacidil and RP 49356 have also been demonstrated to activate a sulphonylurea-sensitive potassium conductance (Quasthoff *et al.*, 1989). However, it is not as yet clear which type of potassium channel is affected by these drugs in the CNS (Alzheimer *et al.*, 1988) or smooth muscle (Weston, 1989). Although it has been little used in CNS preparations the direct approach of single channel recording offers the best opportunity to determine the target site (channel) of these compounds and perhaps indicate the possible mechanisms of action.

Single channel recording techniques have identified three distinct potassium channels in neurones in the ventromedial hypothalamic nuclei (VMHN) the activity of which is modulated by intracellular levels of ATP. The ATP-sensitive K^+ channel is inhibited by increasing levels of intracellular ATP or by the sulphonylurea tolbutamide and is located in glucose receptive neurones where it contributes to resting membrane potential (Ashford *et al.*, 1990a,b). This channel closes under conditions of elevated extracellular glucose (Ashford *et al.*, 1990a) causing membrane depolarization in a manner analogous to that demonstrated in the pancreatic β-cell (Ashcroft *et al.*, 1988). An ATP-activated K^+-channel which in recordings from inside-out patches responds to elevated intracellular ATP levels with a large increase in channel activity, can be isolated from glucose sensing neurones which respond to elevated extracellular glucose with

[1] Author for correspondence.

a hyperpolarization possibly mediated by the opening of these channels (Rowe *et al.*, 1992). A third type of ATP-sensitive K^+ channel is sometimes observed in patches excised from VMHN neurones. This channel has been classed, according to its conductance (250 pS), as a maxi-calcium-activated potassium channel. However, this channel also shows sensitivity to changes in intracellular ATP (Treherne & Ashford, 1991). A separate large conductance (160 pS) Ca^{2+}-activated K^+-channel has also shown to be present in glucose-receptive neurones of the VMHN, and its activity is not sensitive to intracellular ATP levels (Treherne & Ashford, 1991).

As the ATP-K^+ channel is the target channel for cromakalim in cardiac muscle (Sanguinetti *et al.*, 1985) and various potassium channel openers can, at high concentrations, inhibit insulin release from the pancreatic β-cell by increasing ATP-sensitive potassium channel activity (Garrino *et al.*, 1989) the aim of the present experiments was to examine the effects of various potassium channel openers and this benzopyran molecule in particular on the membrane potential and spontaneous action potential firing rate of intact VMHN neurones and on the ATP-modulated potassium channels that can be recorded from isolated membrane patches.

Methods

Electrical recording and analysis

Coronal slices (350 μm thick) of hypothalamus were cut from brains of male Sprague-Dawley rats (80–200 g weight) with a Vibratome (Oxford Instruments). The slices were maintained at room temperature in artificial cerebrospinal fluid (ACSF) bubbled with 95% O_2 and 5% CO_2. For intracellular recording, the slices were transferred to a recording chamber where they were superfused with ACSF at 37°C. Electrodes were filled with 1 M potassium acetate and had d.c. resistances of 100–150 MΩ when measured in physiological saline. A period of 30 min was allowed for equilibration following impalement. Input resistances were derived from the slope of the current-voltage plot obtained by measuring the electronic potential during current injection. Pulses, of greater than 100 ms duration, were applied in order to ensure complete capacitance saturation of the membrane. All experiments were performed at 37°C. These recording procedures have been described in full elsewhere (Boden & Hill, 1988).

For single channel recording from VMHN neurones, cells were acutely dissociated from the VMHN isolated from rat hypothalamic slices. The nuclei were incubated with 0.5 mg ml^{-1} collagenase (Clostridiopaptidase A. Boehringer, Mannheim) and 1 mg ml^{-1} trypsin (Type XII, Sigma, Poole, Dorset) in ACSF at room temperature for 1–2 h and then triturated by the use of flame polished Pasteur pipettes. The dispersed cells were transferred onto Falcon 3001 dishes (35 mm) and left for 30 min to adhere. Single channel currents were recorded, at room temperature (21°C), from cell-attached and inside-out membrane patches by standard patch-clamp recording procedures (Hamill *et al.*, 1981). Current recordings were made with an Axopatch 2D patch clamp amplifier and stored on magnetic tape (Racal 4DS) for later reproduction of figures and analysis. The potential across the membrane is described with the usual sign convention for membrane potential (i.e. inside negative). The data were analysed for current amplitude and open-state probability P_{open} by computer (Apricot XEN-i 286/45) as described previously (Sturgess *et al.*, 1988; Kozlowski *et al.*, 1989). All data in the text are presented as mean values ± s.e.mean.

Solutions

The ACSF contained (in mM): NaCl 128.0, KCl 5.0, NaH_2PO_4 1.2, $CaCl_2$ 2.4, $MgCl_2$ 1.3, $NaHCO_3$ 26.0, D-glucose 10.0, pH 7.4. Before single channel recordings the cells were washed with normal physiological salt solution (PSS) consisting of (mM): NaCl 135.0, KCl 5.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0 pH 7.4 with NaOH. For cell-attached and inside-out recordings the patch pipette contained (mM): KCl 140.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0, pH 7.2 with KOH and the bathing solution was either the normal external PSS (cell-attached recordings) or an intracellular solution (inside-out recordings) containing (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 0.9, EGTA 1.0, HEPES 10.0, pH 7.2 with KOH (free Ca^{2+} concentration of 0.8 μM). Free calcium concentrations were controlled and changed by use of EGTA, and determined by the 'METLIG' metal ion/ligand binding programme (P. England & R. Denton, University of Bristol).

Drugs

All potassium channel openers were made up in a 10^{-2} M stock solution in 70% ethanol. Diazoxide, minoxidil sulphate and tolbutamide were obtained from Sigma Chemicals, Poole, Dorset. For brain slice experiments cromakalim was made up as a 10^{-2} M stock in dried dimethylsulphoxide (DMSO) and diluted to the required concentration in ACSF. We acknowledge kind gifts of pinacidil from Leo Pharmaceuticals, Ballerup, Denmark and cromakalim and BRL38227 from SmithKline Beecham Pharmaceuticals, Welwyn, Herts.

Results

Inside-out patches were obtained from isolated VMHN neurones bathed in symmetrical potassium solutions. In approximately one third of patches obtained from the VMHN cells ($n = 28$) the ATP-K^+ channel was observed and identified by its conductance (146 pS) and sensitivity to ATP. The ATP-K^+ channel can be isolated from glucose-receptive neurones that respond to elevated extracellular glucose with a depolarization mediated by closure of these ATP-K^+ channels (Ashford *et al.*, 1990a). ATP caused a concentration-dependent reduction in channel activity with an IC_{50} of approximately 3 mM, with channel activity exhibiting no sensitivity to calcium (Ashford *et al.*, 1990a). To reduce the activity of the large conductance (160 pS) calcium-activated potassium channel observed in most patches, the free calcium concentration was reduced to very low (nM) levels. Figure 1 shows a typical trace showing activity of an ATP sensitive K^+ channel recorded from an inside-out patch isolated from a VMHN neurone. In the absence of ATP the channel had a P_{open} of 0.863. The addition of 3 mM ATP caused a reduction in channel activity to an open probability of 0.110. BRL38227 was then applied concomitant with 3 mM ATP at a concentration of 30 μM and caused no observable change in channel activity (P_{open}; 0.110). The effects of ATP were readily reversed on washing (P_{open}; 0.870). BRL38227 was also applied under identical conditions at a concentration of 100 μM in three further experiments and no activation of channel activity was observed. Likewise, no other potassium channel opener tested on ATP-K^+ channel activity recorded from inside-out VMHN membrane patches, inhibited submaximally by 3 mM ATP, produced any observable activation. The openers tested were cromakalim (100 μM, $n = 2$), pinacidil (30 μM, $n = 2$, and 100 μM, $n = 2$), minoxidil sulphate (100 μM, $n = 3$) and diazoxide (100 μM, $n = 2$). For example, the typical open state probability of the ATP-K^+ channel in such an experiment was; control 0.774, 3 mM ATP 0.250, 3 mM ATP + 100 μM pinacidil 0.246, wash 0.800. In order to determine whether the KCO's had an underlying effect on ATP-K^+ channel activity, they were applied to the isolated inside-out patch in the absence of ATP. For exam-

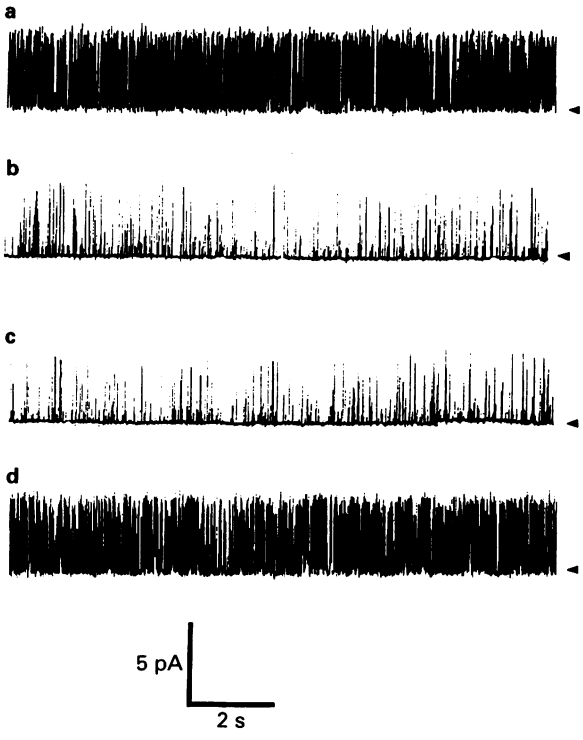

Figure 1 Single channel currents recorded from a single inside-out patch, excised from a VMHN neurone, held at a membrane potential of +30 mV. The recording pipette contained (in mM). KCl 140, $MgCl_2$ 1 and $CaCl_2$ 1, and the bath (in mM) KCl 140, $MgCl_2$ 1, EGTA 1 (with a free calcium concentration of <10 nM). The arrow denotes the channel in its closed state. Upward deflections indicate outward currents. (a) Channel activity in absence of ATP. (b) Application of 3 mM ATP to the bathing solution inhibits channel activity. (c) In the continued presence of ATP, BRL38227 at a concentration of 30 μM does not alter channel activity. (d) Wash demonstrates reversibility of effect of ATP. The values of P_{open} were as follows: control 0.863; 3 mM ATP 0.110; 3 μM BRL38227 + 3 mM ATP 0.110; wash 0.870.

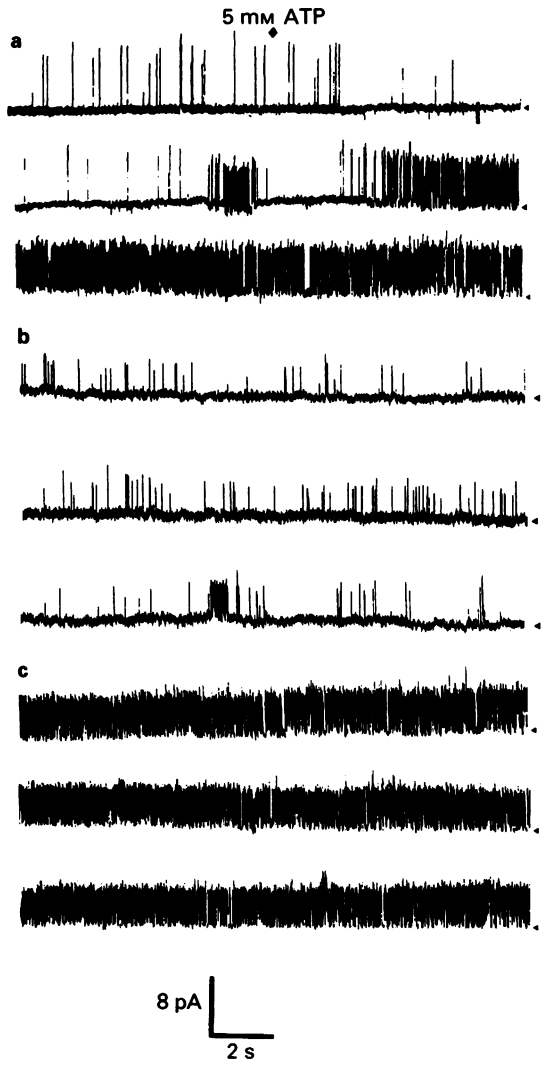

Figure 2 The lack of effect of BRL38227 on an ATP-activated K^+ channel recorded from an inside-out patch excised from a VMHN neurone. The patch potential was +40 mV, and the solutions were as follows (in mM): pipette, KCl 140, $MgCl_2$ 1, $CaCl_2$ 1; bath, KCl 140, $MgCl_2$ 1, free Ca^{2+} <10 nM. The channel closed state is denoted by the arrow. (a) Consecutive recordings showing activation of 5 mM ATP (start addition at diamond). P_{open} in absence of ATP, 0.030, after application of ATP, 0.680. Effects of ATP fully reversible on washing (not shown; P_{open} 0.03). (b) Effect on ATP-activated K^+ channel of BRL38227 (30 μM) in the absence of ATP. P_{open} values were; 0.020 control, 0.025 BRL38227, 0.025 wash. (c) Effect of BRL38227 on channel activated by 5 mM ATP. The values of the open probability were control 0.820; BRL38227 0.816; wash 0.819.

ple, there was no observable inhibition of ATP-K^+ channel activity induced by BRL38227 at either concentration tested (30 μM, $n = 3$ and 100 μM, $n = 3$). In a typical experiment the P_{open} was 0.668 in the absence of applied ATP, 0.666 in the presence of 100 μM BRL38227, and 0.666 after washout of the BRL38227.

There is a separate population of neurones that can be isolated from the lateral hypothalamic area (LHA), and, to a lesser extent from the VMHN, which respond to an increase in extracellular glucose with a hyperpolarization and have thus been termed glucose-sensing neurones (Oomura *et al.*, 1974). Following cell-attached recording and excision of the patch into the inside-out configuration an ATP-activated potassium channel is observed from such cells (Rowe *et al.*, 1992). In the present series of experiments, this channel had a conductance of 137.4 ± 3.6 pS ($n = 10$) in symmetrical potassium solutions and could be activated in a concentration-dependent manner by ATP. Application of ATP caused a rapid and marked increase in channel open probability (Figure 2a) which was fully reversible on washing. This ATP-activated K^+ channel was identified (by its conductance and low P_{open} in the absence of ATP) in approximately 10% of patches isolated from VMHN neurones. In the absence of applied ATP the P_{open} was 0.031 ± 0.016 ($n = 10$). Figure 2b shows the lack of effect of BRL38227 at a concentration of 30 μM on channel activity with the patch membrane held at a potential of + 40mV in the un-activated state (i.e. 0 mM ATP). No change in channel activity was observed when BRL38227 was applied at this concentration ($n = 3$) or at a higher concentration (100 μM, $n = 2$). Other potassium channel openers tested (pinacidil and cromakalim, both at 100 μM with $n = 2$, data not shown) also failed to alter channel activity under these conditions. Figure 2c shows a typical example of BRL38227 when applied to an ATP-activated K^+ channel after activation by 5 mM ATP. In this case the membrane potential was also held at + 40mV, and the channel P_{open} increased from 0.030 to 0.820 in the presence of the ATP. BRL38227, at 30 μM had no inhibitory effect on the channel under these conditions. This was repeated on a further three separate inside-out patches with the same results. In order to show that BRL38227 had no effect on the sensitivity of the channel to ATP, the channel was activated by 5 mM ATP and this was then washed off, with a subsequent reversal of effect (P_{open} values; control 0.023, 5 mM ATP 0.760, wash 0.030). BRL38227 was then applied prior to re-application of ATP, and no alteration in the respon-

siveness of the channel to ATP was observed (P_{open} values; 30 μM BRL38227 0.028, 30 μM BRL38227 + 5 mM ATP 0.800).

Another potassium-selective channel that may be isolated from VMHN neurones is a large-conductance (250 pS) calcium-activated potassium channel the activity of which is also modulated by ATP (Treherne & Ashford, 1991). This channel is distinct from the 160 pS calcium-activated K^+ channel observed in VMHN neurones in that it is not thought to be present in glucose-receptive neurones and is not ATP-sensitive (Treherne & Ashford, 1991). Figure 3 shows the channel activity of this Ca^{2+}–K^+ channel in an inside-out patch held at a membrane potential of −30mV. Note the lack of effect of BRL38227 (60 μM), under conditions of free calcium levels of either 0.8 μM or <10 nM, which are associated with different levels of P_{open}. When the free calcium level was elevated to 0.2 mM it can be seen that there were, in fact, five 250 pS calcium activated K^+ channels in the patch of membrane. BRL38227, at a concentration of 30 μM ($n = 2$) was also applied to the 160 pS Ca^{2+}–K^+ channel in inside-out membrane patches isolated from glucoreceptive VMHN neurones without effect on channel activity.

Recently, BRL38227 has been shown to modulate some metabolic processes including phosphoinositide turnover and calcium mobilization (Challis *et al.*, 1992; Bray *et al.*, 1991). In order to demonstrate that this compound not only had no effect on ATP-K^+ channels but also had no indirect effect on cellular activity via alteration of intracellular metabolic processes, the racemic mixture cromakalim (50 μM) was tested on glucose-receptive neurones by intracellular recording from intact rat VMHN slices maintained at 37°C. Figure 4 shows a typical pen recorder trace from a glucose-receptive neurone in a rat VMHN slice. The potassium channel opener had no effect on either the resting membrane parameters or spontaneous action potential firing rate of glucose-receptive neurones bathed in normal (10 mM glucose) ACSF (Figure 4a). Subsequent removal of glucose from the ACSF produced the expected hyperpolarization and decrease in input resistance, effects which could be reversed by reapplication of glucose (Figure 3b) or the sulphonylurea tolbutamide at a concentration of 100 μM (Figure 3c). Cromakalim had no effect on a further 4 neurones, all of which were shown to be glucose-receptive. Cromakalim (50 μM; $n = 3$) and diazoxide (60 μM; $n = 2$) were also without effect in experiments using glucose-receptive neurones in the presence of 10 mM glucose or which had been pretreated with tolbutamide (100 μM) in an attempt to ensure that the ATP-K^+ channels were mostly closed in the neuronal membrane. Furthermore, BRL38227 (30 μM, $n = 3$ and 90 μM, $n = 3$) applied to isolated glucose-receptive VMHN neurones in the cell-attached configuration (at 21°C) had no effect on the rate of firing of these neurones (data not shown).

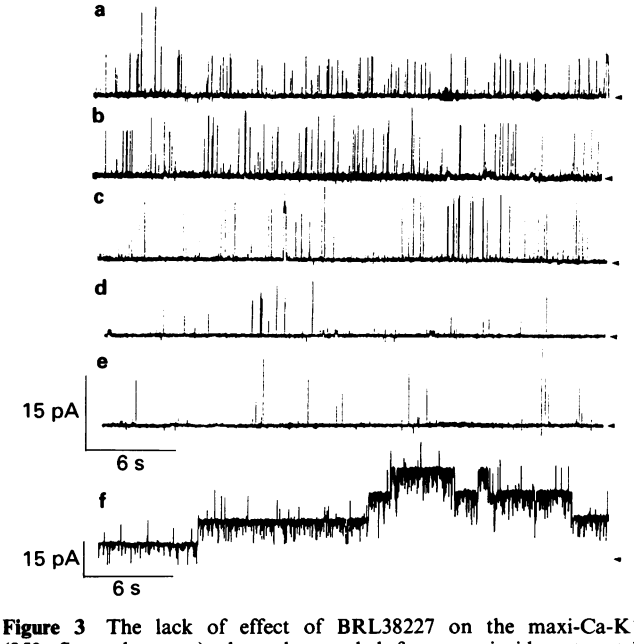

Figure 3 The lack of effect of BRL38227 on the maxi-Ca-K^+ (250 pS conductance) channel recorded from an inside-out patch excised from a VMHN neurone. Membrane potential was −30 mV in all traces, the arrow denotes the zero current level. (a) Control trace with free calcium concentration of 0.8 μM. (b) Addition of 60 μM BRL38227. (c) Washout of BRL38227. (d) Calcium concentration reduced to <10 nM free, with associated reduction in channel activity. (e) Application of BRL38227 (60 μM) at low calcium concentrations is also without effect on channel activity. (f) In absence of BRL38227, elevation of free calcium levels to 0.2 mM causes the simultaneous opening of five channels in the patch. Note the change in the ordinate scale. The values for the P_{open} were as follows; control 0.110; BRL38227 0.110; wash, 0.112; <10 nM calcium 0.010; BRL38227 0.010; 0.2 mM calcium 0.885.

Discussion

Three distinct potassium channels the activity of which may be modulated by intracellular ATP can be recorded from membrane patches isolated from neurones of the ventromedial hypothalamus, the ATP-sensitive K^+ channel, the ATP-activated K^+ channel and a large conductance (250 pS) calcium-activated K^+ channel. A fourth channel, a large-conductance (160 pS) calcium-activated K^+ channel is not sensitive to intracellular ATP. The ATP-K^+ channel from the VMHN has a clear physiological role in regulating the membrane potential of these cells according to the level of extracellular glucose. However, there are many differences between the two channels with regard to conductance (146 pS in the VMHN, approximately 60 pS in the β cell; Ashford *et al.*, 1990a; Ashcroft *et al.*, 1988), rectification properties (absent in the VMHN, present in the β-cell; Ashford *et al.*, 1990a; Ashcroft *et al.*, 1988) and the form of ATP more potent at inhibiting the channel (MgATP for the VMHN, ATP^{4-} for the β-cell; Rowe & Ashford, 1991; Ashcroft & Kakei, 1989). Pharmacologically, the most interesting difference so far reported is that the first generation sulphonylurea tolbutamide inhibits the channel indirectly in the VMHN (i.e. not in isolated membrane patches) whereas in the β-cell this inhibition may also be observed in isolated membrane patches (Ashford *et al.*, 1990b). As the sulphonylureas have been shown to antagonize the effects of the potassium channel openers in various peripheral tissues (heart; Fosset *et al.*, 1988; Ripoll *et al.*, 1990; pancreatic β-cells; Zünkler *et al.*, 1988; Garrino *et al.*, 1989; central neurones; Ben-Ari, 1989, Politi & Rogawski, 1991), sulphonylurea binding is often used as a means of identifying ATP-K^+ channels (and so, presumably, one of the sites of action of cromakalim) in the central nervous system (for examples Bernardi *et al.*, 1988; Miller *et al.*, 1991). The results shown in this paper demonstrate the lack of effect of potassium channel openers (BRL38227 in particular) on the ATP-sensitive K^+ channel from VMHN neurones, using either single channel or intact cell recording techniques. Previous work has shown that the sulphonylureas depolarize glucose-receptive neurones in the VMHN by a blockade of the ATP-K^+ channel (although indirectly; Ashford *et al.*, 1990b) indicating that the ATP-K^+ channel isolated from this brain region is fundamentally different from those found in the heart and pancreatic β-cell in its pharmacology, and that differences in the association between sulphonylurea receptor sites and ATP-K^+ channel exist between different tissues. Therefore it is possible that ambiguities may occur when using the sulphonylureas to identify brain ATP-K^+ channels by radioligand binding (for instance, the VMHN shows a relatively low specific tritiated glibenclamide binding and yet possesses ATP-K^+ channels at a high density; Treherne & Ashford, 1991).

The ATP-activated potassium channel represents a second

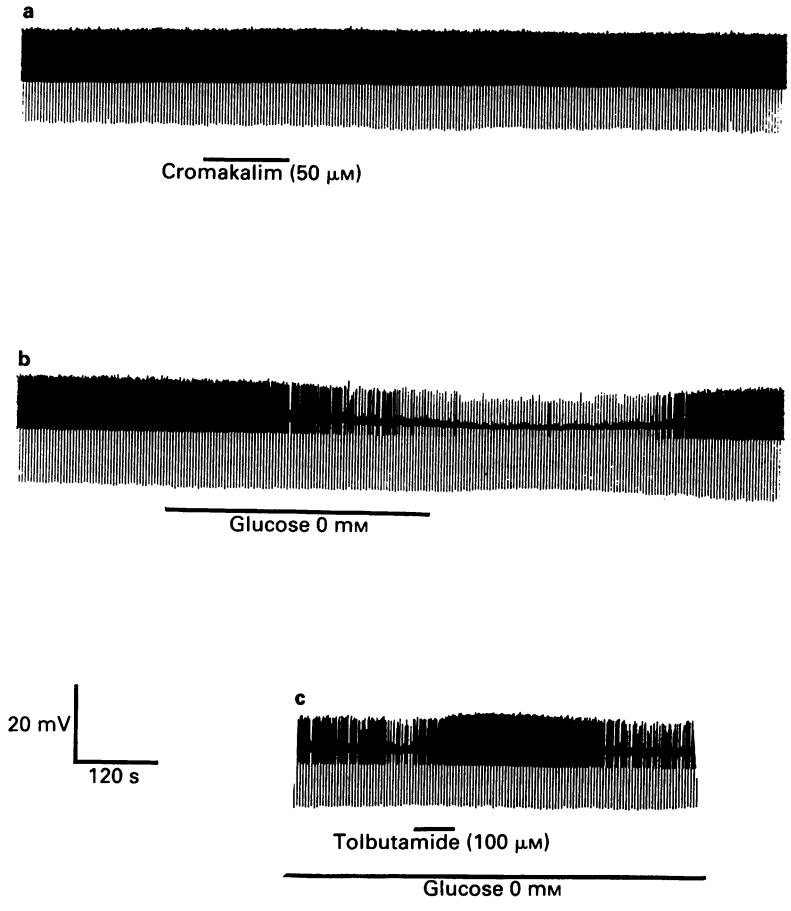

Figure 4 Lack of effect of cromakalim on membrane properties of a glucose-receptive neurone in the rat ventromedial hypothalamus. Resting potential of the neurone was −52 mV. (a) A 2 min application of cromakalim (50 μM) did not change the membrane potential or firing rate of the cell. (b) Removal of glucose from the perfusing ACSF for 5 min hyperpolarized the neurone concomitant with a decrease in input resistance and reduction in spontaneous action potential firing, all of which were reversed on return to normal (10 mM glucose) ACSF. (c) During a second period in the absence of glucose the neurone was challenged for 1 min with the sulphonylurea tolbutamide (100 μM) which produced a membrane depolarization and increase in action potential firing, effects which were fully reversed on return to drug-free ACSF.

link between internal cellular metabolism and neuronal excitability in VMHN neurones. The role of this channel is not clear at present although it is tempting to speculate that it provides a glucose-sensing mechanism. This channel has also been identified, albeit at low densities, in the cortex (Ashford & Treherne, 1989). Although the physiology of the channel has not been characterized fully it is clearly shown from the present study that BRL38227, cromakalim, and pinacidil had no effect on this channel.

The large conductance (250 pS) calcium-activated potassium channel that can be identified in non-glucose-receptive neurones from the VMHN may also provide a link between cell metabolism and neuronal excitability. A similar channel, also sensitive to calcium and ATP, and of a similar conductance, was reported to be isolated from a vascular smooth muscle preparation (Gelband *et al.*, 1989). However, Klöckner & Isenberg (1992), have shown that the ATP sensitivity was due to chelation of calcium by the ATP, and that the channel showed no sensitivity to ATP when the calcium was buffered more effectively. The Ca-K^+ channel from the VMHN is sensitive to ATP because ATP causes an inhibition in channel activity in conditions of high calcium where chelation would not reduce the free calcium concentration to below maximally stimulating levels (Treherne & Ashford, 1991). ATP sensitivity has also been demonstrated for calcium-activated potassium channels isolated from the *Amphiuma* renal distal tubule (Hunter & Giebisch, 1988) and from respiratory epithelial cells (Kunzelmann *et al.*, 1989). Thus, there is evidence for a role for the calcium-activated K^+ channels in responding to elevated extracellular glucose. The channel in the VMHN showed no sensitivity to BRL38227 but it remains to be seen if similar channels sensitive to both calcium and ATP exist elsewhere in the brain. The other (160 pS) Ca^{2+}-K^+ channel that may be recorded from neurones is found almost exclusively in glucose-receptive cells. This channel is insensitive to ATP or tolbutamide applied to the cytoplasmic aspect of the patch isolated in inside-out patches (Treherne & Ashford, 1991) and from the present study we have shown that it is also insensitive to BRL38227.

BRL38227 applied to isolated inside-out membrane patches has not been shown to activate any of the channels modulated by intracellular nucleotides found in the VMHN. However, there are reports that BRL38227 can alter intracellular calcium levels or IP_3 release, thus altering cell excitability through mechanisms other than potassium channel opening. For instance, Bray *et al.* (1991) reported that in calcium-free conditions, BRL38227 inhibited the release of calcium from, or the refilling of, calcium stores within smooth muscle cells. Challiss *et al.* (1992) have also reported that BRL38227 can inhibit histamine-induced IP_3 release from airway smooth muscle. However, in cell-attached recordings from isolated neurones, and, more importantly, intracellular studies performed in ventromedial hypothalamic slice preparations maintained at 37°C, cromakalim had no effect on the activity of glucose-receptive neurones regardless

of whether the majority of the ATP-sensitive K^+ channels were open or closed. This demonstrates that this potassium channel opener has no effect on intact neurones which have not been subjected to the isolation procedures used in the single channel recording experiments.

In conclusion, BRL38227 has been shown not to activate any of the nucleotide-sensitive potassium channels in the VMHN. However, cromakalim has been reported to have effects in a number of other CNS preparations (Tricklebank *et al.*, 1988; Gandolfo *et al.*, 1989a,b; Schmid-Antomarchi *et al.*, 1990; Politi *et al.*, 1991). There are a number of possibilities that may explain this dichotomy. For example, there may be subtypes of ATP-sensitive K^+ channel throughout the CNS, with different biophysical properties, and perhaps different physiological functions, and only a certain type is activated by BRL38227; or, alternatively, BRL38227 may act in the CNS on a channel that is insensitive to intracellular nucleotides but sensitive to sulphonylureas. What is clear from the present study is that the sulphonylurea-sensitive channel in the VMHN is not activated by BRL38227 or any of the other potassium channel openers tested, indicating important differences in the pharmacology of ATP-sensitive K^+ channels between peripheral tissues and the CNS.

This work was supported by the M.R.C.

References

ALZHEIMER, C. & TEN BRUGGENCATE, G. (1988). Actions of BRL34915 (cromakalim) upon convulsive discharges in guinea pig hippocampal slices. *Naunyn-Scmiedebergs Arch. Pharmacol.*, **337**, 429–434.

ARONSON, J.K. (1992). Potassium channels in nervous tissue. *Biochem. Pharmacol.*, **43**, 11–14.

ASHCROFT, F.M., ASHCROFT, S.J.H. & HARRISON, D.E. (1988). Properties of single potassium channel modulated by glucose in rat pancreatic beta cells. *J. Physiol.*, **400**, 501–527.

ASHCROFT, F.M. & KAKEI, M. (1989). ATP-sensitive K^+ channel in rat pancreatic beta cells: modulation by ATP and Mg^{2+} ions. *J. Physiol.*, **416**, 349–367.

ASHFORD, M.L.J., BODEN, P.R. & TREHERNE, J.M. (1990a). Glucose-induced excitation of hypothalamic neurones is mediated by ATP-sensitive K^+ channels. *Pflügers Arch.*, **412**, 479–483.

ASHFORD, M.L.J., BODEN, P.R. & TREHERNE, J.M. (1990b). Tolbutamide excites rat glucoreceptive ventromedial hypothalamic neurones by indirect inhibition of ATP-K^+ channels. *Br. J. Pharmacol.*, **101**, 531–540.

ASHFORD, M.L.J. & TREHERNE, J.M. (1989). Adenosine triphosphate increases the activity of a potassium channel recorded from neonatal rat cultured neurones. *J. Physiol.*, **409**, 54P.

BEN-ARI, Y. (1989). Effect of glibenclamide, a selective blocker of an ATP-K^+ channel, on the anoxic response of hippocampal neurones. *Pflügers Arch.*, **414**, S111–S114.

BERNARDI, H., FOSSET, M. & LAZDUNSKI, M. (1988). Characterisation, purification, and affinity labelling of the brain [^{3}H]glibenclamide-binding protein, a putative neuronal ATP-regulated K^+ channel. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9816–9820.

BODEN, P.R. & HILL, R.G. (1988). Effects of cholecystokinin and related peptides on neuronal activity in the ventromedial nucleus of the rat hypothalamus. *Br. J. Pharmacol.*, **94**, 246–252.

BRAY, K.M., WESTON, A.H., DUTY, S., NEWGREEN, D.T., LONGMORE, J., EDWARDS, G. & BROWN, T.J. (1991). Differences between the effects of cromakalim and nifedipine on agonist-induced responses in rabbit aorta. *Br. J. Pharmacol.*, **102**, 337–344.

CHALLISS, R.A.J., PATEL, N. & ARCH, J.R.S. (1992). Comparative effects of BRL 38227, nitrendipine and isoprenaline on carbachol- and histamine-stimulated phosphoinositide metabolism in airway smooth muscle. *Br. J. Pharmacol.*, **105**, 997–1003.

ESCANDE, D., THURINGER, D., LEGUERN, S. & CAVERO, I. (1988). The potassium channel opener cromakalim (BRL34915) activates ATP-dependent K+ channels in isolated cardiac myocytes. *Biochem. Biophys. Res. Commun.*, **154**, 620–625.

FAN, Z., NAKAYAMA, K. & HIRAOKA, M. (1990). Pinacidil activates the ATP-sensitive K+ channel in inside-out and cell-attached patch membranes of guinea-pig ventricular myocytes. *Pflügers. Arch.*, **415**, 387–394.

FOSSETT, M., DE WEILLE, J.R., GREEN, R.D., SCHMID-ANTOMARCHI, H. & LAZDUNSKI, M. (1988). Antidiabetic sulfonylureas control action potential properties in heart cells via high affinity receptors that are linked to ATP-dependent K^+ channels. *J. Biol. Chem.*, **263**, 7933–7936.

GANDOLFO, G., GOTTESMAN, C., BIDARD, J-N. & LAZDUNSKI, M. (1989a). K^+ channel openers prevent epilepsy induced by the bee venom peptide MCD. *Eur. J. Pharmacol.*, **159**, 329–330.

GANDOLFO, G., ROMETTINO, S., GOTTESMAN, C., VAN LUIJTELLAR, G., COENEN, A., BIDARD, J-N. & LAZDUNSKI, M. (1989b). K^+ channel openers decrease seizures in genetically epileptic rats. *Eur. J. Pharmacol.*, **167**, 181–183.

GARRINO, M.G., PLANT, T.D. & HENQUIN, J.C. (1989). Effects of putative activators of K^+ channels in mouse pancreatic beta cells. *Br. J. Pharmacol.*, **98**, 957–965.

GELBAND, C.H., SILBERBERG, S.D., GROSCHNER, K. & VAN BREMEN, C. (1990). ATP inhibits smooth muscle Ca^{2+}-activated K^+ channels. *Proc. R. Soc. B.*, **242**, 23–28.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HUNTER, M. & GIEBISCH, G. (1988). Calcium-activated K-channels of *Amphiuma* early distal tubule: inhibition by ATP. *Pflügers Arch.*, **412**, 331–333.

KLÖCKNER, U. & ISENBERG, G. (1992). ATP suppresses activity of Ca^{2+}-activated K^+ channels by Ca^{2+} chelation. *Pflügers Arch.*, **420**, 101–105.

KOZLOWSKI, R.Z., HALES, C.N. & ASHFORD, M.L.J. (1989). Dual effects of diazoxide on ATP-K^+ currents recorded from an insulin-secreting cell line. *Br. J. Pharmacol.*, **97**, 1039–1050.

KUNZELMANN, K., PAVENSTADT, H. & GREGER, R. (1989). Characterisation of potassium channels in respiratory cells. II. Inhibitors and regulation. *Pflügers Arch.*, **414**, 297–303.

MILLER, R.J. (1990). Glucose-regulated potassium channels are sweet news for neurobiologists. *Trends. Neurosci.*, **13**, 197–199.

MILLER, J.A., VELAYO, N.L., DAGE, R.C. & RAMPE, D. (1991). High affinity [^{3}H] glibenclamide binding sites in rat neuronal and cardiac tissue: localisation and developmental characteristics. *J. Pharmacol. Exp. Ther.*, **256**, 358–364.

OOMURA, Y., OOYAMA, H., SUGIMORI, M., NAKAMURA, T. & YAMADA, Y. (1974). Glucose inhibition of the glucose-sensitive neurone in the rat lateral hypothalamus. *Nature*, **247**, 284–286.

POLITI, D.M.T. & ROGAWSKI, M.A. (1991). Glyburide-sensitive K^+ channels in cultured rat hippocampal neurons: activation by cromakalim and energy-depleting conditions. *Mol. Pharmacol.*, **40**, 308–315.

QUASTHOFF, S., SPULER, A., LEHMANN-HORN, F. & GRAFE, P. (1989). Cromakalim, pinacidil and RP 49356 activate a tolbutamide-sensitive K^+ conductance in human skeletal muscle fibres. *Pflügers. Arch.*, **414**, S179–S180.

RIPOLL, C., LEDERER, W.J. & NICHOLS, C.G. (1990). Modulation of ATP-sensitive K+ channel activity and contractile behaviour in mammalian ventricle by the potassium channel openers cromakalim and RP49356. *J. Pharmacol. Exp. Ther.*, **255**, 429–435.

ROBERTSON, D.W. & STEINBERG, M.I. (1990). Potassium channel modulators: scientific applications and therapeutic promise. *J. Med. Chem.*, **33**, 1529–1540.

ROWE, I.C.M. & ASHFORD, M.L.J. (1991) The effects of Mg^{2+} ions on the inhibition of the ATP-sensitive K^+ channel by adenosine nucleotides in rat isolated hypothalamic neurones. *J. Physiol.*, **446**, 274P.

ROWE, I.C.M., TREHERNE, J.M. & ASHFORD, M.L.J. (1992). The effects of adenosine nucleotides on the ATP-activated potassium channel in rat isolated hypothalamic neurones. *J. Physiol.* (in press).

SANGUINETTI, M.C., SCOTT, A.L., ZINGARO, G.J. & SIEGL, P.K.S. (1988). BRL34915 (cromakalim) activates ATP-sensitive K^+ current in cardiac muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8360–8364.

SCHMID-ANTOMARCHI, H., AMOROSO, S., FOSSETT, M. & LAZDUNSKI, M. (1990). K^+ channel openers activate brain sulfonylurea-sensitive K^+ channels and block neurosecretion. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 3489–3492.

STURGESS, N.C., KOZLOWSKI, R.Z., CARRINGTON, C.A., HALES, C.N. & ASHFORD, M.L.J. (1988). Effects of sulphonylureas and diazoxide on insulin-secretion and nucleotide-sensitive channels in an insulin-secreting cell line. *Br. J. Pharmacol.*, **95**, 83–94.

TREHERNE, J.M. & ASHFORD, M.L.J. (1991). Calcium-activated potassium channels in rat dissociated ventromedial hypothalamic neurones. *J. Neuroend.*, **3**, 323–329.

TRICKLEBANK, M.D., FLÖCKHART, G. & FREEDMAN, S.B. (1988). The potassium channel activator, BRL 34915, antagonises a behavioural response to the muscarinic receptor agonist, pilocarpine. *Eur. J. Pharmacol.*, **151**, 349–350.

WESTON, A.H. (1989). Smooth muscle K^+ channel openers; their pharmacology and clinical potential. *Pflügers. Arch.*, **414**, S99–S105.

ZÜNKLER, B.J., LENZEN, S., MÄNNER, K., PANTEN, U. & TRUBE, G. (1988). Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic beta cells. *Naunyn-Scmiedebergs Arch. Pharmacol.*, **337**, 225–230.

(Received June 24, 1992
Revised August 5, 1992
Accepted August 6, 1992)

Br. J. Pharmacol. (1992), **107**, 1075–1082

Involvement of cyclic GMP in non-adrenergic, non-cholinergic inhibitory neurotransmission in dog proximal colon

Sean M. Ward, *Hugh H. Dalziel, *Michael E. Bradley, *Iain L.O. Buxton, Kathleen Keef, *David P. Westfall & [1]Kenton M. Sanders

Departments of Physiology and *Pharmacology, University of Nevada School of Medicine, Reno, NV 89557, U.S.A.

1 Nitric oxide (NO) may serve as a non-adrenergic, non-cholinergic (NANC) neurotransmitter released from enteric inhibitory nerves in the gastrointestinal tract. We tested whether guanosine 3':5'-cyclic monophosphate (cyclic GMP) may serve as a second messenger in transducing the NO signal into inhibitory junction potentials (i.j.ps) and relaxation in the canine proximal colon.

2 The membrane permeable analogue of cyclic GMP, 8-bromo cyclic GMP (8-Br-cyclic GMP) mimicked the effects of NO by hyperpolarizing cells near the myenteric border of the circular muscle layer and shortening slow waves in cells near the submucosal surface of the circular muscle layer. 8-Br-cGMP also inhibited spontaneous phasic contractions.

3 The specific cyclic GMP phosphodiesterase inhibitor, M&B 22948, hyperpolarized cells near the myenteric border and prolonged the duration of i.j.ps. M&B 22948 also inhibited phasic contractile activity.

4 Methylene blue failed to reduce significantly the amplitude and duration of i.j.ps and had variable effects on contractions.

5 Cyclic GMP levels were assayed in unstimulated muscles and in muscles exposed to exogenous NO and electrical field stimulation. Both stimuli hyperpolarized membrane potential, inhibited contractions, and elevated cyclic GMP levels.

6 Treatment of muscles with L-N^G-nitroarginine methyl ester (L-NAME) increased spontaneous contractile activity and lowered cyclic GMP levels. The inhibitory effect of M&B 22948 on contractions was greatly reduced after muscles were treated with L-NAME.

7 These data support the concept that the effects of NANC nerve stimulation and NO (which may be one of the enteric inhibitory transmitters) may be mediated by cyclic GMP.

Keywords: Nitric oxide; non-adrenergic, non-cholinergic nerves; colonic motility; cyclic GMP; enteric nervous system, methylene blue, gastrointestinal motility

Introduction

Nitric oxide (NO) may serve as a neurotransmitter in non-adrenergic, non-cholinergic (NANC) responses in the oesophagus (Murray *et al.*, 1991; Tottrup *et al.*, 1991), stomach (Desai *et al.*, 1991; Boeckxstaens *et al.*, 1991), small bowel (Toda *et al.*, 1990; Stark *et al.*, 1991), ileocolonic sphincter (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990; Ward *et al.*, 1992b), colon (Dalziel *et al.*, 1991; Thornbury *et al.*, 1991; Ward *et al.*, 1992a; Huizinga *et al.*, 1992) and internal anal sphincter (Rattan & Chakder, 1991). In many of these muscles relaxation of tonic contraction or reduction in the amplitude of phasic contractions are mediated via hyperpolarization responses known as inhibitory junction potentials (i.j.ps; Burnstock *et al.*, 1963; 1966). I.j.ps are thought to be due to a transient increase in potassium conductance (Tomita, 1972), and repetitive electrical field stimulation can lead to summation of i.j.ps, prolonged hyperpolarization, and sustained inhibition of contractile activity (e.g. Thornbury *et al.*, 1991). At least one K channel has been shown to be activated by NO stimulation (Thornbury *et al.*, 1991), but the transduction mechanism that causes this effect is unknown. In vascular smooth muscles, NO binds to a haeme group and activates soluble guanylate cyclase (Craven & DeRubertis, 1978). The production of guanosine 3':5'-cyclic monophosphate (cyclic GMP), and perhaps phosphorylation of cellular proteins by cyclic GMP-dependent protein kinase, is thought to transduce the NO signal and produce relaxation of smooth muscle cells (Rapoport & Murad, 1983).

Previous studies have also shown that electrical field stimulation evokes cyclic GMP formation in the lower oesphageal sphincter (Torphy *et al.*, 1986), and it is well known that elevation of cyclic GMP causes relaxation of a variety of smooth muscles (e.g. Barnette *et al.*, 1989; Katsuki *et al.*, 1977; Rattan & Moummi, 1988). Recent evidence has shown that cystamine and methylene blue can block hyperpolarization responses to sodium nitroprusside and electrical field stimulation in the opposum oesphagus (Du & Conklin, 1992). These observations suggest that cyclic GMP formation may mediate responses to NO (and therefore enteric inhibitory nerve responses) in gastrointestinal muscles. In the present study this hypothesis was tested by measuring the electrical and mechanical effects of elevation of cyclic GMP with the membrane permeable form of cyclic GMP, 8-bromo cyclic GMP and by delaying the metabolism of cyclic GMP with a specific cyclic GMP phosphodiesterase inhibitor, M&B 22948 (Weishaar *et al.*, 1986). The effects of enteric inhibitory nerve stimulation, inhibitors of NO synthesis, and exogenous NO on cyclic GMP levels in muscles of the canine proximal colon were also examined.

Methods

Mongrel dogs of either sex were anaesthetized with sodium pentobarbitone (45 mg kg^{-1}). The abdomen of each animal was opened and a segment of proximal colon, 4–12 cm from the ileocolonic sphincter, was removed. The colonic segment was opened along the mesenteric border and faecal material was removed by washing with Krebs-bicarbonate solution.

[1] Author for correspondence.

The resulting sheet was pinned out in a dissecting dish. Muscle strips (1 mm by 10 mm) were cut parallel to the longitudinal muscle fibres for electrophysiological studies (see Smith *et al.*, 1987a,b) and parallel to the circular fibres for mechanical/biochemical studies, and mucosal tissues were removed. The muscles were maintained in Krebs-bicarbonate solution at 37.5 ± 0.5°C. The Krebs-bicarbonate solution used in this study contained (in mM): NaCl 110, KCl 4.6, $CaCl_2$ 2.5, $NaHCO_3$ 24.8, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 5.6. When equilibrated with 95% O_2/5% CO_2, this solution had a pH of 7.3–7.4. Atropine, phentolamine and propranolol (all at 10^{-6} M) were routinely added to block muscarinic receptors and adrenoceptors ('NANC solution'). In the presence of these agents, responses elicited by electrical field stimulation (EFs) were blocked by tetrodotoxin (10^{-6} M) and were therefore regarded as enteric inhibitory nerve responses.

Electrophysiology experiments

The electrophysiological chamber was continuously perfused with Krebs-bicarbonate solution, and muscles were allowed to equilibrate for at least 2 h before intracellular recordings were initiated. Smooth muscle cells near either the submucosal or myenteric borders of the circular muscle layer were impaled with glass microelectrodes to measure enteric inhibitory nerve responses (Smith *et al.*, 1987a,b). Transmembrane potential was measured with a standard electrometer (WPI M-7000), and outputs were displayed on an oscilloscope. Signals were recorded on magnetic tape and chart paper. Electrical field stimulation (EFS) was delivered as square wave pulses (0.5 ms duration, supramaximal voltage at a variety of frequencies) from a Grass S44 stimulator coupled via a stimulus isolation unit (Grass SIU5) to platinum wire electrodes placed on either side of the muscle strips.

Mechanical and biochemical experiments

Muscle strips, cut parallel to the circular fibres, were mounted in jacketed tissue baths under a resting tension of 1 g and allowed to equilibrate for 60–90 min. This degree of resting tension has been shown to produce optimal tension in canine colonic circular muscles (Keef *et al.*, 1991). During the equilibration period, some muscles developed spontaneous phasic contractions and others were mechanically quiescent. EFS (0.5 ms, supramaximal voltage at selected frequencies) was performed in a similar manner to that used in electrophysiological experiments. The Krebs-bicarbonate solution bathing the preparations was changed every 15 min. Mechanical responses were recorded with force transducers (Grass FT03) and a polygraph chart recorder. Mechanical responses were tabulated by measuring the area under the traces for a 3 min period before and one minute after addition of drugs. Effects are expressed as percentage change from control activity per minute.

To assay cyclic GMP levels, muscles were snap frozen by quickly removing the tissue holder from the tissue bath and freeze clamping the tissue with flat tongs that had been cooled in liquid N_2. Frozen muscles were stored at −85°C and later assayed for cyclic GMP content (see below).

In some experiments the muscle strips were bisected, creating strips consisting of the submucosal and myenteric halves of the circular muscle layer. The myenteric strips contained the myenteric plexus and the longitudinal muscle layer, and submucosal strips contained submucosal elements including enteric ganglia as confirmed histologically. These 'isolated region' muscle strips were used to measure cyclic GMP levels in response to nitric oxide (NO) or EFS. These muscles were also used to compare the inhibitory effects of NO on contractions of the 2 regions. These muscles were pre-stimulated with ACh (3×10^{-7} M; in the presence of tetrodotoxin, 10^{-6} M), and the effects of various concentrations of NO on phasic contractions were studied.

Cyclic GMP determination

Cyclic GMP was assayed by an enzyme immunoassay method (Caymen Chemical Company, Ann Arbor, MI, U.S.A.). Samples were prepared for assay by homogenization in 6% TCA with glass Duall tissue grinders followed by extraction with water-saturated diethyl ether. Aqueous phases were then lyophilized to dryness and resuspended in 1.0 M potassium phosphate buffer (pH 7.4) before addition to duplicate microtiter plate wells. Cyclic GMP levels in samples and standards were detected following competition between cyclic GMP and the acetylcholinesterase-linked cyclic GMP tracer for specific antiserum binding sites. The antiserum complex, linked to acetylcholinesterase, was used to cleave Ellmans reagent, and absorbance was measured at 412 nm. Cyclic GMP content of samples was determined from a standard curve constructed from determination of known amounts of cyclic GMP added to the plate. Levels of cyclic GMP are expressed as pmol cyclic GMP mg^{-1} protein (determined by method of Bradford, 1976). Duplicate variation in the cyclic GMP assay was less than 3%.

Drugs and active agents

Nitric oxide: Stock solutions of NO were prepared by bubbling ice-cold, deoxygenated (sonication under vacuum followed by purging with 100% N_2 gas) distilled water with NO gas (99% pure) to give a saturated solution (1–1.5 mM; Ignarro *et al.*, 1987). In tension experiments NO was delivered to the tissues by addition of the appropriate volume of stock solution directly to the tissue chamber. The stated concentrations of NO have not been corrected for breakdown and therefore may be somewhat overestimated. Addition of water alone instead of NO solution had no effect on electrical or mechanical activity.

Other drugs: L-N^G-nitroarginine methyl ester (L-NAME), L-N^G-monomethyl arginine (L-NMMA) and acetylcholine (ACh) (Sigma) were made in stock solutions at 10^{-1} M. Propranolol (Sigma) was obtained as the hydrochloride salt. Atropine (Sigma) was used as the sulphate salt and phentolamine (Ciba Geigy) as the mesylate salt. Substance P (Sigma) was dissolved in phosphate buffer (10^{-4} M). M&B 22948 (zaprinast; a gift from Rhone-Poulenc Rorer, Dagenham, England) was dissolved in 0.1 N NaOH at a concentration of 10^{-2} M. Methylene blue and tetrodotoxin (TTX) were also obtained from Sigma. LY-83583 (6-anilo-5,8-quinolinedione; CalBiochem) was dissolved in a stock solution (10^{-2} M). Stock solutions were diluted to desired concentrations with Krebs-bicarbonate solution.

Data analysis

Statistical significance of differences between the means of data groups was determined by Student's *t* test for paired or unpaired data, as appropriate.

Results

Control electrical and mechanical responses

Cells near the myenteric border had average resting membrane potentials (RMP) of - 45 ± 2 mV ($n = 24$ preparations) and exhibited small spontaneous electrical oscillations as previously described (Smith *et al.*, 1987b). Electrical field stimulation (EFS) induced hyperpolarization or inhibitory junction potentials (i.j.ps).

Cells at the submucosal border had more negative RMPs, averaging (−82 ± 2 mV, $n = 12$) and exhibited spontaneous slow wave activity as previously described (cf. Smith *et al.*, 1987a). EFS reduced the amplitude of slow waves during stimulation, and a 'rebound excitation' followed the period of stimulation (Ward *et al.*, 1992a).

The majority of circular muscle strips were spontaneously

mechanically active and exhibited small phasic contractions at the same frequency as the electrical slow waves (i.e. 5–6 cycles per min). These contractions were often irregular in amplitude and rarely exceeded 10% of the maximum contractile amplitude produced with ACh (10^{-4} M; Keef *et al.*, 1991). EFS (5–20 Hz) in the absence of antagonists consistently gave rise to an excitatory contractile response ($n = 9$). In the presence of muscarinic receptor and adrenoceptor antagonists ('NANC solution'), EFS inhibited spontaneous contractions.

Effects of 8-bromo cyclic-GMP

Muscles were exposed to the membrane permeable analogue of cyclic GMP, 8-bromo cyclic GMP (8-Br-cyclic GMP) to determine whether increasing cyclic GMP would mimic the effects of NO. 8-Br-cyclic GMP (10^{-3} M) caused hyperpolarization of cells near the myenteric border averaging 23 ± 3 mV (from -50 ± 3.8 mV to -73 ± 2.8 mV; $n = 5$ $P < 0.005$). Oscillatory electrical activity was superimposed upon the hyperpolarization, this activity increased in amplitude from an average of 9.2 ± 1.5 mV prior to the addition of 8-Br-cyclic GMP to 32 ± 4.0 mV in the presence of 8-Br-cyclic GMP ($P < 0.005$; $n = 5$). Although the absolute amplitude of oscillatory activity increased in the presence of 8-Br-cyclic GMP, the maximum depolarization level reached during oscillations was reduced (see Figure 1a), suggesting that 8-Br-cyclic GMP would inhibit phasic contractions (Barajas-Lopez & Huizinga, 1989). This type of electrical activity has also been observed in response to NO, the NO-thiol conjugate, *S*-nitrosocysteine, and during sustained EFS (Thornbury *et al.*, 1991).

8-Br-cyclic GMP did not significantly hyperpolarize cells near the submucosal border (Figure 1b), which may have been due to the negative RMPs of these cells (-83 ± 2 mV; $n = 6$). 8-Br-cyclic GMP decreased the frequency (from 4.6 ± 0.2 cycles min^{-1} to 3.0 ± 0.3 cycles min^{-1}; $n = 6$; $P < 0.005$) and duration of slow waves (from 4.3 ± 0.2 s to 2.9 ± 0.4 s; $n = 6$; $P < 0.05$; Figure 1b). The amplitude of slow waves was unchanged by this compound. The effects of 8-Br-cyclic GMP persisted for at least 20–30 min after removal of the drug from the bath.

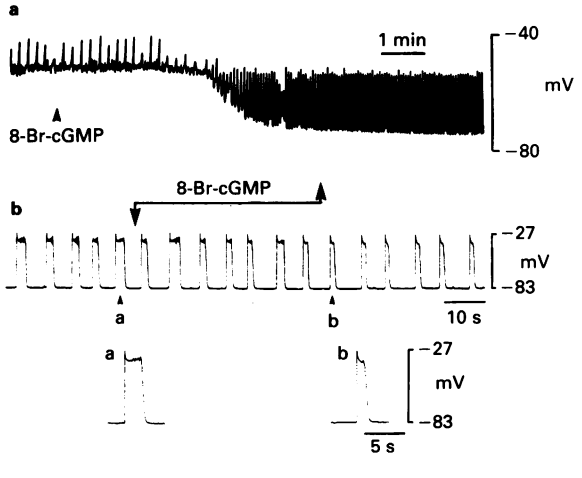

Figure 1 Effect of 8-Br-cyclic GMP (8-B-cGMP) on electrical activities of a cell near the myenteric border (a) and a cell near the submucosal surface (b). In the myenteric cell, membrane potential was about -50 mV and small electrical oscillations were superimposed. Addition of 8-Br-cyclic GMP (at arrow, 10^{-3} M) caused a large hyperpolarization and larger amplitude oscillations were superimposed. Despite the large amplitude of these events, at their peaks they did not reach even the former resting potential level. (b) Shows slow waves recorded from a submucosal cell. Addition of 8-Br-cyclic GMP (10^{-3} M) had little or no effect on resting potential but shortened the duration of slow waves.

A separate series of experiments tested the effects of 8-Br-cyclic GMP (10^{-4} M) on the contractile activity of circular muscle strips. 8-Br-cyclic GMP (10^{-4} and 10^{-3} M) inhibited contractile activity in these muscles by an average of $51 \pm 12\%$ ($n = 5$) and $86 \pm 3\%$ ($n = 6$), respectively. The effects of 8-Br-cyclic GMP were persistent, and at least 30 min were required after removal of the drug (10^{-3} M) for full restoration of control activity. Figure 2 shows the inhibitory effects of 8-Br-cyclic GMP on contractile activity.

Effects of M&B 22948

In cells near the myenteric border M&B 22948 (10^{-4} M) caused hyperpolarization averaging 11 ± 4 mV (i.e. from -41 ± 1.9 mV under control conditions to -52 ± 4 mV after the addition of M&B 22948; $n = 8$, $P < 0.05$). In the 2 preparations, where M&B 22948 produced the largest hyperpolarizations, electrical oscillations were superimposed upon the hyperpolarizations (Figure 3a). These events were similar to the responses caused by nitric oxide, S-nitrosocysteine, and EFS at 1 Hz (see Thornbury *et al.*, 1991). At the submucosal border M&B 22948 (10^{-4} M) did not significantly alter resting membrane potential, slow wave upstroke and plateau amplitude, slow wave duration or frequency ($n = 4$; Figure 3b).

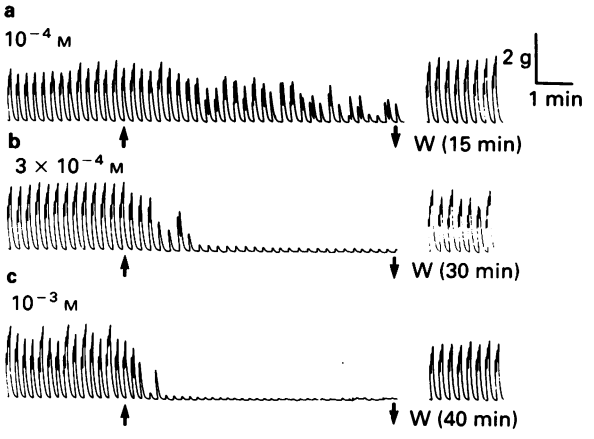

Figure 2 Effects of 8-Br-cyclic GMP on mechanical activity of colonic muscles. Spontaneously active muscles were exposed to 10^{-4} to 10^{-3} M 8-Br-cyclic GMP for 10 min. Representative responses to 3 concentrations are shown. 8-Br-cyclic GMP reduced the amplitude of contractions in a concentration-dependent manner. Panels to right of each trace show restoration of control activity after wash periods noted in figure.

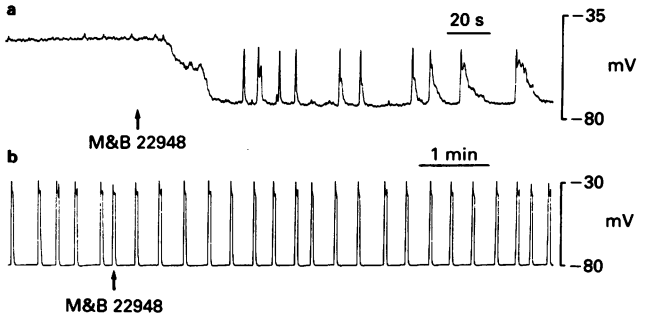

Figure 3 Effect of M&B 22948 (10^{-4} M) on electrical activities of cells near myenteric (a) and submucosal (b) boundaries of the circular muscle layer. Addition of M&B 22948 in (a) caused a large hyperpolarization similar to the effects of 8-Br-cyclic GMP. Large amplitude oscillations were superimposed upon the hyperpolarization response. M&B 22948 had little or no effect on slow waves at the submucosal border (b). All effects were reversible upon washout of the drug.

I.j.ps were elicited by trains of stimuli (1–10 pulses at 20 Hz) before and after incubation with M&B 22948 (10^{-4} M; 10 min). M&B 22948 significantly increased the duration of i.j.ps (e.g. from 3.5 ± 0.24 s to 10.8 ± 1.9 s with 6 pulse trains (0.5 ms at 20 Hz) $n = 6$; $P < 0.05$; see Figure 4). There was not a significant change in the absolute amplitude of i.j.ps in the presence of M&B 22948, but this result is somewhat difficult to interpret since this compound also hyperpolarized membrane potential. M&B 22948 also caused a change in the shape of i.j.ps (see Figure 4a). After M&B 22948, i.j.ps, reached a maximum level of hyperpolarization, partially repolarized, and then settled into a sustained 'plateau' hyperpolarization. Repolarization occurred in 2 phases, an initial rapid component, followed by a slow component with a time-constant of several seconds.

M&B 22948 (10^{-4} M) essentially abolished mechanical activity in intact strips of muscle ($n = 6$). This effect persisted while the drug was present, but was reversible upon washout.

Effects of methylene blue

Others have shown that methylene blue inhibits the soluble guanylate cyclase in smooth muscles (Ignarro *et al.*, 1986). Methylene blue (10^{-5} to 10^{-4} M) added for up to 1.5 h did not significantly affect membrane potential in cells near the myenteric border (−46 ± 3 mV to −41 ± 2 mV; 100 μM) or i.j.p. amplitude and duration (i.e. 29 ± 4 mV to 27 ± 3 mV and 4.0 ± 0.4 s to 4.0 ± 0.4 s, respectively; P values > 0.5; $n = 7$), (Figure 5). We also tested the effects of LY-83583 (10^{-5} M; an inhibitor of soluble guanylate cyclase; Mulsch *et al.*, 1988) on i.j.ps. In 5 preparations, this compound failed to reduce i.j.p. amplitude or duration.

In addition to a lack of effects on i.j.ps, methylene blue (10^{-4} M) also had no significant effect on hyperpolarization

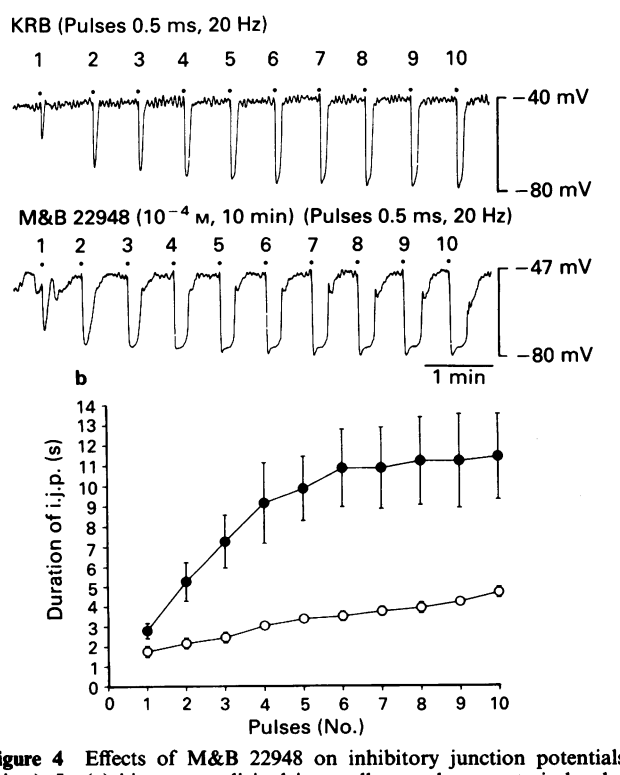

Figure 4 Effects of M&B 22948 on inhibitory junction potentials (i.j.ps). In (a) i.j.ps were elicited in a cell near the myenteric border by electrical field stimulation. Trace shows responses to short trains consisting of the number of pulses indicated. Addition of M&B 22948 (10^{-4} M) caused hyperpolarization (about 7 mV in this muscle) and an increase in the duration of the i.j.ps. (b) Shows a summary plot of i.j.p. duration as a function of the number of pulses in the stimulus train ($n = 6$): (○) control; (●) M&B 22948 (10^{-4} M).

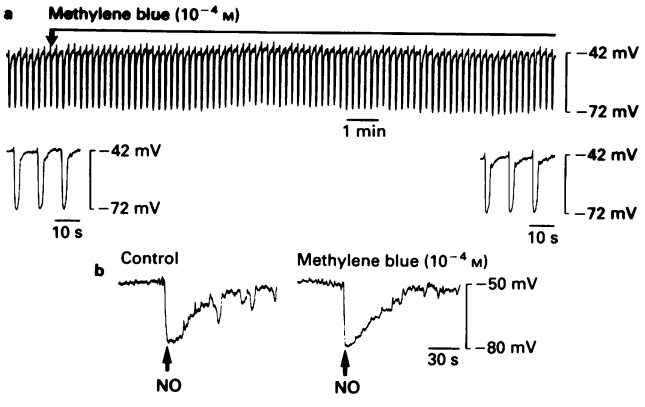

Figure 5 Effects of methylene blue on i.j.ps; (a) shows continuous recording after exposure to methylene blue (10^{-4} M). In this cell methylene blue caused depolarization. Methylene blue did not significantly affect the amplitude or duration of i.j.ps. (see text for averaged values). (b) Methylene blue did not affect hyperpolarization induced by nitric oxide.

responses to NO (5 μl bolus application to recording chamber; $n = 4$). In these experiments resting potential averaged −45 ± 2.1 mV and −43 ± 2.5 mV before and in the presence of methylene blue, respectively. NO caused an average 27 ± 2.5 mV hyperpolarization before methylene blue and 28.3 ± 1.4 hyperpolarization in the presence of the drug.

The effects of methylene blue were not tested at the submucosal border, because we have previously shown that this compound depolarizes these cells due to non-specific effects, making interpretation of results very difficult (Sanders *et al.*, 1989). Methylene blue had variable effects on mechanical activity, which may have been due to the non-specific actions of this compound.

Effect of NO on cyclic GMP levels and mechanical activity

NO (10^{-5} M; muscles taken for assay 15 s after addition of NO) significantly increased cyclic GMP content (Figure 6) and caused concentration-dependent relaxation of colonic muscles (Figure 7).

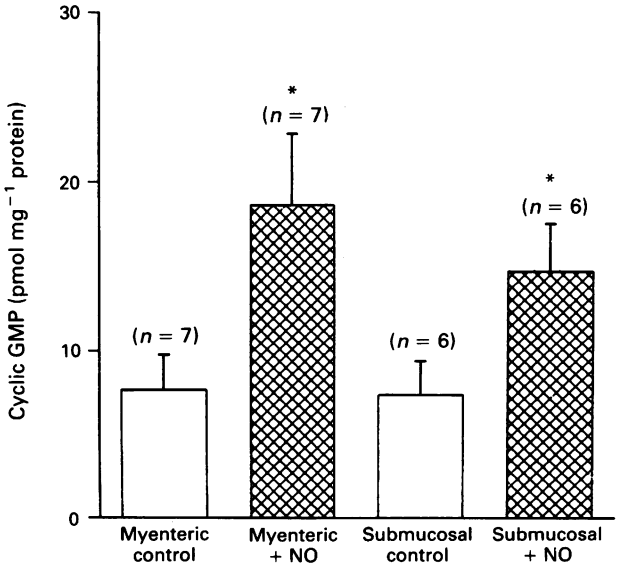

Figure 6 Effects of NO on cyclic GMP levels in colonic circular muscles. Cyclic GMP was measured in isolated strips dissected from the myenteric and submucosal halves of the circular layer. NO (10^{-5} M) caused a significant increase in cyclic GMP in both regions ($P < 0.05$).

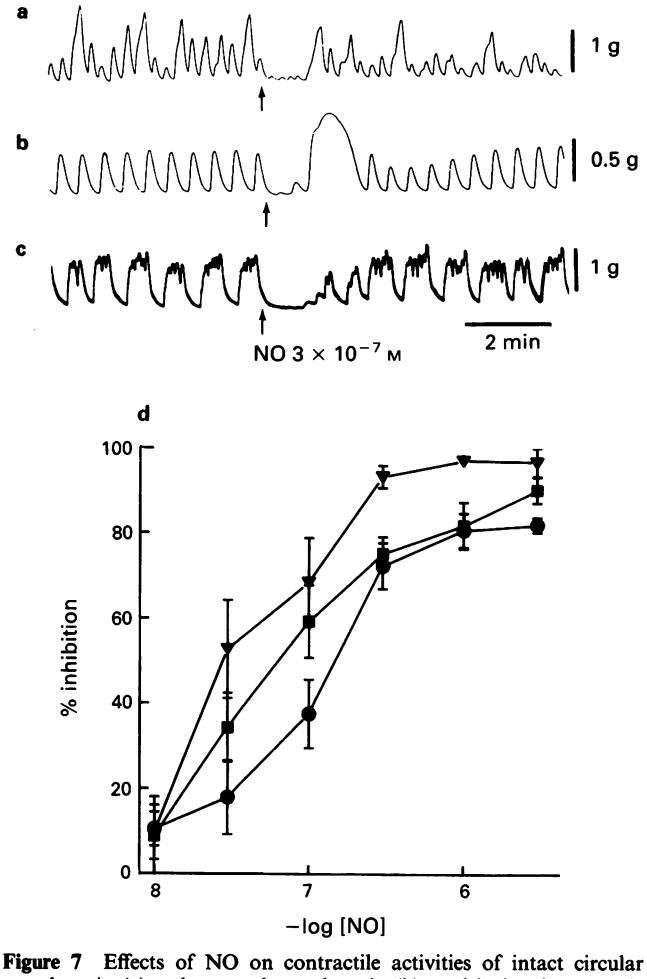

Figure 7 Effects of NO on contractile activities of intact circular muscle strip (a), submucosal muscle strip (b), and isolated myenteric muscle strip (c). Muscles were prestimulated with acetylcholine (3×10^{-7} M), and tetrodotoxin (10^{-6} M) was included in the bath solution. In each panel addition of NO (3×10^{-7} M) caused an interruption in the normal pattern of contractile activity. In (b), as the inhibitory effects of NO diminished there is a rebound contraction; (d), shows concentration-response relationships for myenteric (▼), submucosal (■) and intact (●) circular muscles. Data are averages and s.e.mean (vertical bars) from 6 experiments for each curve.

In some experiments muscles were bisected into the myenteric and submucosal regions (see Methods) and changes in cyclic GMP levels in each region in response to NO were assayed. Figure 6 shows that NO produced significant and similar increases in cyclic GMP levels in both myenteric and submucosal regions. NO also inhibited contractile activity in both regions (Figure 7). The concentration-response curve for the effects of NO on intact muscles was shifted to the right with respect to the responses of isolated myenteric and submucosal muscles, because NO may not have penetrated the intact muscles as completely before it was inactivated.

Effect of EFS on cyclic GMP levels

EFS (5 Hz, 0.5 ms, supramaximal voltage) for 15 s caused cessation of mechanical activity in intact muscle strips. Assay of cyclic GMP in intact muscles failed to detect significant changes in cyclic GMP levels in response to EFS. We also tested the effects of EFS on regional muscle strips consisting of: (i) the circular muscle along the submucosal surface of the circular layer, and (ii) circular muscle near the myenteric border. After equilibration and in the presence of the 'NANC solution', phasic contractions were stimulated with substance P (10^{-7} M). After 5 min in substance P, EFS (5 Hz, 0.5 ms, supramaximal voltage) was applied for 15 s. Cyclic GMP levels were elevated by EFS (Table 1). The increase in cyclic GMP produced by EFS was enhanced by M&B 22948.

Effect of L-NAME on spontaneous activity

Exposure of intact muscles to the NO-synthase inhibitor, L-NAME (10^{-4} M) significantly enhanced the amplitude of spontaneous contractions by an average of 256 ± 46% (n = 8; $P<0.05$; Figure 8). L-NAME reduced cyclic GMP levels in 4 of 6 muscles studied (Figure 8).

After inhibition of spontaneous contractile activity with M&B 22948, L-NAME (10^{-4} M; $n = 3$) also reversed the effects of M&B 22948 by more than 95% (Figure 9). When

Table 1 Effects of NANC nerve stimulation on cyclic GMP levels in colonic muscles

	Control	*NANC stimulation*
Tissues		
Myenteric		
Basal	177 ± 75	*392 ± 50
+M&B22948	75 ± 43	**257 ± 92
Submucosal		
Basal	117 ± 33	**242 ± 52
+M&B22948	81 ± 9.6	*391 ± 115

Cyclic GMP was measured in substance P-stimulated segments of colonic smooth muscle with or without NANC nerve stimulation (5 Hz) in the presence and absence of the cyclic GMP-phosphodiesterase inhibitor M&B 22948 (10^{-4} M) as described in Methods. Contraction was monitored and tissues frozen in Liq. N_2 at 15 s following EFS or at matched times for control tissues. Values are expressed as the mean ± s.e. of three experiments.
* Significant difference from control: $P \leq 0.05$. **Significant difference from control: $P \leq 0.03$.

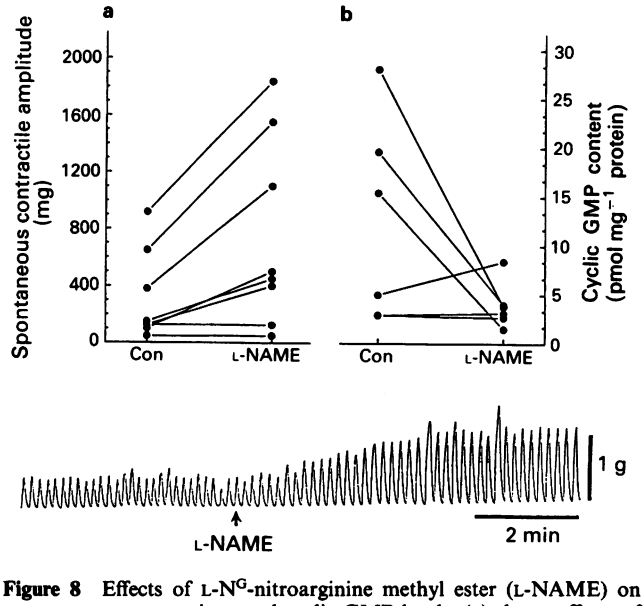

Figure 8 Effects of L-N^G-nitroarginine methyl ester (L-NAME) on spontaneous contractions and cyclic GMP levels: (a) shows effects of L-NAME (10^{-4} M) on the average spontaneous contractile amplitude; see example trace below graphs. L-NAME increased the force of phasic contractions from an average amplitude of 312 ± 111 mg to 752 ± 253 mg ($n = 8$). In another series of experiments, muscles were frozen for cyclic GMP assay before and 25 min after exposure to L-NAME (10^{-4} M; b). L-NAME decreased cyclic GMP in 4 of 6 of the muscles studied. Data suggest that some muscles may have spontaneous release of NO and therefore elevated levels of cyclic GMP. Block of NO synthesis can decrease production of cyclic GMP and decrease tonic inhibition.

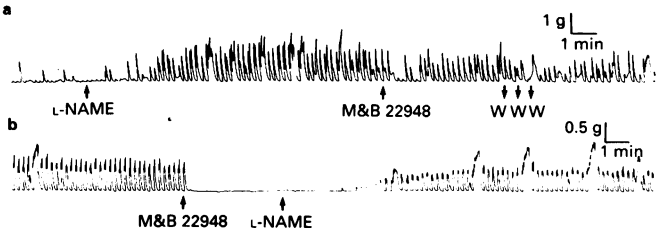

Figure 9 L-N^G-nitroarginine methyl ester (L-NAME) prevents or reverses the effects of M&B 22948: (a) shows spontaneous contractile activity enhanced by L-NAME. After L-NAME, M&B 22948 caused much less reduction in contractile amplitude than in muscles untreated by L-NAME (see Figure 5 and b). (b) Shows inhibition of effects by M&B 22948. L-NAME reversed the inhibitory effect of M&B 22948. These data suggest that effects of M&B 22948 depend upon NO synthesis, and suggest that NO effects are mediated via cyclic GMP.

M&B 22948 (10^{-4} M) was added after treatment with L-NAME ($n = 4$) contractile amplitude was reduced by $33 \pm 4\%$ (Figure 9a), whereas in muscles untreated with L-NAME, this concentration of M&B 22948 abolished mechanical activity (Figure 9b and see section above on M&B 22948).

Discussion

The results of this study provide additional support for the hypothesis that NO is an essential component of enteric inhibitory neurotransmission in the canine gastrointestinal tract. Previous studies showed that EFS increased cyclic GMP levels in the bovine retractor penis muscle (Bowman & Drummond, 1984), corpus cavernosum smooth muscle (Ignarro *et al.*, 1990), and oesophageal muscles (Torphy *et al.*, 1986). The present results add to this concept, suggesting that cyclic GMP may be involved in transducing enteric inhibitory neurotransmission in the proximal colon. This idea is supported by the following: (i) NO inhibited mechanical activity (we showed previously that NO hyperpolarized membrane potential; see Thornbury *et al.*, 1991) and elevated cyclic GMP, (ii) cyclic GMP (delivered as its membrane permeable analogue) mimicked the hyperpolarization and mechanical effects of NO and EFS, (iii) M&B 22948, a selective cyclic GMP-phosphodiesterase inhibitor, inhibited mechanical activity, hyperpolarized RMP, and prolonged i.j.ps. These observations satisfy several of the major criteria necessary for establishing cyclic GMP as a second messenger in enteric inhibitory neurotransmission in the canine colon (see discussion in Conklin & Du, 1992).

Few studies have previously shown an increase in cyclic GMP during EFS in GI muscles. We found that it was difficult to demonstrate changes in cyclic GMP in strips of colonic muscle consisting of the full thickness of the muscularis. This may have been due to a localization of the elevation of cyclic GMP within cells or tissues. We have previously reported that the enteric inhibitory innervation is functionally directed toward specific 'pacemaker regions' within the muscularis of the canine proximal colon (Smith *et al.*, 1989), and recent morphological studies have demonstrated nitric oxide synthase-like immunoreactivity in nerves near the submucosal and myenteric pacemaker regions (Ward *et al.*, 1992c). Changes in cyclic GMP were demonstrable in full thickness muscles when exogenous NO was applied (e.g. we observed a 2.5 fold change in cyclic GMP levels), but it was more difficult to demonstrate significant changes in cyclic GMP levels when nerves were activated. This suggests the possibility that NO released from nerves may reach only a fraction of the effector cells. As we have discussed previously, inhibitory innervation of the pacemaker regions is sufficient to reduce the amplitude and durations of slow waves and therefore to reduce the amplitude of phasic contractions throughout the circular muscle layer (Smith *et al.*, 1989). Because responses to NO released from nerves could be specifically directed at pacemaker regions, we also analyzed cyclic GMP changes in muscles dissected from the pacemaker regions. In these strips EFS produced significant elevation in cyclic GMP levels and these effects were augmented by M&B 22948.

Different basal levels of cyclic GMP were observed in experiments that tested effects of NO versus EFS. There are 2 possible explanations for these differences in basal cyclic GMP: (i) the EFS experiments were performed in the presence of substance P which may lower cyclic GMP levels; (ii) cyclic GMP was normalized against protein content of samples and the relative protein content of the thin (regional) muscle strips may have been different from that in full-thickness or bisected muscle strips (as used in experiments depicted in Figures 6 and 8).

Others have reported that methylene blue, which is known to inhibit soluble guanylate cyclase in smooth muscles (Ignarro *et al.*, 1986), reduces enteric inhibitory nerve-mediated responses in gastrointestinal muscles (Conklin & Du, 1992; Huizinga *et al.*, 1992). In contrast we found that methylene blue had no effect on i.j.ps. These results seem contradictory to the involvement of guanylate cyclase in enteric inhibitory nerve responses, but there are also other explanations for the lack of effects of methylene blue as discussed below.

Cyclic GMP is produced by guanylate cyclases (for review see Tremblay *et al.*, 1988), and several investigators have shown that the enhancement in cyclic GMP levels resulting from stimulation with NO is due to activation of guanylate cyclase (e.g. Mittal & Murad, 1977). Methylene blue has been used frequently as a tool to determine whether cyclic GMP is involved in the transduction of agonist responses because it can inhibit the soluble isoforms of guanylate cyclase (Ignarro *et al.*, 1986). The pharmacology of methylene blue is complex and in some tissues other effects may accompany the inhibition of cyclic GMP formation. For example, we have shown that methylene blue (10^{-5} M) depolarizes colonic muscle cells near the submucosal surface of the circular muscle layer (Sanders *et al.*, 1989). This effect does not appear to be due to an inhibition of spontaneous cyclic GMP production because, in the present study, we found that M&B 22948 (which would tend to preserve spontaneously produced cyclic GMP) had little effect on cells near the submucosal border. Huizinga and co-workers (1992) have reported that methylene blue reduces nerve-mediated relaxations of colonic muscle strips but their data are less than convincing because: (i) methylene blue (used at 5×10^{-5} M) caused a significant potentiation of contractions (which is consistent with our finding that methylene blue depolarizes these muscles), and (ii) it is difficult to tell whether the failure of nerve stimulation to block contractions was due to a block of cyclic GMP formation or because there was insufficient inhibitory transmitter released by nerve stimulation to reverse the depolarization caused by methylene blue. In the present study we examined the effects of methylene blue on cells near the myenteric border of the circular layer because the membrane potentials of these cells are not greatly affected by this compound (Sanders *et al.*, 1989). Methylene blue did not significantly affect i.j.ps during exposures of up to 1.5 h.

It is also possible that in some preparations NO effects could be mediated by particulate forms of guanylate cyclase, which are not inhibited by methylene blue. For example, Horio & Murad (1991) have recently purified a particulate form of guanylate cyclase from retinal rod outer segments that is activated by sodium nitroprusside and NO. Others have also reported activation of particulate forms of G cyclase by NO and SNP (Lad & White, 1979; Waldman *et al.*, 1982), although one has to be somewhat cautious about some of the earlier studies because it is possible that a small

amount of soluble guanylate cyclase could have been trapped in the preparation of the particulate form of the enzyme (see Tremblay *et al.*, 1988).

At present the mechanisms by which cyclic GMP causes cellular responses in colonic smooth muscles is not fully understood. The electrical responses are likely to be due to changes in membrane conductance and cyclic GMP is known to regulate ion channels in other cells either directly (Fesenko *et al.*, 1985) or via activation of cyclic GMP-dependent protein kinase (Paupardin-Tritsch *et al.*, 1986). The relaxation response could be mediated by mechanisms in addition to the electrical effects. For example, cyclic GMP-dependent mechanisms affect the Ca^{2+} sensitivity of contractile proteins in gastrointestinal muscles (e.g. Ozaki *et al.*, 1992), and the rate of Ca^{2+} uptake or extrusion may also be regulated by cyclic GMP (Lincoln, 1989).

In summary, our findings are consistent with the hypothesis that cyclic GMP may be a second messenger that transduces the enteric inhibitory transmitter signal into i.j.ps and relaxation in the proximal colon. There is growing evidence that at least a portion of the enteric inhibitory signal arises from release of NO, or a related compound. NO increased cyclic GMP levels in colonic muscle tissues and produced hyperpolarization and relaxation. Some pharmacological tools known to affect levels of cyclic GMP altered electrical and mechanical responses in ways consistent with mechanisms involving cyclic GMP.

This project was supported by a Program Project Grant from the National Institutes of Health (DK 41315) and a Research Grants (HL 38126 to D.P.W. and HD 26227 to I.L.O.B.). The authors acknowledge the technical assistance of Jeff Weinert.

References

BARAJAS-LOPEZ, C. & HUIZINGA, J.D. (1989) Different mechanisms of contraction generation in circular muscle of canine colon. *Am. J. Physiol.*, **256**, G570–G580.

BARNETTE, M., TORPHY, T.J., GROUS, M., FINE, C. & ORMSBEE, H.S. (1989). Cyclic GMP: a potential mediator of neurally- and drug-induced relaxation of opossum lower esophageal sphincter. *J. Pharmacol. Exp. Ther.*, **249**, 524–528.

BOECKXSTAENS, G.E., PELCKMANS, P.A., BOGERS, J.J., BULT. H., DE MAN, J.G., OOSTERBOSCH, L., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991). Release of nitric oxide upon stimulation of nonadrenergic noncholinergic nerves in the rat gastric fundus. *J. Pharmacol. Exp. Ther.*, **256**, 441–447.

BOECKXSTAENS, G.E., PELCKMANS, P.A., BULT, H., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990). Non-adrenergic non-cholinergic relaxation mediated by nitric oxide in the ileocolonic junction. *Eur. J. Pharmacol.*, **190**, 239–246.

BOWMAN, A. & DRUMMOND, A.H. (1984). Cyclic GMP mediates neurogenic relaxation in the bovine retractor penis muscle. *Br. J. Pharmacol.*, **81**, 665–674.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

BULT, H., BOECKXSTAENS, G.E., PELCKMANS, P.A., JORDAENS, F.H., VAN MAERCKE, Y.M. & HERMAN, A.G. (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature*, **345**, 346–347.

BURNSTOCK, G., CAMPBELL, G., BENNETT, M. & HOLMAN, M.E. (1963). Inhibition of the smooth muscle of the taenia coli. *Nature*, **200**, 581–582.

BURNSTOCK, G., CAMPBELL, G. & RAND, M.J. (1966). The inhibitory innervation of the taenia of the guinea-pig caecum. *J. Physiol.*, **182**, 504–526.

CONKLIN, J.L. & DU, C. (1992). Guanylate cyclase inhibitors: effect on inhibitory junction potentials is esophageal smooth muscle. *Am. J. Physiol.*, **263**, 687–690.

CRAVEN, P.A. & DE RUBERTIS, F.R. (1978). Restoration of the responsiveness of purified guanylate cyclase to nitrosoguanidine, nitric oxide, and related activators by heme and heme proteins: Evidence for the involvement of the paramagnetic nitrosylheme complex in enzyme activation. *J. Biol. Chem.*, **253**, 8433–8443.

DALZIEL, H.H., THORNBURY, K.D., WARD, S.M. & SANDERS, K.M. (1991). Involvement of nitric oxide synthetic pathway in inhibitory junction potentials in canine proximal colon. *Am. J. Physiol.*, **260**, G789–G792.

DESAI, K.M., SESSA, W.C. & VANE, J.R. (1991). Involvement of nitric oxide in the reflex relaxation of the stomach to accommodate food or fluid. *Nature*, **351**, 477–479.

FESENKO, E.E., KOLESNIKOV, S.S. & LYUBARSKY, A.L. (1985). Induction by cyclic GMP of cationic conductances in plasma membrane of retinal rod outer segment. Nature, **313**, 310–313.

HORIO, Y. & MURAD, F. (1991). Purification of guanylyl cyclase from rod outer segments. *Biochim. Biophys. Acta*, **1133**, 81–88.

HUIZINGA, J.D., TOMLINSON, J. & PINTIN-QUEZADA, J. (1992). Involvement of nitric oxide in nerve-mediated inhibition and action of vasoactive intestinal peptide in colonic smooth muscle. *J. Pharmacol. Exp. Ther.*, **260**, 803–808.

IGNARRO, L.J., BUSH, P.A., BUGA, G.M., WOOD, K.S., FUKUTO, J.M. & RAJFER, J. (1990). Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Commun.*, **170**, 843–850.

IGNARRO, L.J., BYRNS, R.E., BUGA, G.M. & WOOD, K.S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical those of nitric oxide radical. *Circ. Res.*, **61**, 866–879.

IGNARRO, L.J., HARBISON, R.G., WOOD, K.S. & KADOWITZ, P.J. (1986). Dissimilarities between methylene blue and cyanide on relaxation and cyclic GMP formation in endothelium-intact intrapulmonary artery caused by nitrogen oxide-containing vasodilators and acetylcholine. *J. Pharmacol. Exp. Ther.*, **236**, 30–36.

KATSUKI, S., ARNOLD, W.P. & MURAD, F. (1977. Effects of sodium nitroprusside, nitroglycerin, and sodium azide on levels of cyclic nucleotides and mechanical activity of various tissues. *J. Cyclic Nucleotide Res.*, **3**, 239–247.

KEEF, K.D., WARD, S.M., STEVENS, R.J., FREY, B.W. & SANDERS, K.M. (1992). Electrical and mechanical effects of acetylcholine and substance P on sub-regions of canine colon. *Am. J. Physiol.*, **262**, G298–G308.

LAD, P.J. & WHITE, A.A. (1979). Activation of particulate guanylate cyclase by nitroprusside and MNNG after filipin treatment. *J. Cyclic Nuclotide Res.*, **5**, 315–525.

LINCOLN, T.M. (1989). Cyclic GMP and mechanism of vasodilation. *Pharmacol. Ther.*, **41**, 479–502.

MITTALL, C.K. & MURAD, F. (1977). Activation of guanylate cyclase by superoxide dismutase and hydroxyl radical: A physiological regulator of guanosine 3',5'-monophosphate formation. *Proc. Nat. Acad. Sci. U.S.A.*, **74**, 4360–4364.

MULSCH, A., BUSSE, R., LIEBAU, S. & FOSTERMANN, U. (1988). LY83583 interferes with the release of endothelium derived relaxing factor and inhibits soluble guanylate cyclase. *J. Pharmacol. Exp. Ther.*, **247**, 283–288.

MURRAY, J., DU, C., LEDLOW, A., BATES, J.N. & CONKLIN, J.L. (1991). Nitric oxide: mediator of nonadrenergic, noncholinergic responses of opossum esophageal muscle. *Am. J. Physiol.*, **261**, G401–G406.

OZAKI, H., BLONDFIELD, D.P., HORI, M., PUBLICOVER, N.G., KATO, I. & SANDERS, K.M. (1992). Spontaneous release of nitric oxide inhibits electrical Ca^{2+} and mechanical transients in canine gastric smooth muscle. *J. Physiol.*, **445**, 231–247.

PAUPARDIN-TRITSCH, D., HAMMOND, C., GERSCHENFELD, H.M., NAIRN, A.C. & GREENGARD, P. (1986). cGMP-dependent protein kinase enhances Ca^{2+} current and potentiates serotonin-induced Ca^{2+} current increase in snail neurons. *Nature*, **323**, 812–814.

RAPOPORT, R.M. & MURAD, F. (1983). Agonist induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cyclic GMP. *Circ. Res.*, **52**, 352–357.

RATTAN, S. & CHAKDER, S. (1991). Role of nitric oxide as a mediator of internal anal sphincter relaxation. *Gastroenterology*, **100**, A485.

RATTAN, S. & MOUMMI, C. (1988). Influence of stimulators and inhibitors of cyclic nucleotides on lower esophageal sphincter. *J. Pharmacol. Exp. Ther.*, **248**, 703–709.

SANDERS, K.M., BURKE, E.P. & STEVENS, R.J. (1989). Effects of methylene blue on rhythmic activity and membrane potential in the canine proximal colon. *Am. J. Physiol.*, **256**, G779–G784.

SMITH, T.K., REED, J.B. & SANDERS, K.M. (1987a). Origin and propagation of electrical slow waves in circular muscles of the canine proximal colon. *Am. J. Physiol.*, **252**, C215–C224.

SMITH, T.K., REED, J.B. & SANDERS, K.M. (1987b), Interaction of two electrical pacemakers in muscularis of canine proximal colon. *Am. J. Physiol.*, **252**, C290–C299.

SMITH T.K., REED, J.B. & SANDERS, K.M. (1989). Electrical pacemakers of the canine proximal colon are functionally innervated by inhibitory motor neurons. *Am. J. Physiol.*, **256**, C466–C477.

STARK, M.E., BAUER, A.J. & SZURSZEWSKI, J.H. (1991). Effect of nitric oxide on circular muscle of the canine small intestine. *J. Physiol.*, **444**, 743–761.

THORNBURY, K.D., WARD, S.M., DALZIEL, H.H., CARL, A., WESTFALL, D.P. & SANDERS, K.M. (1991). Nitric oxide mimics non-adrenergic, non-cholinergic hyperpolarization in gastrointestinal muscles. *Am. J. Physiol.*, **261**, G553-G557.

TODA, N., BABA, H. & OKAMURA, T. (1990). Role of nitric oxide in non-adrenergic, non-cholinergic nerve-mediated relaxation in dog duodenal longitudinal muscle strips. *Jpn. J. Pharmacol.*, **53**, 281–284.

TOMITA, T. (1972). Conductance change during the inhibitory junction potential in the guinea-pig taenia coli. *J. Physiol.*, **225**, 693–703.

TORPHY, T.J., FINE, C.F., BURMAN, M., BARNETTE, M.S. & ORMSBEE, H.S. (1986). Lower esophageal spincter relaxation is associated with increased cyclic nucleotide content. *Am. J. Physiol.*, **251**, G786–G793.

TOTTRUP, A., SVANE, D. & FORMAN, A. (1991). Nitric oxide mediating NANC inhibition in opossum lower esophageal sphincter. *Am. J. Physiol.*, **260**, G385–G389.

TREMBLAY, J., GERZER, R. & HAMET, P. (1988). Cyclic GMP in cell function. In: *Advances in Second Messenger and Phosphoprotein Research*, Volume 22. ed. Greengard, P. & Robison, G.A. pp. 319–383. New York: Raven press.

WALDMAN, S.A., LEWICKI, J.S., BRANDWEIN, H.J. & MURAD, F. (1982). Partial purification and characterization of particulate guanylate cyclase from rat liver after solubilization with trypsin. *J. Cyclic Nucleotide Res.*, **8**, 359–370.

WARD, S.M., DALZIEL, H.H., THORNBURY, K.D., WESTFALL, D. & SANDERS, K.M. (1992a). Non-adrenergic, non-cholineric inhibition and rebound excitation in canine colon depend on nitric oxide. *Am. J. Physiol.*, **262**, G237–G243.

WARD, S.M., MCKEEN, E.S. & SANDERS, K.M. (1992b). Role of nitric oxide in non-adrenergic, non-cholinergic inhibitory junction potentials in canine ileocolonic sphincter. *Br. J. Pharmacol.*, **105**, 776–782.

WARD, S.M., XUE, C., SHUTTLEWORTH, C.W., BREDT, D.S., SNYDER, S.H. & SANDERS, K.M. (1992c). NADPH diaphorase and nitric oxide synthase colocalization in enteric neurons of canine proximal colon. *Am. J. Physiol.*, **263**, G277–G284.

WEISHAAR, R.E., BURROWS, S.D., KOBYLARZ, D.C., QUADE, M.M. & EVANS, D.B. (1986). Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets. Isolation, characterization, and effects of various reference phosphodiesterase inhibitors and cardiotonic agents. *Biochem. Pharmacol.*, **35**, 787–800.

(Received May 7, 1992
Revised July 26, 1992
Accepted August 5, 1992)

Br. J. Pharmacol. (1992), **107**, 1083–1087

Inositol 1,4,5-trisphosphate generation and calcium mobilisation via activation of an atypical P_2 receptor in the neuronal cell line, N1E-115

[1]Philip A. Iredale, *Keith F. Martin, Stephen P.H. Alexander, Stephen J. Hill & David A. Kendall

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH, and *Boots Pharmaceuticals Research Department, Nottingham NG2 3AA

1 Alterations in the levels of intracellular calcium ($[Ca^{2+}]_i$) and D-*myo*-inositol-1,4,5-trisphosphate ($InsP_3$) were measured in the murine neuroblastoma cell line clone, N1E-115, by use of the calcium-sensitive dye, fura-2 and a radioreceptor assay, respectively.

2 Exposure of the cells to ATP (100 μM) elicited rapid and transient increases in $[Ca^{2+}]_i$ and $InsP_3$, with both responses reaching a maximum between 10– 20 s after agonist addition.

3 Investigation of concentration-response data by use of various analogues of ATP suggests the presence of an extracellular receptor which fails to fit into the current classification of purinoceptors.

4 Cross-desensitization experiments suggest that the same receptor can also be activated by the structurally different pyrimidine base, UTP.

5 Application of the tumour-promoting agent, β-phorbol-12,13 dibutyrate (PDBu) caused a reduction in the increases in both $[Ca^{2+}]_i$ and $InsP_3$, suggesting a role for protein kinase C in feedback inhibition of purinoceptor responses in this cell line.

6 In summary, we present the first evidence for the existence of an atypical purinoceptor on a cell line of CNS origin. This receptor is linked to stimulation of phosphoinositide turnover and subsequent mobilisation of intracellular calcium.

Keywords: ATP; nucleotides; P_2-purinoceptor; intracellular calcium; phorbol esters; fura-2

Introduction

The existence of purinoceptors has been accepted for many years and it was Burnstock (1978) who first proposed a division of these receptors into two classes: P_1-purinoceptors, now sub-divided into A_1 and A_2-adenosine receptors and P_2-purinoceptors which are activated by ATP and ADP. ATP exerts many biological effects, e.g. contraction and relaxation of smooth muscle (Burnstock & Kennedy, 1985), via P_2-purinoceptors and its extracellular effects in cultured cell lines have been thoroughly investigated (Hallam & Pearson, 1986; Phaneuf *et al.*, 1987; Rice & Singleton, 1987; Ehrlich *et al.*, 1988; Cowen *et al.*, 1990; Kastritsis *et al.*, 1992; Koike *et al.*, 1992; Sato *et al.*, 1992). A sub-classification of P_2-purinoceptors into P_{2X} and P_{2Y} has been proposed (Burnstock & Kennedy, 1985) based on the rank order of potency of ATP analogues in a number of tissues. In general, the rank order of potency at P_{2X} is α,β-methylene ATP (α,β-MeATP) > β,γ-methylene ATP > ATP = ADP > 2-methylthio ATP (2-MeSATP) and at P_{2Y}, 2-MeSATP > ATP = ADP > α,β,MeATP > β,γ,-methylene ATP.

However, the existence of P_2-purinoceptors other than the P_{2X} and P_{2Y} subtypes has been proposed. For example, a receptor activated by ADP but not ATP in platelets has been designated P_{2T} (Gordon, 1986) and a P_{2Z}-purinoceptor responsible for permeabilization of mast cells has been identified (Cockroft & Gomperts, 1980). More recently, studies in a number of cell lines have produced evidence of purinoceptors with pharmacological profiles that are not consistent with the current classification (Allsup & Boarder, 1990; Cowen *et al.*, 1990; Brown *et al.*, 1991; Murrin & Boarder, 1992). ATP and the pyrimidine UTP, for instance, appear to regulate the activity of phospholipase C via a common 5′ nucleotide receptor in human epithelial cells and in several cell lines (Forsberg *et al.*, 1987; Fine *et al.*, 1989; Pfeilschifter, 1990; Brown *et al.*, 1991).

The murine neuroblastoma cell line clone N1E-115 is known to possess a number of receptors linked to mobilisation of intracellular calcium (Monck *et al.*, 1990; Iredale *et al.*, 1991), and in this study we have investigated the effects of ATP and a number of its analogues. We present here, the first evidence for the existence of an atypical P_2-purinoceptor in a neuronal cell line, linked to the production of D-*myo*-inositol-1,4,5-trisphosphate ($InsP_3$) and subsequent mobilization of intracellular calcium ($[Ca^{2+}]_i$).

Methods

Cell culture

N1E-115 cells, passages 30–47, were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagles Medium (with glutamine) containing 5% foetal calf serum without antibiotics.

Calcium measurements

$[Ca^{2+}]_i$ was measured as previously described (Iredale *et al.*, 1992). Briefly, the monolayers from two near confluent flasks were detached with Pucks D1 solution (composition, mM: glucose 5.5, KCl 5.4, sucrose 58.4, Na_2HPO_4 0.17, NaCl 138 and KH_2PO_4 0.22) and resuspended in a simple saline HEPES buffer (mM: $CaCl_2$ 2, NaCl 145, glucose 10, KCl 5, $MgSO_4$ 1 and HEPES 10, pH 7.45). This was followed by incubation with fura-2 acetoxy methyl ester (5 μM) at 37°C (in the presence of 5% foetal calf serum) for 20 min and for a further 5 min following a three fold dilution (to ensure maximum hydrolysis of ester to the acid form). At the end of

[1] Author for correspondence.

this loading period, excess dye was removed by centrifugation, the cells resuspended in fresh buffer (no serum), and left at room temperature until use. Each calcium time course was preceded by a rapid spin in a microcentrifuge followed by resuspension in fresh buffer.

All experiments were carried out with a Perkin Elmer LS 50 Spectrometer, with excitation ratioing between 340 and 380 nm, recording at 500 nm. The time course for each calcium measurement was 200 s with drugs added in 10 μl (bradykinin 16 μl) aliquots.

Calibration

At the end of each time course, ionomycin (20 μM) was added followed by EGTA (6.25 mM, pH greater than 8.5) in order to calculate R_{max} and R_{min}. Autofluorescence was determined in a separate cuvette following the addition of manganese (5 mM) after the ionophore ionomycin (20 μM). From these values and those obtained with fura-2 free acid, $[Ca^{2+}]_i$ was calculated according to the method of Grynciewicz et al. (1985).

Measurement of $InsP_3$

This was carried out with minor modifications of the radioreceptor method as previously described (Challiss et al., 1988). Briefly, N1E-115 cells (approx 5×10^5 cells ml^{-1}) were preincubated in simple saline buffer for 10 min at 37°C. Aliquots of the cell suspension were removed at intervals, and the incubation stopped with ice-cold perchloric acid (7.5%). The agonist was added and further aliquots taken at specific time points (initially every 10 s). The acid-stopped samples were neutralized with a calibrated amount of $KHCO_3$ (1.2 M) and the protein separated by centrifugation. $InsP_3$ was quantified in the supernatant layer by a radioreceptor assay using a bovine adrenal-cortical binding protein at 4°C. Authentic $InsP_3$ (10^{-10}–10^{-14} mol) in neutralized perchloric acid buffer was used to construct a standard curve for displacement of bound [^{3}H]-$InsP_3$. The bound [^{3}H]-$InsP_3$ was separated by rapid filtration and quantified by liquid-scintillation spectrometry.

The protein pellet was digested in NaOH (0.5 M) and the protein content estimated by the method of Bradford (1976).

Materials

N1E-115 cells were supplied by Porton Down, Wiltshire. Cell culture flasks were obtained from Costar with Dulbecco's Modified Eagle's Medium and foetal calf serum from NBL Ltd. PDBu, was supplied by Sigma, with fura-2 AM and ionomycin from Calbiochem. All nucleotides were supplied by Boehringer Mannheim with $InsP_3$ from Amersham and [^{3}H]-$InsP_3$ from NEN Dupont.

Statistics and data analysis

EC_{50} and IC_{50} (concentrations of drug producing 50% of maximal stimulation and inhibition) values were obtained by computer-assisted curve fitting by using the computer programme Graph-Pad (ISI). Calcium data were captured using the ICBC programme supplied by Perkin-Elmer and imported to the graphics programme Sigma-Plot (Jandel).

Significance testing was carried out by use of an unpaired Student's t test.

Results

The calcium response to ATP

Addition of ATP (100 μM) to populations of fura-2-loaded N1E-115 cells produced a transient increase in $[Ca^{2+}]_i$ of 243 ± 17 nM ($n = 3$) from a resting value of 138 ± 7 nM (Figure 1a). The response reached a maximum between 10–20 s after addition of ATP, and was concentration-dependent with an EC_{50} value of 21 ± 4 μM; $n = 3$ (Figure 1b). When the experiments were repeated under nominally calcium-free conditions there was no significant decrease in the response (86 ± 9% ($n = 3$) of control values), suggesting that intracellular stores were the source of the calcium. Furthermore, when bradykinin (BK; 100 nM) was added approximately 80 s after ATP, the response to the second agonist was progressively increased with decreasing concentrations of ATP suggesting a common, releasable store of calcium (Table 1).

The effects of other ATP analogues

In order to characterize the receptor involved, several other nucleotides were investigated for their ability to mobilize

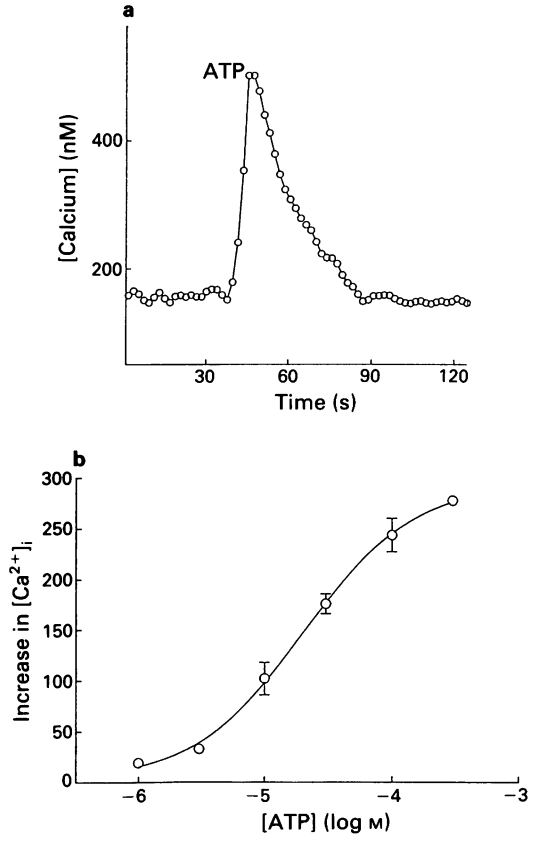

Figure 1 Panel (a) shows the effect of ATP (100 μM) on $[Ca^{2+}]_i$ in populations of fura-2-loaded N1E-115 cells. Fluorescence values were measured following excitation at 340 and 380 nm and the ratio used to determine $[Ca^{2+}]_i$. The graph is typical of two others. Panel (b) shows the concentration-effect relationship for ATP. Data are the means of three separate determinations; vertical error bars represent s.e.mean.

Table 1 The effect of the ATP response on a subsequent addition of bradykinin (BK, 100 nM)

	Increase in $[Ca^{2+}]_i$ (nM)	
ATP (μM)	*ATP*	*BK*
30	192	257
10	134	390
3	41	445
1	22	470

The cells were challenged with a range of concentrations of ATP followed by BK approx. 80 s later.

calcium in N1E-115 cells. Both UTP and ADP elicited significant increases in $[Ca^{2+}]_i$ with maximal responses of approximately 80% of the ATP signal; however the EC_{50} for ADP was approximately 50 times greater than that for ATP (0.9 ± 0.6 mM, $n = 3$; Table 2). Adenosine and AMP were without affect, suggesting that P_1-purinoceptors, which are preferentially activated by these agents, were not involved in the response to ATP. A small response (<10% of the ATP signal) was seen following addition of GTP (100 μM), but CTP (100 μM) showed no significant effect (data not shown).

A number of ATP analogues were also investigated; adenosine 5'-(3-thio)triphosphate (ATPγS) was able to increase $[Ca^{2+}]_i$ with similar potency and efficacy to ATP (Table 2); however the P_{2x}-selective agonist, α,β-MeATP (100 μM), and the P_{2y}-selective agonist, 2MeSATP (100 μM), elicited only very small changes in $[Ca^{2+}]_i$ (<10% of the effect of ATP; data not shown).

Production of inositol 1,4,5-trisphosphate

Mass measurements of $InsP_3$ showed basal levels of 12 ± 2 pmol mg^{-1}, with a transient elevation above basal upon stimulation with ATP (100 μM), of 35 ± 3 pmol mg^{-1} ($n = 5$; Figure 2), peaking at 10–20 s. A similar change was seen following addition of UTP (27 ± 1 pmol mg^{-1} ($n = 3$) above basal). Omission of calcium from the extracellular medium failed to affect significantly the $InsP_3$ response to ATP (32 ± 3 pmol mg^{-1} above basal; $n = 3$).

Phorbol pretreatment

Pre-incubation (for 20 min during the fura-2 loading period) with the phorbol ester, β-phorbol-12,13 dibutyrate (PDBu; 1.5 μM) caused significant reductions in the calcium responses to UTP and ATP (Figure 3). PDBu had similar inhibitory effects on the production of $InsP_3$ (Figure 4).

Evidence for a common ATP/UTP receptor

To determine whether ATP and UTP were acting via the same receptors, the cells were pre-incubated for 20 min with ATPγS (160 μM), the stable analogue of ATP, and were then challenged with a maximally effective concentration of UTP (100 μM). Calcium responses to UTP (compared with control responses to UTP in untreated cells) were significantly reduced by pretreatment with ATPγS (Figure 5) suggesting that a cross-desensitization between the two agonists had occurred. There was, however, no significant change in the response to BK (100 nM) following pre-incubation with ATPγS (89 ± 6% of control; $n = 3$).

Table 2 The relative potencies and efficacies of ATP, its analogues and other nucleotides

Agonist	EC_{50}	*Proportion of ATP response*
ATP	21 ± 4 μM	1.0
ADP	0.9 ± 0.6 mM	0.8
AMP	–	Not detectable
Adenosine	–	Not detectable
ATPγS	30 ± 20 μM	0.8
2MeSATP	–	<0.1
α,βMeATP	–	<0.1
UTP	13 ± 5 μM	0.9
GTP	–	<0.1
GTPγS	–	<0.1
CTP	–	Not detectable

An estimate of the efficacy of each agent was obtained by comparing the maximum calcium responses with that produced by ATP (100 μM). Wherever possible comparisons were made between cells from the same passage on the same day. The results are the mean of three separate determinations. For abbreviations, see text.

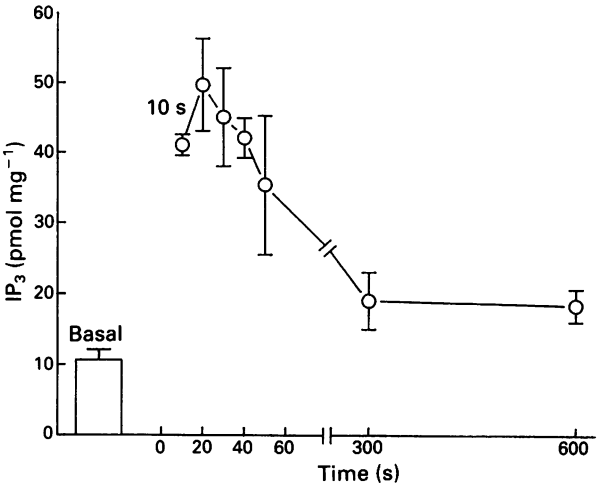

Figure 2 The time course for D-*myo*-inositol-1,4,5-trisphosphate ($InsP_3$) generation following the addition of ATP (100 μM). Basal measurements are represented by the histogram. The graph shows $InsP_3$ formation (pmol mg^{-1}) as a function of time. Data are mean of three separate determinations; vertical error bars represent s.e.mean.

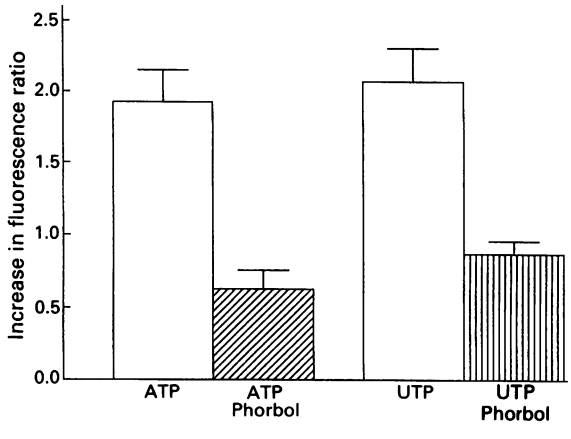

Figure 3 The effect of β-phorbol-12,13 dibutyrate (PDBu, 1.5 μM) on the calcium responses (fluorescence ratios shown) to ATP (100 μM) and UTP (100 μM). Cells were pre-incubated with the phorbol ester for 25 min, which was present for the remainder of the experiment, resulting in significant reductions ($P < 0.01$) in the responses to both agents. Data are the means of three separate determinations; error bars represent s.e.mean.

Discussion

The existence of 'purinergic' nerves was suggested by the observations that a non-cholinergic/non-adrenergic component of the autonomic nervous system was associated with smooth muscle in the gastrointestinal tract and elsewhere. ATP was suggested to be the neurotransmitter involved, following its detection after nerve stimulation, and the presence at neuroeffector junctions of ATP metabolizing enzymes (Burnstock, 1971). Furthermore, ATP has been found to be co-released from cholinergic nerve endings isolated from the CNS (Richardson & Brown, 1978). It has been suggested that phosphoinositide hydrolysis is associated with purinoceptor stimulation in neuronally-derived preparations (Allsup & Boarder, 1990), but there have been relatively few studies of the expected increases in $[Ca^{2+}]_i$.

In the N1E-115 neuroblastoma cells addition of ATP resulted in a transient increase in the production of $InsP_3$ with a concomitant mobilization of calcium from intracel-

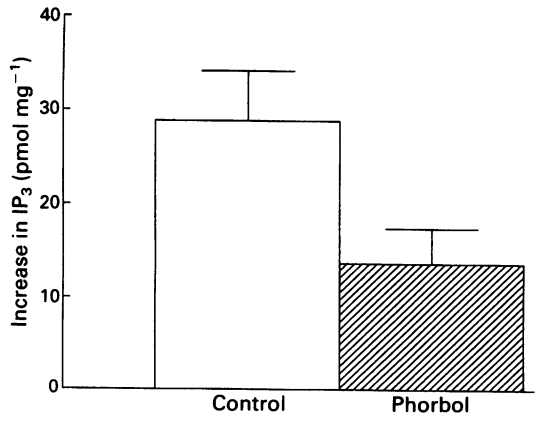

Figure 4 The effect of β-phorbol-12,13 dibutyrate (PDBu, 1.5 μM) on D-*myo*-inositol-1,4,5-trisphosphate ($InsP_3$) generation following stimulation with ATP (1.5 μM). Cells were pre-incubated with the phorbol ester for 25 min which was present for the remainder of the experiment, resulting in significant reductions ($P<0.05$) in the production of $InsP_3$ following agonist stimulation. The increase in production of $InsP_3$ (pmol mg^{-1}) following stimulation with ATP is plotted along the y axis. Data are mean of three separate experiments; error bars represent s.e.mean.

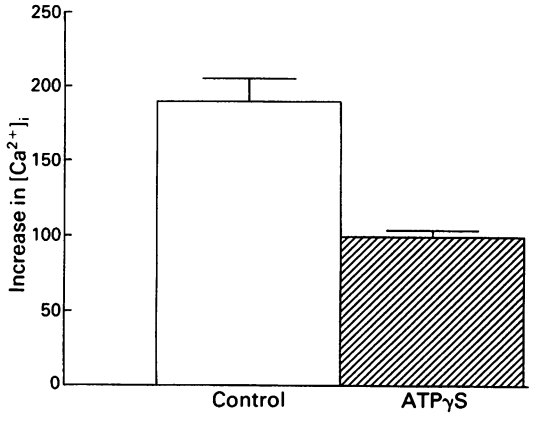

Figure 5 Cross-desensitization of the calcium repsonse to UTP (100 μM). The cells were pre-incubated for 30 min with the stable ATP analogue adenosine 5'-(3-thio) triphosphate (ATPγS, 160 μM). The open column shows the control response to UTP. The hatched column shows the effect of the same concentration of UTP after the cells were pretreated with ATPγS. Significant ($P<0.01$) reductions in the calcium response were seen. Data are the means of three separate determinations. Error bars represent s.e.mean.

lular stores. However, the pharmacological profile of the receptor mediating these effects is different from that described for P_2-purinoceptor subtypes (Burnstock, 1978; Gordon, 1986).

It would appear unlikely that a P_1-purinoceptor is involved in the response to ATP, since neither adenosine nor AMP elicited any significant calcium increases. Furthermore, it is unlikely that the actions of ATP are mediated via one of its hydrolysed breakdown products because ADP was much less potent and, AMP and adenosine were ineffective. It would also appear that the possession of a triphosphate group by the agonist is not sufficient for activity because, although UTP was as efficacious and potent as ATP, GTP exhibited only slight activity and CTP was unable to increase $[Ca^{2+}]_i$ even at very high concentrations (Table 2). However, the presence of the triphosphate moiety may increase agonist potency because ADP showed only slightly reduced efficacy, but had approximately 50 times lower potency than ATP. This order of potency also suggests that the effect of ATP is mediated via a P_2 rather than a P_1 receptor.

The current sub-classification of P_2-purinoceptors into P_{2X} and P_{2Y} is based upon a rank order of agonist potencies for stable analogues of ATP (Burnstock *et al.*, 1985). In the N1E-115 cell line, 2MeSATP (the highly potent P_{2Y}-purinoceptor agonist) and α,β-MeATP (the highly potent P_{2X}-purinoceptor agonist) were able to elicit only very small increases in $[Ca^{2+}]_i$. Indeed, of the ATP analogues investigated, only ATPγS was able to increase $[Ca^{2+}]_i$ with similar potency and efficacy to ATP. This is therefore evidence for the existence in the N1E-115 cells of a novel receptor subtype similar to the 5' nucleotide receptor of human airway epithelial cells (Brown *et al.*, 1991).

The lack of selective antagonists for P_2-receptors makes further classification of the subtype difficult. Although suramin has been reported to antagonize both P_{2X}- and P_{2Y}-mediated responses (Hoiting *et al.*, 1990; Hoyle *et al.*, 1990) and the atypical nucleotide receptor in PC12 cells (Murrin & Boarder, 1991) its effects on the P_2-purinoceptor in N1E-115 cells were difficult to assess due to non-specific disruption of the cells (data not shown).

UTP was able to increase $InsP_3$ production and mobilize $[Ca^{2+}]_i$ with similar efficacy and slightly increased potency compared with ATP. The structural differences between the pyrimidine (UTP) and purine (ATP) bases raises the question of whether the two agonists act via the same nucleotide receptor and previous studies have suggested the existence of separate purine and pyrimidine receptors (Stutchfield & Cockroft, 1990; von Kugelan & Starke, 1990), although other workers have suggested that a single receptor recognizes both structures (Pfeilschifter, 1990; Brown *et al.*, 1991; Murrin & Boarder, 1992). The evidence from the present study in N1E-115 cells suggests that UTP and ATP mobilize calcium via activation of the same receptor population. Pre-incubation with ATPγS, the poorly-hydrolysable analogue of ATP, resulted in substantial reductions in calcium responses to maximally effective concentrations of UTP (Figure 5) compared with those elicited in untreated control cells. There was, however, no significant change in the calcium response to bradykinin in the cells previously exposed to ATPγS. This finding indicates that the reduced response was not a consequence of the depletion of calcium stores. Furthermore, both agonists showed similar sensitivities to pre-incubation with the phorbol ester PDBu (Figure 3).

The inhibition of the nucleotide response by phorbol esters is typical of many receptor-mediated phosphoinositidase C signal transduction pathways (Nishizuka, 1986). Indeed, other receptors linked to mobilization of $[Ca^{2+}]_i$ in N1E-115 cells are subject to PDBu pretreatment via activation of protein kinase C (Iredale *et al.*, 1991). A number of possible targets for subsequent phosphorylation exist, but the similarly sized reduction in $InsP_3$ production compared with inhibition of calcium mobilization (Figures 4 and 5) suggest a site on the receptor-G protein-phosphoinositidase C complex.

In summary, these data provide the first evidence for the existence of an atypical, P_2-purinoceptor subtype in a neuronal cell line. This receptor is linked to $InsP_3$ production and subsequent calcium mobilization. It is activated by both ATP and UTP with similar potencies and efficacies and resembles the 5' nucleotide receptor previously described in human airway epithelial and PC12 cells, but until a suitable antagonist is found more detailed classification is difficult.

A physiological role for ATP as a signal molecule in nervous tissues is rapidly becoming established. Storage of ATP and co-release with other classical neurotransmitters is well documented (Gordon, 1986; Richardson & Brown, 1987). Cells of nervous system origin grown in culture, whilst providing useful biochemical information, are subject to certain non-physiological conditions and stimuli which often lead to changes in their phenotype. Thus, although it is desirable to exercise caution in extrapolating from cell lines to native tissue, it appears likely that activation of phos-

phoinositidase C (and the ensuing biochemical consequences) may well be one mechanism of signal transduction for ATP in the central nervous system.

Supported by a SERC/CASE award with Boots Pharmaceuticals (P.A.I.) and by the Wellcome Trust (S.P.H.A.).

References

ALLSUP, D.J. & BOARDER, M.R. (1990). Comparison of P_2-purinergic receptors of aortic endothelial cells with those of the adrenal medulla: evidence for heterogeneity of receptor subtype and inositol phosphate response. *Mol. Pharmacol.*, **38**, 84–91.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Anal. Biochem.*, **72**, 248–254.

BROWN, H.A., LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (1991). Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5'-nucleotide receptor in human airway epithelial cells. *Mol. Pharmacol.*, **40**, 648–655.

BURNSTOCK, G. (1971). Neural nomenclature. *Nature*, **229**, 282–283.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptors. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach.* ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G. & KENNEDY, C (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CHALLISS, R.A.J., BATTY, I.H. & NAHORSKI, S.R. (1988). Mass measurements of inositol (1,4,5) trisphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarisation. *Biochem. Biophys. Res. Commun.*, **157**, 684–691.

COCKROFT, S. & GOMPERTS, B.D. (1980). The ATP^4-receptor of rat mast cells. *Biochem. J.*, **188**, 789–798.

COWEN, D.S., SANDERS, M. & DUBYAK, G. (1990). P_2-purinergic receptors activate a guanine nucleotide-dependent phospholipase C in membranes from HL-60 cells. *Biochem. Biophys. Acta*, **1053**, 195–203.

EHRLICH, Y.H., SNIDER, R.M., KORNECKI, E., GARFIELD, M.G. & LENOX, R.H. (1988). Modulation of neuronal signal transduction systems by extracellular ATP. *J. Neurochem.*, **50**, 295–301.

FINE, J., COLE, P. & DAVIDSON, J.S. (1989). Extracellular nucleotides stimulate receptor-mediated calcium mobilisation and inositol phosphate production in human fibroblasts. *Biochem. J.*, **263**, 371–376.

FORSBERG, E.J., FEVERSTEIN, G., SHOHAMI, E. & POLLARD, H.B. (1987). Adenosine triphosphate stimulates inositol phospholipid metabolism and prostacycline formation in adrenal medullary endothelial cells by means of P_2-purinergic receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 5630–5634.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biochem. Sci.*, **260**, 2440–3450.

HALLAM, T.J. & PEARSON, J.D. (1986). Exogenous ATP raises cytoplasmic free calcium in fura-2 loaded piglet aortic endothelial cells. *FEBS Lett.*, **207**, 95–99.

HOITING, B., MOLLEMAN, A., NELEMANS, A. & DEN HERTOG, A. (1990). P_2-purinoceptor-activated membrane currents and inositol tetrakisphosphate formation are blocked by suramin. *Eur. J. Pharmacol.*, **181**, 127–131.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonises responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617–621.

IREDALE, P.A., RUCK, A., KENDALL, D.A., MARTIN, K.F. & HILL, S.J. (1991). Histamine-induced changes in intracellular calcium in undifferentiated N1E-115 cells. *Biochem. Soc. Trans.*, **19**, 6S.

IREDALE, P.A., MARTIN, K.F., HILL, S.J. & KENDALL, D.A. (1992). Comparison of calcium mobilisation responses to different agonists in undifferentiated N1E-115 cells: modulatory effects of phorbol esters. *Br. J. Pharmacol.*, **105**, 119P.

IREDALE, P.A., MARTIN, K.F., HILL, S.J. & KENDALL, D.A. (1992). Agonist-induced changes in $[Ca^{2+}]_i$ in N1E-115 cells: differential effects of bradykinin and carbachol. *Eur. J. Pharmacol.*, **226**, 163–168.

KASTRITSIS, C.H.C., SALM, A.K. & MCCARTHY, K. (1992). Stimulation of the P_{2Y} purinergic receptor on type 1 astroglia results in inositol phosphate formation and calcium mobilisation. *J. Neurochem.*, **58**, 1277–1284.

KOIKE, M., KASHIWAGURA, T. & TAKEGUCHI, T. (1992). Gluconeogenesis stimulated by extracellular ATP is triggered by the initial increase in the intracellular Ca^{2+} concentration of the periphery of hepatocytes. *Biochem. J.*, **283**, 265–272.

MONCK, J.R., WILLIAMSON, R.E., ROGULJA, I., FLUHARTY, S.J. & WILLIAMSON, J.R. (1990). Angiotensin II effects on the cytosolic free Ca^{2+} concentration in N1E-115 neuroblastoma cells: kinetic properties of the Ca^{2+} transient measured in single fura-2-loaded cells. *J. Neurochem.*, **54**, 278–287.

MURRIN, R.J.A. & BOARDER, M.R. (1992). Neuronal 'nucleotide' receptor linked to phospholipase C and phospholipase D? Stimulation of PC12 cells by ATP analogues and UTP. *Mol. Pharmacol.*, **41**, 561–568.

NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. *Science* (Wash. DC), **233**, 305–312.

PFEILSCHIFTER, J. (1990). Comparison of extracellular ATP and UPT signalling in rat renal mesangial cells. *Biochem. J.*, **272**, 469–472.

PHANEUF, S., BERTA, P., CASANOVA, J. & CAVADORE (1987). ATP stimulates inositol phosphates accumulation and calcium mobilisation in a primary culture of rat aortic myocytes. *Biochem. Biophys. Res. Commun.*, **143**, 454–460.

RICE, W.R. & SINGLETON, F.M. (1987). P_{2Y}-purinoceptor regulation of surfactant secretion from isolated alveolar type II cells is associated with mobilisation of intracellular calcium. *Br. J. Pharmacol.*, **91**, 833–838.

RICHARDSON, P.J. & BROWN, S.J. (1987). ATP release from affinity-purified rat cholinergic nerve terminals. *J. Neurochem.*, **48**, 622–630.

SATO, K., OKAJIMA, F. & KONDO, Y. (1992). Extracellular ATP stimulates three different receptor-signal transduction systems in FRTL-5 thyroid cells. *Biochem. J.*, **283**, 281–287.

STUCHFIELD, J. & COCKROFT, S. (1990). Undifferentiated HL60 cells respond to extracellular ATP and UTP by stimulating phospholipase C activation and exocytosis. *FEBS Lett.*, **262**, 256–258.

VON KUGELGEN, I., BULTMANN, R. & STARKE, K. (1990). Interaction of adenine nucleotides, UTP and suramin in mouse vas deferens: suramin-sensitive and suramin-insensitive components in the contractile effect of ATP. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **342**, 198–205.

(Received June 19, 1992
Revised August 6, 1992
Accepted August 7, 1992)

Br. J. Pharmacol. (1992), **107**, 1088–1091

Pterins inhibit nitric oxide synthase activity in rat alveolar macrophages

Philippe G. Jorens, [1]Frans J. van Overveld, *Hidde Bult, Paul A. Vermeire & *Arnold G. Herman

Departments of Respiratory Medicine and *Pharmacology, University of Antwerp, UIA, Universiteitsplein 1, B-2610 Antwerp, Belgium

1 The synthesis of nitrite and citrulline from L-arginine by immune-stimulated rat alveolar macrophages and the modulation of this synthesis were studied. 2,4-Diamino-6-hydroxypyrimidine (DAHP), 6**R**-5,6,7,8-tetrahydro-L-biopterin (BH_4) and L-sepiapterin were potent inhibitors of the recombinant interferon-γ induced production of nitrogen oxides in intact cultured cells with I_{50} values for BH_4 and L-sepiapterin of approximately 10 μM. They were equally effective in inhibiting the induced production of citrulline. This inhibitory effect was concentration-dependent for all three modulators investigated.

2 The inhibitory effects were not dependent on incubation times of either 24 or 48 h, on the immune-stimulus used (lipopolysaccharide, interferon-γ), or whether these stimuli were added during or after the induction period.

3 Pterin-6-carboxylic acid (PCA), which cannot be converted into BH_4, and methotrexate (MTX), which inhibits dihydrofolatereductase but not *de novo* biosynthesis of BH_4, did not change the production of nitrite.

4 The data indicate that DAHP, an inhibitor of the *de novo* biosynthesis of the co-factor BH_4, blocks the nitric oxide synthase activity in intact cells. Since the pterins BH_4 and L-sepiapterin blocked the L-arginine dependent production of nitrite and citrulline, the activity of nitric oxide synthase in phagocytic cells may be regulated by metabolic endproducts of the *de novo* biosynthesis of BH_4.

Keywords: Nitric oxide (NO); nitrite; citrulline; alveolar macrophages; arginine; tetrahydrobiopterin; pterins

Introduction

In immune-activated macrophages recombinant interferon-γ (rIFN-γ) and bacterial lipopolysaccharide (LPS) induce a soluble nitric oxide synthase, that is L-arginine (L-Arg)-dependent (Hibbs *et al.*, 1987; Stuehr & Marletta, 1987). The products of this pathway are citrulline and the highly reactive and unstable nitric oxide which decomposes rapidly into nitrite (NO_2^-) and nitrate (NO_3^-). The quantification of these two degradation products indirectly reflects nitric oxide production (Albina *et al.*, 1989). This metabolic pathway has been demonstrated in many types of murine macrophages (Albina *et al.*, 1989; 1990; Billiar *et al.*, 1989; Keller *et al.*, 1990) as well as in certain macrophage cell lines (Tayeh & Marletta, 1989; Di Rosa *et al.*, 1990). Recent work in our laboratory showed that rIFN-γ and LPS can also stimulate rat alveolar macrophages to release L-Arg-dependent nitric oxide and nitrite (Jorens *et al.*, 1991). *In vitro* studies with nitric oxide synthase from cytosol of partially purified murine macrophages unmasks a need for co-factors, such as reduced glutathione, reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and 6**R** 5,6,7,8-tetrahydro-L-biopterin (BH_4) (Kwon *et al.*, 1989; Stuehr *et al.*, 1989; 1990; Tayeh & Marletta, 1989), among which BH_4 belongs to a group of molecules characterized by a common 2-amino-4-hydroxypteridine structure, called the pterins. A recent study indicates that pterins can modulate the L-Arg-dependent NO_2^- production when administered to intact murine fibroblasts in culture after activation with rIFN-γ (Werner-Felmayer *et al.*, 1990). The effect of BH_4 and other pterins on the nitric oxide-synthase activity in intact macrophages has not been reported.

We therefore examined the extracellular administration of several pterins and a modulator of BH_4 biosynthesis on the induction and release of NO_2^- and citrulline from immune-activated rat alveolar macrophages.

Methods

Bronchoalveolar lavage (BAL)

Specific pathogen-free male Wistar rats (Proefdierencentrium Leuven, Belgium; weight 200–250 g) were killed; after exposure of their trachea a sterile catheter (outer diameter, 2 mm, inner diameter 1 mm) was introduced. For lavage, 50 ml of sterile Dulbecco's phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} was used. Introduction (in 5 ml aliquots) and suction of the solution were done manually.

Cell culture

Immediately after lavage, the lavage fluid was centrifuged (10 min, 1400 r.p.m., room temp.). The cell pellet was suspended in Dulbecco's modified Eagle's medium without phenol red (DMEM) supplemented with 5% heat-inactivated, low endotoxin foetal calf serum (FCS), as well as streptomycin 100 μg ml^{-1} and penicillin 100 u ml^{-1}. Cells were allowed to adhere to plastic in sterile 24 well dishes (Nunc, Roskilde, Denmark) for 90 min at 37°C, with 0.5×10^6 macrophages added to each well. Nonadherent cells were removed by three washings with prewarmed DMEM. The adherent population always contained more than 95% macrophages as determined by morphological evaluation of May-Grunwald Giemsa staining of methanol-fixed cells of the adherent population of macrophages after culture, and biochemical criteria by the non-specific esterase staining specific for macrophages in culture (Hayhoe & Fletmans, 1982).

[1] Author for correspondence.

Cell stimulation

In duplicate wells, 1050 µl of DMEM with 5% FCS and antibiotics were added. The appropriate stimuli and/or modulating compounds were dissolved in 50 µl DMEM and added to the wells, which were incubated for 24 or 48 h (37°C, 5% CO_2). Cell viability was assessed by trypan blue exclusion; it always exceeded 95%.

Assay for NO_2^- and citrulline

Nitric oxide synthase activity was measured by determining NO_2^- and citrulline on cell-free supernatant after the incubation time reported. Experiments were also conducted in alveolar macrophages pretreated with rIFN-γ or LPS. In that case, the medium was removed after 12 h, the cells were washed with prewarmed medium and after the next 24 h release of nitrite was measured in fresh medium, to which pterins were added.

Nitrite (NO_2^-) was determined by a spectrophotometric assay based on the Griess reaction (Schmidt *et al.*, 1988). Briefly, 90 µl 6.5 M HCl and 90 µl 37.5 mM sulphanilic acid were added to 900 µl supernatant. After 10 min, 90 µl 12.5 mM N-(1-naphthyl)-ethylene diamine HCl was added. After 30 min absorbance was measured at 540 nm. None of the used stimuli, pterins or other agents, interfered with the NO_2^- assay in its linear range (0.5–100 µM).

Citrulline was measured with a Biotronik LC 6000E amino acid analyser (Biotronik, Maintal, Germany). A classical elution procedure for the separation of the amino acids in physiological fluids and tissues was used, based on the automatic recording of the absorbance of the effluent of culture medium from the ion exchange columns (Spackman *et al.*, 1958).

Materials

2,4-Diamino-6-hydroxypyrimidine (DAHP), *Escherichia coli* lipopolysaccharide serotye O111:B4 (LPS), N-(1-naphthyl)-ethylenediamine HCl, pterin-6-carboxylic acid (PCA), penicillin, streptomycin, sulphanilic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.); Dulbecco's modified Eagle's medium without phenol red (DMEM), heat-inactivated foetal calf serum (FCS), Dulbecco's phosphate buffered saline without calcium and magnesium (Gibco Ltd, Paisley, United Kingdom). 6**R** 5,6,7,8-tetrahydro-L-biopterin (BH_4) and L-sepiapterin were purchased from B. Schircks Labs. (Jona, Switzerland). Methotrexate (MTX) (Cyanamid Benelux, Mont-Saint Guibert, Belgium) and rat recombinant interferon-γ (rIFN-γ) (P.H. van der Meide, TNO, Rijswijk, The Netherlands) were kindly provided as indicated in parentheses.

Statistics

Statistical analysis was performed with Student's *t* test (two-tailed) for unpaired data. $P<0.05$ was considered as statistically significant. Results are expressed as mean ± s.e.mean with *n* denoting the number of experiments performed.

Results

LPS (500 ng ml^{-1}) and rIFN-γ (50 u ml^{-1}) induced a time-dependent release of NO_2^-, amounting to 27.8 ± 0.8 nmol per 10^6 cells 24 h^{-1} ($n = 5$) and 48.2 ± 1.3 nmol per 10^6 cells 48 h^{-1} ($n = 5$) after stimulation with 50 u ml^{-1} rIFN-γ. With NO_2^- production a high concentration of citrulline also appeared in the incubation medium (Table 1). Without stimulation alveolar macrophages did not release more than 0.8 nmol NO_2^- per 10^6 cells 24h^{-1} ($n = 5$).

Table 1 Effect of L-sepiapterin, 6**R** 5,6,7,8-tetrahydro-L-biopterin (BH_4) and 2,4-diamino-6-hydroxypyrimidine (DAHP) on NO_2^- and citrulline production by 10^6 alveolar macrophages after challenge with recombinant interferon-γ (rIFN-γ, 50 u ml^{-1})

Modulator	*Concentration* (nmol ml^{-1})	*NO_2^-* (nmol 48h^{-1})	*Citrulline* (nmol 48h^{-1})
Control		48.2 ± 1.3	76.3 ± 3.2
L-Sepiapterin	100	7.6 ± 0.4***	16.6 ± 1.0***
L-Sepiapterin	30	15.3 ± 0.5***	34.2 ± 2.1***
BH_4	100	8.7 ± 0.6***	13.5 ± 0.5***
DAHP	100	19.6 ± 1.4***	33.8 ± 1.3***
DAHP	30	28.4 ± 0.6***	47.5 ± 1.1**

Rat alveolar macrophages were incubated for 48 h in DMEM in the presence of 5% FCS. Data represent mean ± s.e.mean for 5 experiments.
Significance levels are shown as **$P<0.01$; ***$P<0.001$, versus control stimulation with rIFN-γ (50 u ml^{-1}).

Modulation of NO_2^- and citrulline production

The synthesis of NO_2^- by alveolar macrophages following stimulation with rIFN-γ (50 u ml^{-1}), was significantly inhibited by the addition of DAHP, in a concentration range from 3×10^{-6} to 1×10^{-3} M (Figure 1). The I_{50} value for DAHP was 6×10^{-5} M. The pterins BH_4 and L-sepiapterin, also in a concentration range from 3×10^{-6} to 1×10^{-3} M, inhibited the rIFN-γ-induced NO_2^- production in a concentration-dependent manner (Figure 1) with an I_{50} of 1×10^{-5} M. Lower concentrations of these 3 agents (10^{-11}–10^{-6} M) did not influence NO_2^- production. Moreover, citrulline production was also significantly inhibited by these agents (Table 1). Addition of MTX and PCA in a comparable concentration-range as for the three former agents (1×10^{-7}–1×10^{-3} M) did not significantly alter the rIFN-γ-and LPS-induced NO_2^- production ($n = 5$).

Addition of sepiapterin (1×10^{-6}–3×10^{-3} M) to incubations with DAHP (1×10^{-5}–1×10^{-4} M) did not reverse the inhibition of DAHP on rIFN-γ-and LPS-induced NO_2^- production ($n = 5$).

Only L-sepiapterin concentrations of $> 1 \times 10^{-3}$ M showed some interference with the nitrite assay in its linear range (5×10^{-7} to 1×10^{-4} M) with a L-sepiapterin concentration of 3×10^{-3} M showing a 30% decrease in absorption ($n = 5$). Therefore, the determination of nitrite in the supernatants of cells cultured in the presence of L-sepiapterin concentrations $> 1 \times 10^{-3}$ M was corrected for interference with the assay and data in Figure 1 are presented with these corrections taken into account.

The inhibitory effect is independent of incubation-time or stimulus investigated

The observed inhibition of rIFN-γ induced NO_2^- production was not limited to incubations during 48 h. A comparable degree of inhibition was observed when the supernatant of 24 h cultures was investigated (Table 2). Moreover, this inhibitory effect was also observed when LPS was used as a stimulus (Table 2).

Effect of pterins when added after the induction period

In other experiments, alveolar macrophages were pretreated with rIFN-γ (50 u ml^{-1}) of LPS (500 ng ml^{-1}) during 12 h; this exceeds by far the 3–6 h, necessary to induce NO_2^- production in these cells (Jorens *et al.*, 1991). After removal of the stimulus by vigorous washing of these cells, DAHP (1×10^{-4} M), BH_4 (1×10^{-4} M) or L-sepiapterin (1×10^{-4} M) were added. The production of NO_2^- was also significantly inhibited during the subsequent 24 h (Table 2).

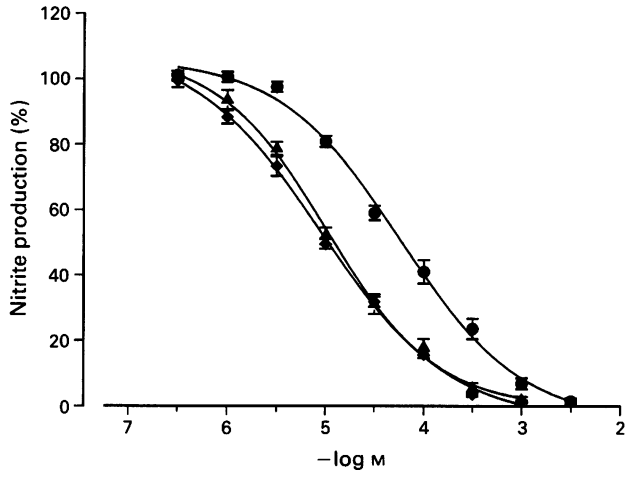

Figure 1 The effects of DAHP (●), BH_4 (▲) and L-sepiapterin (◆) on rIFN-γ (50 u ml^{-1}) induced NO_2^- production by alveolar macrophages during 48 h of incubation. Data are expressed as relative production (%) as compared to control incubations with rIFN-γ alone. Points show mean of 6 observations, with s.e.mean as vertical bars.

Discussion

DAHP, L-sepiapterin and BH_4 inhibited cytokine- and LPS-induced nitric oxide synthase activity in rat alveolar macrophages, as measured by NO_2^- and citrulline production. This inhibition was dependent on concentration, but not dependent on incubation time over the range studied. It was also of equal magnitude for the three agents and it was observed for both enzyme activity inducing cytokine rIFN-γ and LPS studied.

For its activity, nitric oxide synthase of murine macrophages requires a low molecular weight co-factor, which has been shown to be BH_4 (Kwon *et al.*, 1989; Tayeh & Marletta, 1989). The *de novo* biosynthesis of this pterine in mammalian cells starts with GTP, which is cleaved by GTP-cyclohydrolase I (EC 3.5.4.16) to 7,8-dihydro-neopterin biphosphate (Gal *et al.*, 1978). The latter is further converted by a sequence of two enzymes, 6-purovyltetrahydrobiopterin synthase and sepiapterin reductase into BH_4, the active form of the co-factor (Werner *et al.*, 1991). Sepiapterin can be converted to BH_4 by a different salvage pathway in many cells (Nichol *et al.*, 1985).

In our model of intact alveolar macrophages in culture, the inhibitor of GTP cyclohydrolase I, DAHP was able to inhibit this NO_2^- and citrulline production. Although *in vitro* studies have shown that GTP cyclohydrolase I derived from rat liver can be inhibited noncompetitively by its metabolic end-product BH_4 and by sepiapterin (Shen *et al.*, 1988), we are unaware of any reports on the effects of these agents on BH_4 dependent metabolic processes in intact cells. Moreover, the observed I_{50} values for BH_4 and sepiapterin on the nitrite production in intact phagocytic cells (I_{50} approx 1×10^{-5} M) were very close to the reported I_{50} value for both of these reduced pterins ($1.3 \pm 0.2 \times 10^{-5}$ M) for GTP cyclohydrolase I activity *in vitro* (Shen *et al.*, 1988). The unconjugated pterin PCA did not influence this induced NO_2^- production in a wide concentration-range, because it cannot be converted to BH_4 due to the lack of the 1',2'-dihydroxypropylside chain at position 6 of the pterin ring (Nichol *et al.*, 1985). Also MTX, which inhibits dihydrofolatereductase but not the *de novo* biosynthesis of BH_4 from GTP (Nichol *et al.*, 1985), showed no influence on the production of NO_2^-.

Some of our data are in contrast with recent observations in intact murine fibroblasts (Werner-Felmayer *et al.*, 1990). Indeed, although DAHP (1×10^{-4} M) also inhibited cytokine-induced NO_2^- production in these fibroblasts, L-sepiapterin (1×10^{-4} M) was without effect and even partially reversed the DAHP (1×10^{-4} M) induced decrease in NO_2^- production when added to these immune-stimulated murine cells. It is not clear why our results with alveolar macrophages are different. This may be attributed to species and cell differences in BH_4 generating systems, as has been observed between murine and human macrophages (Werner *et al.*, 1991). It may also arise from different levels of GTP-cyclohydrolase I in different cell types of one species (Shen *et al.*, 1988) or from the existence of multiple forms of this enzyme in one cell type, as observed in rat liver (Bellahsene *et al.*, 1984).

Our present findings add BH_4, a known cofactor for NO synthase activity *in vitro*, sepiapterin and DAHP as another family of inhibitors of nitric oxide synthase in intact phagocytic cells to the drugs previously reported: the L-arginine analogues N^G-monomethyl-L-arginine and N^G-nitro-L-arginine (Gross *et al.*, 1990; Jorens *et al.*, 1991), N-iminoethyl-L-ornithine (McCall *et al.*, 1991), diphenyliodonium and its analogues as inhibitors of the nucleotide-requiring flavoproteins (Stuehr *et al.*, 1991), serine

Table 2 Effect of L-sepiapterin, 6R 5,6,7,8-tetrahydro-L-biopterin (BH_4) and 2,4-diamino-6-hydroxypyrimidine (DAHP) on recombinant interferon-γ (rIFN-γ) and lipopolysaccharide (LPS) induced NO_2^- production by 10^6 alveolar macrophages, either given before or after the induction period

Stimulus	*Modulator* (10^{-4} M)	NO_2^- (nmol 24 h^{-1}) *before induction*	NO_2^- (nmol 24 h^{-1}) *after induction*
rIFN-γ 50 u ml^{-1}	Control	27.8 ± 0.8	21.9 ± 0.7
	L-Sepiapterin	4.3 ± 0.3***	4.2 ± 0.2***
	BH_4	5.7 ± 0.2***	6.3 ± 0.2***
	DAHP	9.9 ± 0.6***	11.2 ± 0.5**
LPS 500 ng ml^{-1}	Control	19.7 ± 0.5	14.7 ± 0.6
	L-Sepiapterin	2.4 ± 0.3***	3.2 ± 0.4***
	BH_4	4.1 ± 0.4***	5.6 ± 0.6**
	DAHP	6.3 ± 0.3***	7.7 ± 0.2**

Rat alveolar macrophages were incubated for 48 h in DMEM in the presence of 5% FCS. Alveolar macrophages were pretreated with 50 u ml^{-1} rIFN-γ or 500 ng ml^{-1} LPS during 12 h. Then the stimulus was removed and the mentioned modulators were added. Data represent mean ± s.e.mean for 5 experiments.
Significance levels are shown as **$P<0.01$; ***$P<0.001$, versus control stimulation with rIFN-γ (50 u ml^{-1}) or LPS (500 ng ml^{-1}).

protease inhibitors (Kilbourn *et al.*, 1990) and the corticosteriods during the induction phase of the nitric oxide synthase (Di Rosa *et al.*, 1990; Jorens *et al.*, 1991). In summary, the inhibition by BH_4 may suggest that nitric oxide production may be regulated by the metabolic endproducts of GTP cyclohydrolase I. BH_4, DAHP and sepiapterin may be useful for investigating the role of BH_4 requirements and modulation in the biological anti-tumour or anti-microbial function of the nitric oxide synthase pathway in alveolar macrophages, known to play a key role in lung defence mechanisms (Sibille & Reynolds, 1990).

The authors wish to thank Dr B. Marescau, Department of Neurochemistry of the University of Antwerp for technical assistance in the assay of citrulline. Mrs Greta Van De Vijver and Mrs Denise Andries are gratefully acknowledged for typing the manuscript. The authors wish to express their gratitude to Dr P.H. van der Meide (TNO, Rijswijk, The Netherlands) for the gift of rIFN-γ and to Cyanamid Belgium for the gift of methotrexate.

References

ALBINA, J.E., MILLS, C.D., HENRY, W.L. & CALDWELL, M.D. (1989). Regulation of macrophage physiology by L-arginine: role of the oxidative L-arginine deiminase pathway. *J. Immunol.*, **143**, 3641–3646.

ALBINA, J.E., MILLS, C.D., HENRY, W.L. & CALDWELL, M.D. (1990). Temporal expression of different pathways of L-arginine metabolism in healing wounds. *J. Immunol.*, **144**, 3877–3880.

BELLAHSENE, Z., DHONDT, J.L. & FARRIAUX, J.P. (1984). Guanosine triphosphate cyclohydrolase activity in rat tissues. *Biochem. J.*, **217**, 59–65.

BILLIAR, T.R., CURRAN, R.D., STUEHR, D.J., WEST, M.A., BENTZ, B.G. & SIMMONS, R.L. (1989). An L-arginine-dependent mechanism mediates Kupffer cell inhibition of hepatocyte protein synthesis in vitro. *J. Exp. Med.*, **169**, 1467–1472.

DI ROSA, M., RADOMSKI, M., CARNUCCIO, R.R. & MONCADA, S. (1990). Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. *Biochem. Biophys. Res. Commun.*, **172**, 1042–1048.

GAL, E.M., NELSON, J.M. & SHERMAN, A.D. (1978). Biopterin III. Purification and characterisation of enzymes involved in the cerebral synthesis of 7,8-dihydrobiopterin. *Neurochem. Res.*, **3**, 69–88.

GROSS, S.S., STUEHR, D.J., AISAKI, K., JAFFE, E.A., LEVI, R. & GRIFFITH, O.W. (1990). Macrophage and endothelial cell nitric oxide synthesis: cell-type elective inhibition by N^G-amino-arginine, N^G-nitroarginine and N^G-methylarginine. *Biochem. Biophys. Res. Commun.*, **170**, 96–103.

HAYHOE, F.G.J. & FLEMANS, R.J. (1982). *Color Atlas of Hematological Cytology*. pp. 233–234. New York; John Wiley & Sons.

HIBBS, J.B., TAINTOR, R.R. & VANIN, Z. (1987). Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science*, **235**, 473–476.

JORENS, P.G., VAN OVERVELD, F.J., BULT, H., VERMEIRE, P.A. & HERMAN, A.G. (1991). L-arginine-dependent production of nitrogen oxides by rat pulmonary macrophages. *Eur. J. Pharmacol.*, **200**, 205–209.

KELLER, R., GEIGES, M. & KEIST, R. (1990). L-arginine-dependent reactive nitrogen intermediates as mediators of tumour cell killing by activated macrophages. *Cancer Res.*, **50**, 1421–1425.

KILBOURN, R. & LOPEZ-BERESTEIN, G. (1990). Protease inhibitors block the macrophage-mediated inhibition of tumour cell mitochondrial respiration. *J. Immunol.*, **144**, 1042–1045.

KWON, N.S., NATHAN, C.F. & STUEHR, D.J. (1989). Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.*, **264**, 20496–20501.

MCCALL, T.B., FEELISCH, M., PALMER, R.M.J. & MONCADA, S. (1991). Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br. J. Pharmacol.*, **102**, 234–238.

NICHOL, C.A., SMITH, G.K. & DUCH, D.S. (1985). Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Annu. Rev. Biochem.*, **54**, 729–764.

SCHMIDT, H.H.H.M., NAU, H., WITTFORT, W., GERLACH, J., PRESCHER, K.E., KLEIN, M.M., NIROOMAND, F. & BÖHME, F. (1988). Arginine is physiological precursor of endothelium-derived nitric oxide. *Eur. J. Pharmacol.*, **154**, 213–216.

SHEN, R.S., ALAM, A. & ZHANG, Y. (1988). Inhibition of GTP cyclohydrolase I by pterins. *Biochim. Biophys. Acta*, **965**, 9–15.

SIBILLE, Y. & REYNOLDS, H.J. (1990). Macrophages and polymorphonuclear neutrophils in lung defence and injury. *Am. Rev. Respir. Dis.*, **141**, 471–501.

SPACKMAN, D.H., STEIN, W.H. & MOORE, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.*, **30**, 1190–1206.

STUEHR, D.J., GLUFUNMILAYO, A.F., KWON, N.S., GROSS, S.S., GONZALEZ, J.A., LEVI, R. & NATHAN, C.F. (1991). Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. *Faseb J.*, **5**, 98–103.

STUEHR, D.J., KWON, N.S., GROSS, S.S., THIEL, B.A., LEVI, R. & NATHAN, C.F. (1989). Synthesis of nitrogen oxides from L-arginine by macrophage cytosol: requirements for inducible and constitutive components. *Biochem. Biophys. Res. Commun.*, **161**, 420–426.

STUEHR, D.J., KWON, N.S. & NATHAN, C.F. (1990). FAD and GSH participate in macrophage synthesis of nitric oxide. *Biochem. Biophys. Res. Commun.*, **168**, 558–565.

STUEHR, D.J. & MARLETTA, M.A. (1987). Induction of nitrite/nitrate biosynthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. *J.Immunol.*, **139**, 518–525.

TAYEH, M.A. & MARLETTA, M.A. (1989). Macrophage oxidation of L-arginine to nitric oxide, nitrite and nitrate. Tetrahydrobiopterin is required as a cofactor. *J. Biol. Chem.*, **264**, 19654–19658.

WERNER, E.R., WERNER-FELMAYER, G., FUCHS, D., HAUSEN, A. REIBNEGGER, R., YIM, J.J. & WACHTER, H. (1991). Biochemistry and function of pteridine synthesis in human and murine macrophages, *Pathobiology*, **59**, 276–279.

WERNER-FELMAYER, G., WERNER, E.R., FUCHS, D., HAUSEN, A., REIBNEGGER, R. & WACHTER, H. (1990). Tetrahydrobiopterin-dependent formation of nitrite and nitrate in murine fibroblasts. *J. Exp. Med.*, **172**, 1599–1607.

(Received April 29, 1992
Revised July 13, 1992
Accepted August 7, 1992)

Br. J. Pharmacol. (1992), **107**, 1092–1095

Inhibitors of nitric oxide synthase selectively reduce flow in tumour-associated neovasculature

[1]*†Silvia P. Andrade, *Ian R. Hart & †Priscilla J. Piper

*Biology of Metastasis Laboratory, Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London, WC2A 3PX and †Department of Pharmacology, Royal College of Surgeons of England, 35-45 Lincoln's Inn Fields, London WC2A 3PN

1 The effects of L-arginine analogues, N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) and methylene blue on blood flow in a murine adenocarcinoma and melanoma have been investigated.

2 Sponge implants in Balb/c and C57/BL mice were used to host proliferating tumour cells while the washout of ^{133}Xe was employed to assess local blood flow in the implanted sponges.

3 Pharmacological inhibition of nitric oxide (NO) reduced blood flow in both tumours but this effect was reversed by administration of L-arginine.

4 In marked contrast, the effect of these same NO inhibitors on the blood flow in sponge-induced non-neoplastic granulation tissue was negligible.

5 These results strongly suggest that: (a) flow in tumour vessels is modulated by nitric oxide which maintains a dilator tone in neoplastic tissue; (b) the constrictor activity (as monitored by an increase in $t_{\frac{1}{2}}$ of ^{133}Xe) of NO inhibitors may be attributed to the removal of such dilator tone; (c) many of the abnormalities described in tumour vasculature, such as hyporeactivity or unresponsiveness to vasoactive mediators and maximum vasodilatation, may be due to an increase in NO synthesis in cancers.

Keywords: Nitric oxide; tumours; neovasculature; granulation tissue

Introduction

A potential mediator of altered vasoactivity is nitric oxide (NO), a very short-lived substance, which is synthesized from the semi-essential amino acid L-arginine (Palmer *et al.*, 1988a) by NO synthases in the vessel walls (Moncada *et al.*, 1991) and by many other activated cells (Stuehr & Marletta, 1985; Schmidt *et al.*, 1989; Radomski *et al.*, 1990). One enzyme (constitutive) generates low NO concentrations regulating physiological vascular tone, blood pressure and tissue perfusion (Vallance *et al.*, 1989) while another is induced by endotoxin and some cytokines, and may have a pathophysiological role by sustaining release of large amounts of NO leading to a prolonged and profound vasodilatation and hypotension (Moncada *et al.*, 1991). Nitric oxide synthesis is inhibited by L-arginine analogues. N^G-nitro-L-arginine methyl ester (L-NAME; Rees *et al.*, 1990), N^G-monomethyl-L-arginine (L-NMMA; Palmer *et al.*, 1988b) and NO activity is attenuated by other compounds, for example, methylene blue (Martin *et al.*, 1985). The influence of NO as a possible factor mediating the vasodilatation found in tumour vasculature (Peterson, 1991) has been investigated by examining the effects of local injections of NO inhibitors on the blood flow in Colon 26 adenocarcinoma and B16 melanoma in syngeneic mice.

Methods

Animals

Male Balb/c and C57/BL mice weighing 20–28 g were used for all experiments. All animal procedures were carried out under a Project Licence approved by the Home Office, London, UK.

Sponge implants

Polyether polyurethane sponge discs, 4 mm height × 8 mm diameter (Vitafoam Ltd., Manchester, UK) were used as the matrix for vessel growth. To the centre of each disc, one end of a polythene tube 1.2 cm long × 1.2 mm internal diameter (Portex Ltd., Hythe, Kent) was secured with two 5.0 silk sutures (Ethicon, Ltd, UK) in such a way that the tube was perpendicular to the disc face. The sponge discs with cannulae attached were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 min.

Implantation of sponges

Discs were implanted by use of aseptic techniques in mice anaesthetized by intramuscular (i.m.) injection of fentanyl citrate/fluanisone acetate and midazolam hydrochloride (0.5 ml kg^{-1} of each). The dorsal hair was shaved and the skin wiped with 70% ethanol. A 1 cm midline incision was made and through it one subcutaneous pocket was prepared by blunt dissection. A sterilized sponge implant was then inserted in the pocket, its cannula being pushed through a small incision which previously had been made on top of the pocket. The base of the cannula was sutured to the skin. The cannula was then plugged with a smaller piece of sealed polythene tubing. The mid-line incision was closed by 2.5 silk sutures and the animals kept singly with free access to food and water after recovery from the anaesthetic.

Establishment of tumour-bearing implants

Colon 26 (Tsuro *et al.*, 1983) and B16 melanoma (Fidler & Kripke, 1977) cells were cultured in Dulbecco's modification of Eagle's essential medium supplemented with 10% foetal bovine serum and 1% L-glutamine. Once confluent, the monolayer was harvested by incubation for 2 min with trypsin/EDTA (0.025% and 0.02 w/v respectively). The dispersed cells were centrifuged for 10 min and adjusted to

[1] Author for correspondence.

the appropriate concentration in saline; 50 µl of the cell suspension (1×10^6 cells) was injected into the sponges 2 days after their implantation. This procedure yielded a tumour take of 100%, producing progressive growths which were visible around 10 and 12 days after cell injection (i.e. 12–14 days after sponge implantation).

Animals in the control group received an injection of 50 µl saline only.

Determination of blood flow in control and tumour-bearing implants

For determination of local blood flow in the implants, the mice were anaesthetized as before and a 10 µl bolus of radioactive Xenon; (^{133}Xe), was injected into the sponge implant via its cannula which was then quickly plugged. The washout of the radioactive tracer was followed by external detection over 6 min (Andrade *et al.*, 1987). The rate of washout of ^{133}Xe was expressed in terms of its half-life ($t_{1/2}$; time taken for the radioactivity to fall to 50% of its original value). For the skin blood flow measurement 10 µl of ^{133}Xe solution was injected intradermally in a site adjacent to the implants.

Effects of inhibitors of NO on blood flow of tumour-bearing and control implants

The effects of inhibitors of NO synthesis, N^G-nitro-L-arginine methyl ester (L-NAME, 3, 10 and 30 µg) and N^G-monomethyl-L-arginine (L-NMMA, 3 and 30 µg) and methylene blue (0.5 and 5 µg) which inhibit NO activity, on blood flow were examined in control and tumour-bearing implants at day 14 postimplantation (tumour weight = 500–700 mg). For each implant, the untreated $t_{1/2}$ was measured following injection of 50 µl of saline alone and 40 min later the $t_{1/2}$ value was measured following injection of inhibitor (50 µl). Up to three successive ^{133}Xe washout assays, with 45 min intervals, were possible in one implant per day.

Assessment of the effects of L-NAME in presence of L-arginine

L-Arginine, (200 mg kg^{-1} body weight) was given intravenously (i.v.) as a bolus injection immediately before the intra-tumour injection of 30 µg in 50 µl L-NAME, in both tumours at day 14 postimplantation to verify whether this amino acid, in excess, would prevent any decrease in blood flow induced by NO synthase inhibitor (L-NAME) in Colon 26 and B16 tumour blood vessels. For this experiment 2 groups of 8 animals bearing either tumour type were used. At day 14 post sponge implantation (12 days after tumour cell injection into the implants) a control blood flow measurement in anaesthetized mice was carried out in all 16 animals. Later (45–60 min), a bolus injection of L-NAME (30 µg) was given into the tumours followed by ^{133}Xe washout measurement. When the remaining radioactivity in the implants was at the background level, a third blood flow measurement was performed. A bolus i.v. injection of L-arginine was followed by an intra-tumour injection of L-NAME (30 µg) and ^{133}Xe washout was again monitored. The last blood flow measurement was performed to determine the effect of L-arginine on blood flow in the implants. The whole experimental procedure lasted about 4 h and a booster dose of anaesthetic was necessary to complete the session. The animals were killed at the end of experiment.

Pharmacological study of the development of the sensitivity of newly formed blood vessels to NO synthase inhibition

In this set of experiments the blood flow of 2 groups of 8 animals each, either control (non tumour-bearing implants) or tumour (Colon 26-bearing implants) was monitored on three separate occasions (days 8, 11 and 14 postimplantation).

L-NAME (30 µg in 50 µl) was injected into sponges followed by ^{133}Xe washout measurement in both tumour-bearing and control implants to determine when the vessels became sensitive to NO synthase inhibition.

Chemicals

Xenon injection, ^{133}Xe, (10 mCi in 3 ml) was obtained from Amersham International U.K. Hypnorm (315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone acetate) from Janssen Pharmaceuticals, Oxford and Hypnovel (5 mg ml^{-1} of midazolam hydrochloride) from Roche Pharmaceuticals, Welwyn Garden City, UK.

N^G-nitro-L-arginine methyl ester (L-NAME), methylene blue and L-arginine were all obtained from Sigma.

N^G-monomethyl-L-arginine (L-NMMA) was a gift from Dr Salvador Moncada, Wellcome Foundation Ltd.

Statistical analysis

Results are given as mean (± s.e.mean) values from n animals. Comparisons between groups were made with Student's t test for unpaired data and a P value less than 0.05 was considered significant.

Results

Effect of tumour cells on the implant vascularization

In the control implants (non tumour-bearing implants in Balb/c and C57 mice), new vessels grew and ^{133}Xe washout increased steadily during a 14 day-period from a $t_{\frac{1}{2}}$ value of 26 ± 2 min at day 4 to a limiting value of 5 ± 2 min; a value identical to that observed in normal skin vasculature (5 ± 0.5 min). In the presence of tumour cells, $t_{1/2}$ at day 7 postimplantation was 7 ± 1 min in Colon 26 and 8 ± 2 in B16 growths versus 15 ± 1 and 16 ± 2 min for their respective sponge only controls (Table 1). These tumour-induced reductions in $t_{\frac{1}{2}}$ values represent the consequence of tumour angiogenesis.

Effects of inhibition of NO on blood flow of tumour-bearing and control implants

Figure 1a,b,c, illustrates the ability of local injections of L-NAME, L-NMMA and methylene blue to reduce blood flow (increase $t_{1/2}$) over a range of doses in Colon 26 tumours. The control implants (non tumour-bearing) showed a much smaller response to L-NAME and no response to L-NMMA or methylene blue. L-NAME, the most potent inhibitor in reducing blood flow in Colon 26, also significantly reduced the blood flow in B16 tumour implants relative to the control implants (Figure 1d).

Table 1 Effect of tumour cells (Colon 26 and B16) on the implant vascularisation

	$t_{\frac{1}{2}}$(min) ^{133}Xe washout			
Days post-implantation	*Control implants*		*Tumour-bearing implants*	
	Balb/c $n = 10$	C57/BL $n = 10$	Colon 26 $n = 10$	B16 $n = 10$
4	26 ± 2	25 ± 2	24 ± 2	27 ± 2
7	15 ± 2	16 ± 2	7 ± 1*	8 ± 2*
10	8 ± 1	9 ± 2	6 ± 1	7 ± 1
14	5 ± 1	6 ± 1	5 ± 1	5 ± 1

The values in the table are the mean ± s.e.mean from the number of animals (n =). *P<0.05, compared with control implants.

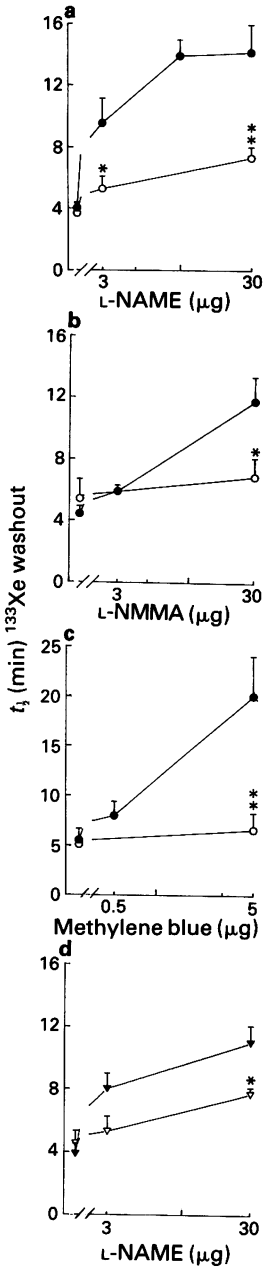

Figure 1 Effects of NO inhibitors on the blood flow of tumour-bearing implants (Colon 26 ● and melanoma ▼) and control implants (open symbols) as determined by washout rate of ^{133}Xe. Values to the left of the break represent ^{133}Xe washout from untreated sponges at day 14 postimplantation. Intra-sponge injections of N^G-nitro-L-arginine methyl ester (L-NAME) (a) N^G-monomethyl-L-arginine (L-NMMA) (b) and methylene blue (c) decreased blood flow, i.e. increased $t_{1/2}$, over a range of doses in Colon 26 tumours. Blood flow in control implants was reduced significantly only with 30 μg of L-NAME. In B16 melanoma (d) $t_{1/2}$ was enhanced 2 and 3 fold with 3 and 30 μg L-NAME respectively.

The values shown represent mean ± s.e.mean (vertical bars) from 6 to 12 animals at each dose.

Comparison between groups was made with Student's t test for unpaired data. $^{**}P<0.001$; $^{*}P<0.05$ compared with control values.

Assessment of the effects of L-NAME on blood flow in the presence of L-arginine

The effect of i.v. injection of L-arginine prevented any significant reduction in blood flow in both types of tumour as shown in Figure 2a,b. This amino acid alone had no effect in either increasing or decreasing blood flow in tumours.

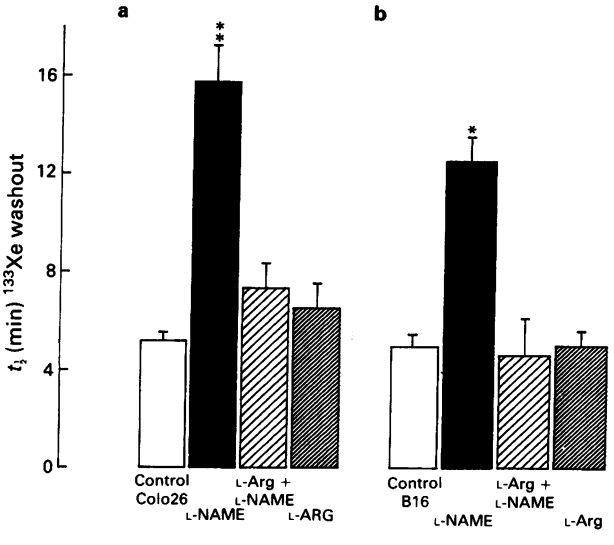

Figure 2 L-Arginine prevents reduction in tumour blood flow induced by N^G-nitro-L-arginine methyl ester (L-NAME) in (a) Colon 26 carcinoma or (b) B16 melanoma. A bolus injection of L-arginine (Arg) 200 mg kg^{-1}, i.v. plus L-NAME (30 μg intra-tumour) given 80–90 min after L-NAME alone (30 μg) which had enhanced $t_{1/2}$ 4 fold, prevented reduction in blood flow. The values shown represent mean ± s.e.mean (vertical bars) from 8 animals in each group; blood flow measurement was made on the same animals on four different occasions. $^{**}P<0.001$; $^{*}P<0.05$ compared with control values.

Pharmacological study of the development of sensitivity of newly formed blood vessels to NO synthase inhibition

Development of sensitivity of the newly formed blood vessels in both the granulation tissue and neoplastic tissue, to NO inhibitors with time was monitored by injecting L-NAME at different stages after sponge implantation. Results showed that despite maximum blood flow being achieved in tumour-bearing implants at day 8 postimplantation ($t_{1/2}$ 7 ± 1 min, similar to that observed in normal skin vessels or comparable to the fully vascularized control implants at day 14) no increase in $t_{1/2}$ was detected following the treatment within this time period. However, a highly significant reduction of blood flow was demonstrated in tumour-bearing implants at days 11 and 14 ($P<0.001$) while normal vessels, i.e. in control sponges, showed only a limited response to L-NAME at day 14 ($P<0.05$), (Table 2).

Table 2 Effects of N^G-nitro-L-arginine methyl ester (L-NAME, 30μg) on the washout of locally injected ^{133}Xe in tumour-bearing and control implants

	$t_{\frac{1}{2}}$(min) ^{133}Xe washout			
Days post-implantation	*Tumour-bearing*		*Control implants*	
	Untreated	*L-NAME*	*Untreated*	*L-NAME*
8	7 ± 1	8 ± 1	14 ± 1	13 ± 1
11	5 ± 1	14 ± 1**	5 ± 1	6 ± 1
14	4 ± 2	16 ± 2**	5 ± 1	7 ± 1*

Results of L-NAME intra-sponge injection at different times during vascular development in control and adeno-carcinoma-bearing implants. Increase in $t_{\frac{1}{2}}$ (decrease in blood flow) observed in tumour-associated blood vessels at day 11 and 14 postimplantation. In contrast, newly formed blood vessels in the control implants responded slightly to L-NAME only on day 14. The values in the table are the mean ± s.e.mean from 8 animals in each group of implants; blood flow measurement were made on the same animal on three different occasions. $^{**}P<0.001$; $^{*}P<0.05$ compared with control values taken 40 min before injection of the drug.

Discussion

We have previously used a combination of the techniques of sponge implantation and ^{133}Xe washout to study neovascularization in rats and mice and to study the pharmacological response of the neovasculature to vasoactive mediators (Andrade *et al.*, 1987; 1991). In this paper we have used these techniques to investigate the response of newly formed blood vessels to pharmacological inhibition of NO in neoplastic and granulation tissue.

Thus, if endogenous NO is formed in the tumour vascular beds promoting increased vasodilatation, inhibition of NO formation would be expected to reduce blood flow. We have shown that L-NAME (inhibitor of NO synthase), applied locally into sponge implants bearing either murine adenocarcinoma or murine melanoma, reduced their blood flow and this effect was reversed by i.v. injection of L-arginine. In marked contrast, the effects of nitric oxide inhibitors on the blood flow of control implants (non-tumour-bearing) were negligible (significant only with L-NAME, 30 μg). L-NAME was approximately 2 times more potent than L-NMMA in decreasing blood flow in Colon 26 tumours. This compound has been shown to be 10 fold more potent in increasing blood pressure in rats (Rees *et al.*, 1990). The fact that no change in tumour blood flow was observed on day 7 post-timplantation after L-NAME injections, despite the fact that maximum blood flow had been achieved, might indicate that the vessels were too immature to constrict or the amount of nitric oxide synthesized at that stage was not high enough to be influenced by the NO synthase inhibitors.

Another specific inhibitor of NO synthase (L-NMMA) and methylene blue, which inhibits NO action, also caused blood flow reduction in Colon 26 adenocarcinoma. These results strongly suggest that flow in tumour vessels is modulated by NO which maintains a dilator tone. The removal of this basal tone could account for the constrictor activity (increase in $t_{\frac{1}{2}}$) of NO inhibitors.

There are several possible explanations for the difference in response of the blood vessels in control and tumour-bearing implants to NO inhibitors. Firstly, the production of NO in granulation tissue may be less than in tumours as a consequence of differences in biochemical pathways. The amino acid composition in wound fluid from subcutaneous implanted sponges in rats showed decreased arginine and elevated ornithine content as a result of the action of macrophage-derived arginase (Albina *et al.*, 1988). Even though arginase activity was also found to be increased in tumours (Yamanaka *et al.*, 1972) it may be that in wound-healing, arginine is required for collagen synthesis rather than NO generation. Thus, in tumours it may be that L-arginine metabolism leads to the predominant formation of NO/citrulline versus urea/ornithine production in wound repair. Secondly, tumour cells might contribute directly to the production of NO within the tumour mass. Human colorectal adenocarcinoma cells have been shown to generate NO (Radomski *et al.*, 1991) and an inducible NO synthase has been found in EMT-6 cells, a spontaneous murine mammary adenocarcinoma cell line (Amber *et al.*, 1988), and it could be that transformed cells generally express the inducible enzymes. An inducible NO synthase has been found to be expressed throughout the vessel wall in response to endotoxin or certain cytokines, and is implicated in a pathophysiological role by sustaining release of large amounts of NO that produce prolonged and profound vasodilatation and hypotension (Moncada *et al.*, 1991). Thus, NO could contribute to many of the abnormalities described in tumour vasculature such as hyporeactivity or unresponsiveness to vasoactive mediators (Wickersham *et al.*, 1977; Andrade *et al.*, 1991) and maximum vasodilatation (Peterson, 1991). The differential response of tumour vessels to inhibitors of NO demonstrated in our experiments may provide new pharmacological approaches to tumour therapy.

References

ALBINA, J.E., MILLS, C.D., BARBUL, A., THIRKILL, C.E., HENRY, W.J.R., MASTROFRANCESCO, B. & CALDWELL, M.D. (1988). Arginine metabolism in wounds. *Am. J. Physiol.*, **254**, E459–E467.

AMBER, I.J., HIBBS, J.B.J.R., TAINTOR, R.R. & VAVRIN, Z. (1988). The L-arginine dependent effector mechanism is induced in murine adenocarcinoma cells by culture supernatant from cytotoxic activated macrophage. *J. Leukocyte Biol.*, **43**, 187–192.

ANDRADE, S.P., BAKHLE, Y.S. & PIPER, P.J. (1991). Decreased response to platelet activating factor (PAF), endothelin-1 (ET-1) and angiotensin II (AII) in tumour blood vessels in mice. *Br. J. Pharmacol.*, **104**, 422P.

ANDRADE, S.P., FAN, T.-P.D. & LEWIS, G.P. (1987). Quantitative in vivo studies on angiogenesis in a rat sponge model. *Br. J. Exp. Pathol.*, **68**, 755–765.

FIDLER, I.J. & KRIPKE, M.L. (1977). Metastasis results from preexisting variant cells within a malignant tumour. *Science*, **197**, 893–895.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glygeryl trinitrate-induced relocation by haemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988b). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **152**, 1251–1256.

PETERSON, H.-I. (1991). Modification of tumour blood flow-a review. *Int. J. Radiat. Biol.*, **60**, 201–210.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). An L-arginine: nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5193–5197.

RADOMSKI, M.W., JENKINS, D.C., HOLMES, L. & MONCADA, S. (1991). Human colorectal adenocarcinoma cells: differential nitric oxide-synthesis determines their ability to aggregate platelets. *Cancer Res.*, **51**, 6073–6078.

REES, D.D., PALMER, R.-M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

SCHMIDT, H.H.H.W., SEIFERT, R. & BOHME, E. (1989). Formation and release of nitric oxide from human neutrophil and HL-60 cells induced by chemotatic peptide, platelet activating factor and leukotriene B_4. *FEBS Lett.*, **244**, 357–360.

STUEHR, D.J. & MARLETTA, M.A. (1985). Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 7738–7742.

TSURO, T., YAMORY, T., NAGANUMA, K., TSUKAGOSHI, I. & SAKURAI, Y. (1983). Characterisation of metastatic clones derived from a metastatic variant of mouse colon adenocarcinoma. *Cancer Res.*, **43**, 5437–5442.

VALLANCE, P., COLLIER, J. & MONCADA, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet*, **ii**, 997–1000.

WICKERSHAM, J.K., BARRET, W.P., FURUKAWA, S.B., PUFFER, W.H. & WARNER, N.E. (1977). An evaluation of the response of the microvasculature in tumours in C3H mice to vasoactive drugs. *Bibliot. Anat.*, **15**, 291–293.

YAMANAKA, H., MAYUZUMI, T., MATSUOKA, M., SHIMAZAKI, J. & SHIDA, K. (1972). Arginase in human urogenital tumours. *Gann.*, **63**, 693–700.

(Received May 22, 1992
Revised July 22, 1992
Accepted August 7, 1992)

Br. J. Pharmacol. (1992), **107**, 1096–1101

L-Leucyl-L-arginine, naltrindole and D-arginine block antinociception elicited by L-arginine in mice with carrageenin-induced hyperalgesia

Atsufumi Kawabata, Yumiko Nishimura & [1]Hiroshi Takagi

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kinki University 3-4-1 Kowakae, Higashi-Osaka 577, Japan

1 Intraplantar injection of carrageenin into the mouse hind paw produced hyperalgesia when measured by the paw pressure test (Randall & Selitto method).

2 Subcutaneous administration of L-arginine (100–1,000 mg kg^{-1}), a possible precursor of kyotorphin which is an endogenous analgesic neuropeptide, inhibited carrageenin-induced hyperalgesia in a dose-dependent manner. This effect was blocked by subcutaneous administration of naloxone, naltrindole, a selective δ-opioid receptor antagonist (enkephalin antagonist), and D-arginine.

3 Intracerebroventricular administration of L-leucyl-L-arginine inhibited the antinociceptive effect of systemically administered L-arginine in hyperalgesic mice.

4 Intracerebroventricular administration of L-arginine (3 and 30 μg per mouse) and kyotorphin (300 ng–3 μg per mouse) produced antinociception in hyperalgesic mice. The antinociceptive effects of L-arginine but not kyotorphin were blocked by intracerebroventricular administration of D-arginine.

5 These results suggest that L-arginine-induced antinociception is mediated by activation of 'kyotorphinergic' nerves followed by activation of the 'opioidergic' (possible 'enkephalinergic') nerves in the central nervous system.

Keywords: L-arginine; kyotorphin; enkephalin; antinociceptive effect; L-leucyl-L-arginine; D-arginine; carrageenin

Introduction

Kyotorphin (L-tyrosyl-L-arginine), an endogenous neuropeptide isolated from bovine brain, produces naloxone-reversible antinociception by enhancing Met-enkephalin release in the brain and spinal cord (Takagi *et al.*, 1979a,b), and is considered to be a neurotransmitter or neuromodulator (Takagi & Ueda, 1988), since it is localized in synaptosomes (Ueda *et al.*, 1982), and released by depolarizing stimuli in a Ca^{2+}-dependent manner (Ueda *et al.*, 1986). It is synthesized from L-tyrosine and L-arginine by a specific enzyme, kyotorphin synthetase, in the presence of ATP and $MgCl_2$ (Ueda *et al.*, 1987), although, like endogenous opioid peptides, it can also be formed from its precursor protein by processing (Yoshihara *et al.*, 1988). In the former pathway, L-arginine may be a rate limiting factor for enzymatic biosynthesis of kyotorphin, since the K_m value for L-arginine is higher than its physiological concentration in the brain. This theory predicts that L-arginine effectively acts as a precursor of kyotorphin *in vivo*, resulting in antinociception.

We have recently demonstrated that subcutaneous administration of L-arginine produced a naloxone-reversible antinociceptive action in rats with carrageenin-induced hyperalgesia (Kawabata *et al.*, 1992). The therapeutic significance of L-arginine is also supported by our clinical finding that intravenous infusion of L-arginine produces potent analgesia in a naloxone-reversible manner in patients with various types of chronic pain (Takagi *et al.*, 1990; Harima *et al.*, 1991).

Here we show that L-arginine-induced antinociception is antagonized by L-leucyl-L-arginine, a kyotorphin receptor antagonist (Ueda *et al.*, 1989), and by naltrindole, a selective δ-opioid antagonist (Portoghese *et al.*, 1988), suggesting that the antinociception is mediated by kyotorphin and enkephalin in the brain, and that D-arginine antagonizes the effect of L-arginine through distinct mechanisms from L-leucyl-L-arginine.

[1] Author for correspondence.

Methods

Animals

Male ddy mice weighing 20–30 g (Japan SLC. Inc.) were given food and water *ad libitum*.

Nociceptive assay

The paw pressure test described by Randall & Selitto (1957) was applied to mice. An analgesia meter was used (MK-300, Muromachi Kikai Co. Ltd, Japan) with a pencil-shaped wooden paw-presser with a dull tip; pressure was gradually applied to the hind paw at an increasing linear rate of 15 g s^{-1}. The weight (g) required to elicit nociceptive responses such as squeak and struggle was determined as a mechanical nociceptive threshold. A cut-off value of 250 g was used to prevent damage to the paw. The nociceptive threshold of each mouse was measured 6–8 times, and only mice with stable thresholds were used in experiments. The control threshold for each mouse was defined as the mean of the values of the last 4 stable thresholds, since the initial 2–4 values were in general, high and unstable. Results are expressed as a percentage of the control threshold. In one experiment, thermal nociception was assayed with a tail flick analgesia meter (MK-330, Muromachi Kikai Co. Ltd., Japan), in which the intensity of the thermal stimulus was adjusted to obtain basal latencies of 2.0–2.5 s.

Induction of hyperalgesia in mice

Hyperalgesia was induced by intraplantar (i.pl.) injection of 25 μl of 1% carrageenin into the right hindpaw. This dose of carrageenin has been reported to elicit paw oedema in the mouse, following a time pattern similar to that seen in the rat (Levy, 1969). The nociceptive threshold was measured at 30 min intervals, unless otherwise stated.

Experimental protocol

Antinociceptive action of systemically administered L-arginine L-Arg (100–1,000 mg kg^{-1}) was given s.c. to mice 30 min after carrageenin treatment. The interaction between L-Arg and three other agents was evaluated according to the following schedules. Naloxone (Nlx, 1 mg kg^{-1}), an opioid antagonist, and naltrindole (NTI, 1 mg kg^{-1}), a δ-selective opioid antagonist, were administered s.c. 30 and 20 min respectively after 1,000 mg kg^{-1} of L-Arg. D-Arg and L-Arg at 1,000 mg kg^{-1} were co-administered s.c. 30 min after carrageenin. Leu-Arg (300 ng per mouse), a KTP antagonist, was given i.c.v. 20 min after 1,000 mg kg^{-1} of L-Arg (50 min after carrageenin).

Antinociception induced by intracerebroventricular administration of L-arginine and kyotorphin L-Arg (30 ng–300 μg per mouse) and KTP (30 ng–3 μg per mouse) were administered i.c.v. to carrageenin-treated (30 min after carrageenin) and non-treated mice. In this case, the nociceptive threshold was assessed 5, 10, 20, 40 and 60 min after i.c.v. injection. D-Arg at doses of 3–30 μg per mouse was co-administered i.c.v. with L-Arg or KTP to carrageenin-treated and non-treated mice.

Control animals received a saline injection s.c., or i.c.v.

Statistical analysis

The results are expressed as means with s.e.mean. Statistical significance between groups was analyzed by Newman-Keuls' multiple comparison test and was set at $P<0.05$.

Chemicals

Naloxone hydrochloride (Nlx), λ-carrageenin as well as kyotorphin (KTP) and L-leucyl-L-arginine (Leu-Arg) as the acetate salt were purchased from Sigma Chem. Co. (U.S.A.). Both L-arginine (L-Arg) and D-arginine (D-Arg) as the hydrochloride salt were from Nacalai Tesque (Japan). Naltrindole hydrochloride (NTI, Sigma) was a gift from Dr K. Takahashi. λ-Carrageenin was dissolved in distilled water and all other chemicals in saline.

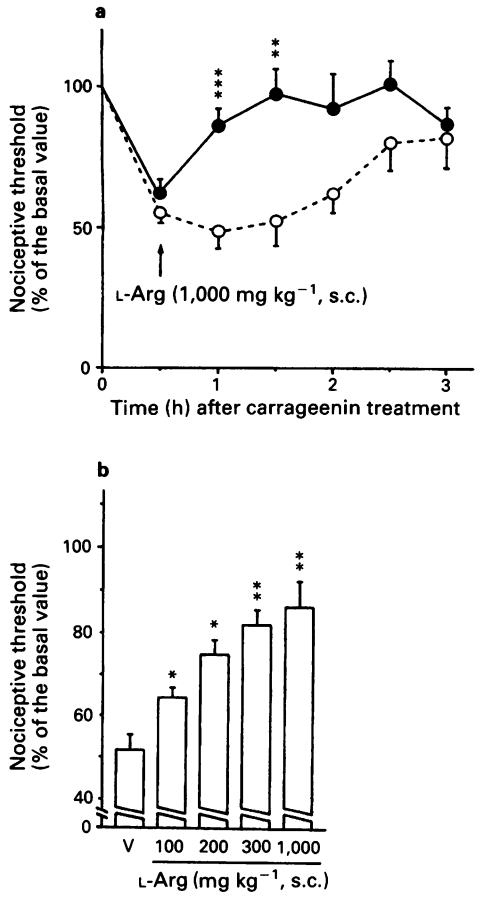

Figure 1 (a) Time-related effects of L-arginine (L-Arg, 1,000 mg kg^{-1}, s.c.) on the nociceptive threshold in carrageenin-induced hyperalgesia in mice. L-Arg was administered s.c. 30 min after carrageenin treatment. (○), Vehicle ($n=7$); (●), L-Arg 1,000 mg kg^{-1} ($n=7$). $^{**}P<0.01$, $^{***}P<0.001$ vs. vehicle. (b) Dose-related antinociceptive effects of L-arginine (100–1,000 mg kg^{-1}) in mice with carrageenin-induced hyperalgesia. Data indicate the nociceptive threshold 30 min after s.c. administration of L-Arg ($n=6$–7) or vehicle ($n=13$) (60 min after carrageenin). $^{*}P<0.05$, $^{**}P<0.01$, vs. vehicle (V).

Results

Carrageenin-induced hyperalgesia and L-arginine (s.c.)-induced antinociception

After an i.pl. injection of carrageenin, the nociceptive threshold of the carrageenin-treated hindpaw decreased almost to 50% of the basal value at 30 min, which was maintained for at least 1–1.5 h, followed by a slow recoevery (Figure 1). That of the contralateral (non-treated) hindpaw showed only a transient and slight tendency toward a decrease (threshold: 79.4 ± 8.7 and 92.4 ± 14.5% at 30 and 60 min after carrageenin, respectively, being not significantly different from the basal value). L-Arg at doses of 100–1,000 mg kg^{-1}, when administered s.c. 30 min after carrageenin, significantly elevated the decreased threshold of the hyperalgesic hindpaw in a dose-dependent manner (Figure 1), without affecting the threshold of the contralateral hindpaw and also without causing any behavioural change. The nociceptive thresholds in intact mice were resistant to the highest dose of L-Arg (1,000 mg kg^{-1}, s.c.). The L-Arg (1,000 mg kg^{-1})-induced antinociception in the hyperalgesic mouse was completely blocked by Nlx (1 mg kg^{-1}, s.c.), when it was administered 30 min after L-Arg (Figure 2).

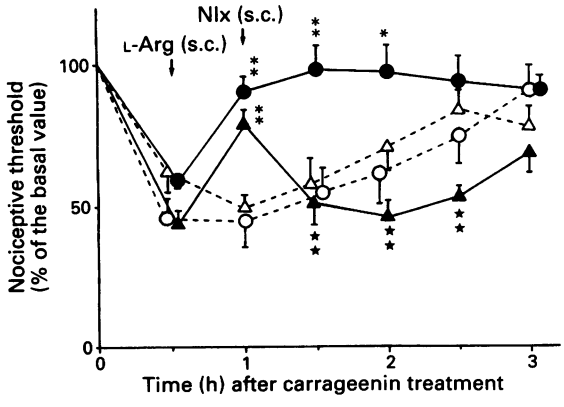

Figure 2 Antagonism by naloxone (Nlx) of L-arginine (L-Arg)-induced antinociception in mice with carrageenin-induced hyperalgesia. L-Arg (1,000 mg kg^{-1}) and Nlx (1 mg kg^{-1}) were administered s.c. 30 and 60 min respectively, after carrageenin treatment. (○) Vehicle + vehicle; (△) vehicle + Nlx; (●) L-Arg + vehicle; (▲) L-Arg + Nlx. $^{*}P<0.05$, $^{**}P<0.01$ vs. vehicle + vehicle; ★★ $P<0.01$ vs. L-Arg + vehicle. $n=6$.

Antagonism by L-leucyl-L-arginine, naltrindole and D-arginine of L-arginine-induced antinociception

In a preliminary experiment, when kyotorphin (KTP, 3 μg per mouse, i.c.v.) and Leu-Arg (300 ng per mouse, i.c.v.), a KTP antagonist, were co-administered, KTP-induced antinociception was completely blocked by Leu-Arg; the threshold at 5 min after administration was 97.5 ± 5.6, 92.9 ± 3.4, 167.9 ± 6.1 and 78.7 ± 9.2%, in groups treated with vehicle, Leu-Arg, KTP and Leu-Arg plus KTP, respectively ($n = 4$), and both the effect of KTP and its antagonism by Leu-Arg were significant ($P<0.01$). The same dose of Leu-Arg completely blocked antinociception induced by L-Arg (1,000 mg kg^{-1}, s.c.) in carrageenin-treated mice, although, when administered alone, it produced no effect on the hyperalgesia (Figure 3). Similarly, naltrindole at 1 mg kg^{-1}, s.c., which completely antagonized KTP (i.c.v.)-induced antinociception in a preliminary experiment, inhibited L-Arg (1,000 mg kg^{-1}, s.c.)-induced antinociception, although alone, it did not show any effect on the hyperalgesia (Figure 3). D-Arg (1,000 mg kg^{-1}, s.c.) blocked the L-Arg (1,000 mg kg^{-1}, s.c.)-induced antinociception, when both agents were co-administered, although D-Arg alone had no antinociceptive activity (Figure 3).

Antinociceptive effects induced by centrally administered L-arginine and kyotorphin

L-Arg, when administered i.c.v. at doses of 3 and 30 μg per mouse, produced rapid and potent antinociception in hyperalgesic mice, which peaked at 5 min (Figure 4a, left). Injections of L-Arg at doses of 30 and 300 μg per mosue i.c.v. induced significant antinociceptive effects even in intact mice, although the effective dose-range was about 10 fold higher than that in hyperalgesic mice. The maximal effect of L-Arg was obtained 20 min after administration in intact mice (Figure 4a, right). In addition, when the effect was evaluated by the tail-flick test, i.c.v. injections of L-Arg at doses of 30 and 300 μg per mouse also exhibited dose-dependent antinociception in intact mice in a similar manner; the threshold 20 min after injection was 2.10 ± 0.06 s, 3.35 ± 0.27 s ($P<0.05$), 4.10 ± 0.65 s ($P<0.01$), in groups treated with vehicle and L-Arg at 30 and 300 μg per mouse, respectively ($n = 4$). L-Arg given s.c. at 1,000 mg kg^{-1} failed to produce such effects (data not shown).

KTP (i.c.v.) at a dose-range of 300 ng–3 μg per mouse produced potent antinociception both in intact and hyperalgesic mice, which peaked at 5 min in both groups of mice. Such effects disappeared 10 min after administration in the former, but persisted even at 70–90 min in the latter (Figure 4b).

Interaction between D-arginine and L-arginine or kyotorphin in the brain

In hyperalgesic mice, L-Arg (3 μg per mouse, i.c.v.)-induced antinociception was completely blocked by co-administration of the same dose of D-Arg which, given alone, did not affect the hyperalgesia (Figure 5a). A similar result was obtained by the co-administration of 30 μg per mouse (i.c.v.) of both drugs to intact mice (Figure 5b).

By contrast, in intact mice, D-Arg even at a dose of 30 μg per mouse (i.c.v.) failed to inhibit the antinociceptive action of KTP (3 μg per mouse, i.c.v.) (data not shown).

Discussion

These results show that carrageenin-induced hyperalgesia in mice is a useful model of persistent pain, which is similar to that in rats: L-Arg (s.c.) elicits naloxone-reversible antinociception in mice with carrageenin-induced hyperalgesia. An i.c.v. injection of a small dose (3 μg per mouse) of L-Arg is also effective in hyperalgesic mice, but not in intact mice. However, in the intact mice, i.c.v. administration of a large dose (30 μg per mouse) of L-Arg elicited antinociception. These results suggest that hyperalgesia elevates the antinociceptive effect of L-Arg, possibly due to the induction of kyotorphin synthetase.

In general, experimental hyperalgesia has been observed during carrageenin-induced inflammation or heat injury in the rat hindpaw (Coderre & Melzack, 1985; Kayer & Guilbaud, 1987), and it involves central and peripheral mechanisms (Coderre & Melzack, 1987; Treede *et al.*, 1992). The peripheral mechanism of carrageenin-induced hyperalgesia is in part due to the increased local formation of bradykinin, since levels of immunoreactive bradykinin increased two fold during carrageenin treatment (Hargreaves *et al.*, 1988). Prostanoids also participate in the peripheral mechanism of pain in that potent suppression by cyclo-

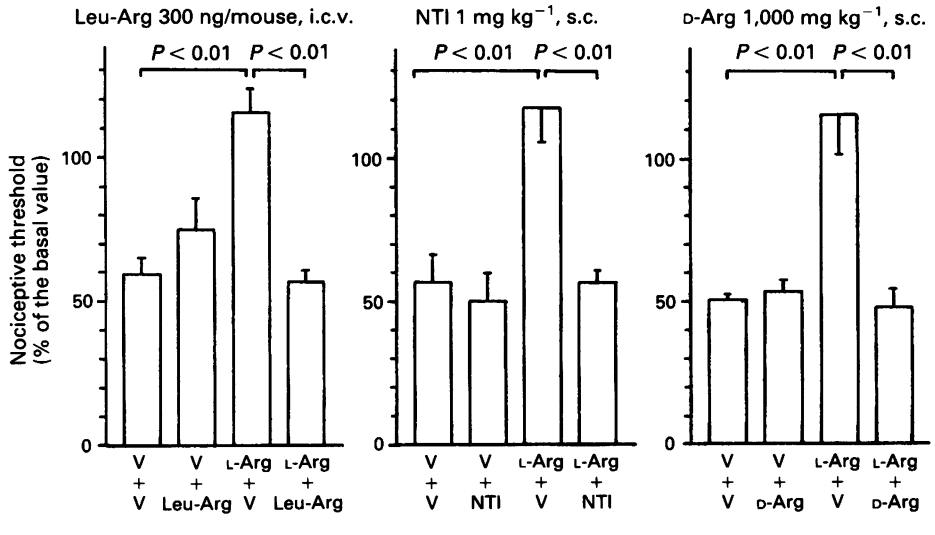

Figure 3 Antagonism of L-arginine (L-Arg)-induced antinociception by L-leucyl-L-arginine (Leu-Arg), naltrindole (NTI) and D-arginine in mice with carrageenin-induced hyperalgesia. L-Arg (1,000 mg kg^{-1}) was administered s.c. 30 min after carrageenin treatment. Leu-Arg (i.c.v.) and NTI (s.c.) were given 20 and 10 min after L-Arg injection, respectively, and D-Arg was co-administered with L-Arg. Data indicate the threshold 60 min after the carrageenin-treatment. V: vehicle. $n = 4$.

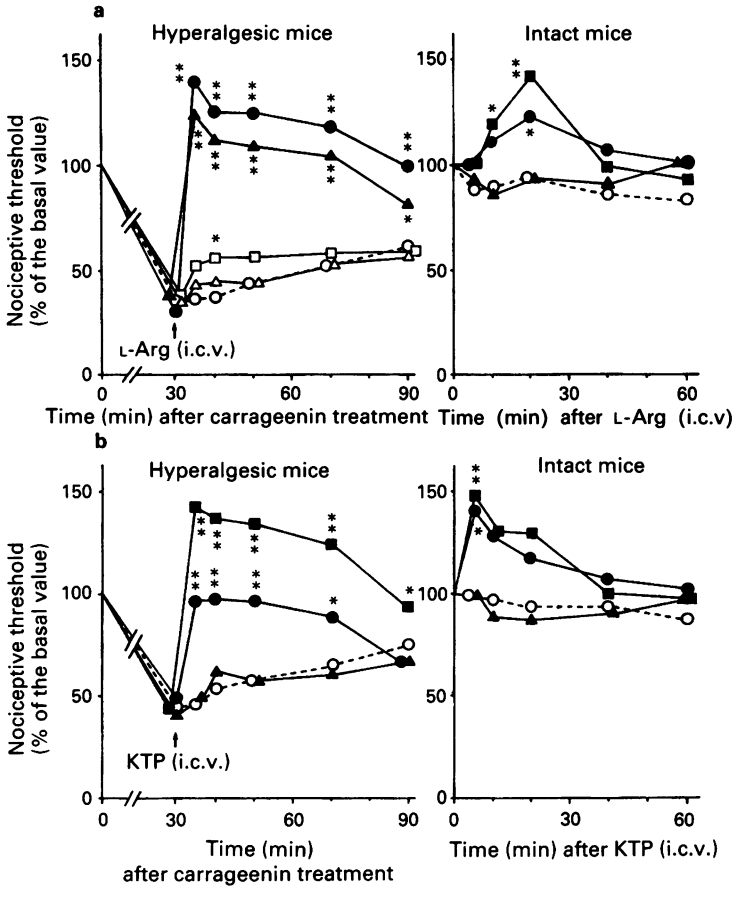

Figure 4 Antinociceptive effects induced by i.c.v. administration of L-arginine (L-Arg) and kyotorphin (KTP) in mice with and without carrageenin-induced hyperalgesia. L-Arg and KTP were administered i.c.v. to hyperalgesic mice (30 min after carrageenin) or to intact mice. (a) (○) Vehicle; (△) L-Arg 30 ng per mouse; (□) L-Arg 300 ng per mouse; (▲), L-Arg 3 μg per mouse; (●), L-Arg 30 μg per mouse; (■), L-Arg 300 μg per mouse. (b) (○) Vehicle; (▲), KTP 30 ng per mouse; (●), KTP 300 ng per mouse; (■), KTP 3 μg per mouse. Data are expressed as means without s.e.mean. $*P<0.05$, $**P<0.01$ vs. vehicle. $n=4$.

oxygenase inhibitors of the hyperalgesia has been reported (Treede *et al.*, 1992). The central mechanism is complex, but a recent study has suggested that C-fibre neuropeptides (e.g. substance P, vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), somatostatin, calcitonin gene-related peptide (CGRP) and galanin) and excitatory amino acids (L-glutamate) are involved in inducing hyperalgesia in the spinal dorsal horn (Coderre & Melzach, 1991). L-Arg appears to inhibit the central neuronal mechanism involving hyperalgesia.

That L-Arg-induced antinociception was blocked by systemic administration of naloxone (Nlx) suggests the involvement of opioid peptides. That L-Arg-induced antinociception was blocked by i.c.v. administration of Leu-Arg, a KTP receptor antagonist, indicates that the antinociception is mediated by KTP formed from L-tyrosine and L-Arg by a KTP synthetase (Ueda *et al.*, 1987). The potency of Leu-Arg as a KTP antagonist is shown by the fact that antinociception induced by an i.c.v. injection of KTP was blocked by an i.c.v. injection of Leu-Arg. As described in the Introduction, L-Arg is a precursor of KTP and administration of L-Arg should increase the KTP level in the brain, which would result in an enhancement of Met-enkephalin-release and antinociception. This possibility is further supported by the present finding that NTI, a selective δ-opioid receptor antagonist, antagonizes the antinociceptive effects of L-Arg as well as KTP. These results suggest the presence of a functional link between the 'kyotorphinergic' system and the 'enkephalinergic' system. The relatively long duration of the antinociceptive action of L-Arg and KTP in hyperalgesia may be due to enhanced recycling of L-Arg; KTP, formed from L-tyrosine and L-Arg in the nerve endings, is released to the synaptic cleft and metabolized by bestatin-sensitive aminopeptidases into L-Arg, which is then incorporated into the nerve endings and re-utilized as a precursor (Ueda *et al.*, 1985; 1986; 1987). Based upon the above evidence, we show a hypothetical scheme of the mechanisms of L-Arg-induced antinociception in Figure 6.

In addition, the role of nitric oxide (NO) formed from L-Arg by a NO synthase in the brain should be considered. According to Bredt & Snyder (1990), purified rat brain NO synthase has high affinity for L-Arg with a K_m of 1.5 μM and it is saturated with L-Arg under normal conditions, since the physiological concentration of L-Arg in the brain is approximately 100 μM (Levy *et al.*, 1967; Norberg & Siesjo, 1975). By contrast, the K_m value of KTP synthetase is 926 μM for L-Arg and 100 μM for L-tyrosine (Ueda *et al.*, 1987), suggesting that KTP synthetase is less active under normal conditions and is activated when sufficient L-Arg accumulates in the brain after it is systemically or i.c.v. administered. Moreover, systemic administration of L-N^G-nitroarginine, a selective NO synthase inhibitor, elicits antinociception in mice by a supraspinal effect which is antagonized by systemic administration of L-Arg (Moore *et al.*, 1991). Spinal inhibition of NO synthase also results in antinociception (Haley *et al.*, 1992). This evidence suggests that the L-Arg-NO pathway in the CNS is not involved in the production of L-Arg-induced antinociception but rather promotes pain transmission at spinal and supraspinal levels.

The peripheral role of the L-Arg-NO pathway in nocicep-

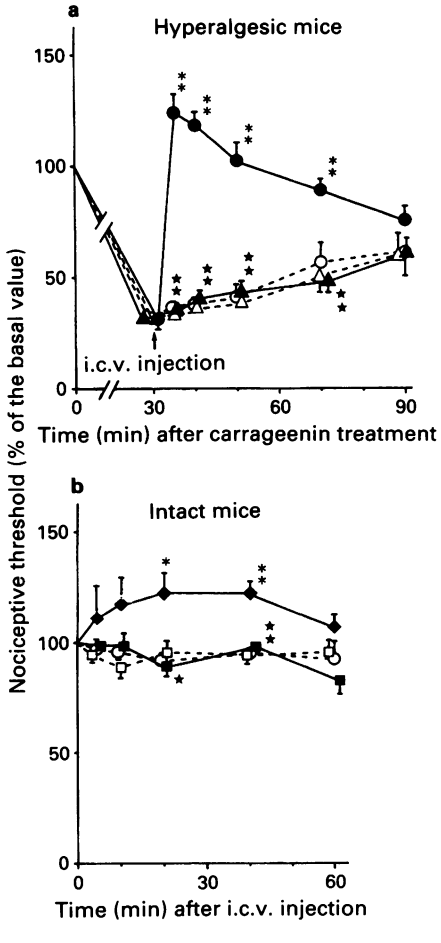

Figure 5 Antagonism of L-arginine (L-Arg, i.c.v.)-induced antinociception in mice with and without carrageenin-induced hyperalgesia by co-administered D-arginine (D-Arg). L-Arg and D-Arg were co-administered i.c.v. to hyperalgesic mice (30 min after carrageenin) or to intact mice. (○), Vehicle; (△), D-Arg 3 μg per mouse; (□), D-Arg 30 μg per mouse; (●), L-Arg 3 μg per mouse; (◆), L-Arg 30 μg per mouse; (▲), L-Arg 3 μg per mouse + D-Arg 3 μg per mouse; (■), L-Arg 30 μg per mouse + D-Arg 30 μg per mouse. $*P<0.05$, $**P<0.01$ vs. vehicle; $\bigstar P<0.05$, $\bigstar\bigstar P<0.01$ vs. L-Arg alone at 3 or 30 μg per mouse. $n=4$.

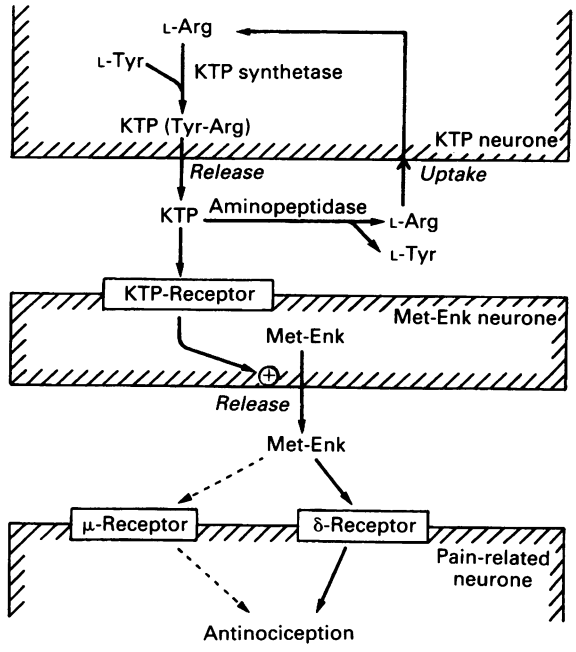

Figure 6 Proposed mechanism of L-arginine-induced antinociception in the central nervous system. Met-Enk, methionine-enkephalin; L-Tyr, L-tyrosine.

tive events is more complex. L-Arg, when injected directly into the carrageenin-treated paw of rats, produces antinociception via the peripheral NO-guanosine 3':5'-cyclic monophosphate (cyclic GMP) pathway (Duarte *et al.*, 1990). However, L-Arg, when injected systemically into rats with a carrageenin-treated hindpaw, produces antinociception which is not mediated by the peripheral NO-cyclic GMP pathway, considering that s.c. L-Arg-induced antinociception was not inhibited by i.pl. methylene blue, a guanylate cyclase inhibitor, and that antinociception elicited by i.pl. L-Arg was reversed by i.pl. methylene blue but was resistant to s.c. Nlx (Kawabata *et al.*, 1992). In contrast, Haley *et al.* (1992) have reported that the number of action potentials of a single dorsal horn neurone, in response to formalin injected into the peripheral receptive field, is reduced by preadministration of L-N^G-nitroarginine into the same site. This suggests that the peripheral L-Arg-NO pathway promotes nociception. Therefore, the peripheral L-Arg-NO system does not appear to be directly involved in the antinociceptive effect of systemic L-Arg in the present study.

A vasodilator effect of the L-Arg-NO system in peripheral blood vessels has been established (Gardiner *et al.*, 1990), which may indirectly contribute to L-Arg (s.c.)-induced antinociception because hypotension in the cat inhibits neural activity in the dorsal horn of the spinal cord (Kitahata, 1975). However, our previous results (Kawabata *et al.*, 1992) do not support this possibility, since L-Arg at 1,000 mg kg^{-1} (s.c.) elicited only a slight and transient hypotension in anaesthetized rats, in contrast to the marked and persistent increase in nociceptive threshold seen in rats with hyperalgesia. With regard to the role of the brain L-Arg-NO system in regulating blood pressure, Mollace *et al.* (1992) have demonstrated that L-Arg at 300 μg per rat (i.c.v.) does not modify blood pressure, and L-N^G-nitroarginine (i.c.v.) causes hypotension in endotoxin-treated but not in control rats. Similarly, Moore *et al.* (1991) have reported that L-N^G-nitroarginine given i.c.v. even at 100 μg per mouse does not significantly alter blood pressure in anaesthetized mice. Therefore, it seems unlikely that centrally mediated circulatory changes exert a role in L-Arg-induced antinociception in mice, although the present study did not actually elucidate whether i.c.v. L-Arg modifies blood pressure or not.

Furthermore, the L-Arg-NO system may also have a role in the modulation of oedema formation by regulating microvascular permeability (Hughes *et al.*, 1990; Ialenti *et al.*, 1992). L-Arg, when injected into the rat paw in combination with carrageenin, enhances increased vascular permeability and oedema volume (Ialenti *et al.*, 1992). However, we have demonstrated that L-Arg administered s.c. 2 h after carrageenin failed to affect the degree of oedema formation but induced definite antinociception (Kawabata *et al.*, 1992). Therefore, systemic L-Arg, after oedema is formed, may be incapable of modifying the inflammation. Also, increases by L-Arg in vascular permeability and oedema volume, if any, appear to promote nociception. In addition, it should be emphasized that the antinociceptive effect of systemic L-Arg is resistant to i.pl. methylene blue as mentioned above (Kawabata *et al.*, 1992). Thus, the L-Arg-NO system in the CNS and peripheral organs appears to play only a minor role, if any, in antinociception induced by systemic and i.c.v. L-Arg.

The mechanism by which D-Arg antagonizes L-Arg-induced antinociception is distinct from those of Leu-Arg and NTI, because D-Arg failed to antagonize KTP-induced antinociception in intact mice. The fact that D-Arg, when

administered i.c.v. as well as s.c. in combination with L-Arg, completely blocked the antinociceptive effect of L-Arg, suggests the existence of an antagonistic site within the CNS, in addition to competitive inhibition by D-Arg of carrier-mediated blood-brain barrier transport of L-Arg. Concerning the mechanism of action of D-Arg in the brain, at least, three mechanisms should be considered: (1) block of L-Arg uptake into central neurones, (2) inhibition of KTP synthetase, and (3) suppression of KTP release from KTP-containing neurones. The first mechanism is probable, since our preliminary experiments do not support the second and third. The first mechanism is being examined in our laboratory.

We thank Morishita Seiyaku Co. Ltd. for financial support. This paper is the second report on 'Pain modulation by neuroactive amino acids'.

References

BREDT, D.S. & SNYDER, S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 682–685.

CODERRE, T.J. & MELZACK, R. (1985). Increased pain sensitivity following heat injury involves a central mechanism. *Behav. Brain Res.*, **15**, 259–262.

CODERRE, T.J. & MELZACK, R. (1987). Cutaneous hyperalgesia: Contribution of the peripheral and central nervous systems to the increase in pain sensitivity after injury. *Brain. Res.*, **404**, 95–106.

CODERRE, T.J. & MELZACK, R. (1991). Central neural mediators of secondary hyperalgesia following heat injury in rats: neuropeptides and excitatory amino acids. *Neurosci. Lett.*, **131**, 71–74.

DUARTE, I.D.G., LORENZETTI, B.B. & FERREIRA, S.H. (1990). Peripheral analgesia and activation of the nitric oxide-cyclic GMP pathway. *Eur. J. Pharmacol.*, **186**, 289–293.

GARDINER, M.S., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1990). Regional haemodynamic changes during oral ingestion of N^G-monomethyl-L-arginine or N^G-nitro-L-arginine methyl ester in conscious Brattleboro rats. *Br. J. Pharmacol.*, **101**, 10–12.

HALEY, J.E., DICKENSON, A.H. & SCHACHTER, M. (1992). Electrophysiological evidence for a role in nitric oxide in prolonged chemical nociception in the rat. *Neuropharmacol.*, **31**, 251–258.

HARGREAVES, K.M., TROULLOS, E.S., DIONNE, R.A., SCHMIDT, E.A., SCHAFER, S.C. & JORIS, J.L. (1988). Bradykinin is increased during acute and chronic inflammation: therapeutic implications. *Clin. Pharmacol. Ther.*, **44**, 613–621.

HARIMA, A., SHIMIZU, H. & TAKAGI, H. (1991). Analgesic effect of L-arginine in patients with persistent pain. *Eur. Neuropsychopharmacol.*, **1**, 529–533.

HUGHES, S.R., WILLIAMS, T.J. & BRAIN, S.D. (1990). Evidence that endogenous nitric oxide modulates oedema formation induced by substance P. *Eur. J. Pharmacol.*, **191**, 481–484.

IALENTI, A., IANARO, A., MONCADA, S. & ROSA, M.D. (1992). Modulation of acute inflammation by endogenous nitric oxide. *Eur. J. Pharmacol.*, **211**, 177–182.

KAWABATA, A., FUKUZUMI, Y., FUKUSHIMA, Y. & TAKAGI, H. (1992). Antinociceptive effect of L-arginine on carrageenin-induced hyperalgesia in rats: possible involvement of central opioidergic systems. *Eur. J. Pharmacol.*, **218**, 153–158.

KAYER, V. & GUILBAUD, G. (1987). Local and remote modification of nociceptive sensitivity during carrageenin-induced inflammation in the rat. *Pain*, **23**, 99–107.

KITAHATA, L.M. (1975). Modes and sites of 'analgesic' action of anesthetics on the spinal cord. *Proc. Int. Un. Physiol.*, **13**, 149–170.

LEVY, L. (1969). Carrageenan paw edema in the mouse. *Life Sci.*, **8**, 601–606.

LEVY, G., KANDERA, J. & LAJITHA, A. (1967). Control of cerebral metabolite levels. I. Amino acid uptake and levels in various species. *Arch. Biochem. Biophys.*, **119**, 303–311.

MOLLACE, V., DE FRANCESCO, E.A. & NISTICO, G. (1992). Evidence that pharmacological manipulation of central L-arginine-NO pathway influences blood pressure and heart rate in rats. *Neurosci. Lett.*, **137**, 87–90.

MOORE, P.K., OLUYOMI, A.O., BABBEDGE, R.C., WALLACE, P. & HART, S.L. (1991). L-N^G-nitroarginine methyl ester exhibits antinociceptive activity in the mouse. *Br. J. Pharmacol.*, **102**, 198–202.

NORBERG, K. & SIESJO, B.K. (1975). Cerebral metabolism in hypoxic hypoxia, II. Citric acid cycle intermediates and associated amino acids. *Brain Res.*, **86**, 45–54.

PORTOGHESE, P.S., SULTANA, M. & TAKEMORI, A.E. (1988). Naltrindole, a highly selective and potent non-peptide δ opioid receptor antagonist. *Eur. J. Pharmacol.*, **146**, 185–186.

RANDALL, L.O. & SELITTO, J.J. (1957). A method for measurement of analgesic activity on inflammed tissue. *Arch. Int. Pharmacodyn.*, **111**, 409–419.

TAKAGI, H., HARIMA, A. & SHIMIZU, H. (1990). A novel clinical treatment of persistent pain with L-arginine. *Eur. J. Pharmacol.*, **183**, 1443.

TAKAGI, H., SHIOMI, H., UEDA, H. & AMANO, H. (1979a). Morphine-like analgesia by a new dipeptide, L-tyrosyl-L-arginine (kyotorphin) and its analogue. *Eur. J. Pharmacol.*, **55**, 109–111.

TAKAGI, H., SHIOMI, H., UEDA, H. & AMANO, H. (1979b). A novel analgesic dipeptide from bovine brain is a possible Met-enkephalin releaser. *Nature*, **282**, 410–412.

TAKAGI, H. & UEDA, H. (1988). Kyotorphin as an endogenous pain modulator. In *The Biowarning System in the Brain*. ed. Takagi, H., Oomura, Y., Ito, M. & Otsuka, M. pp. 139–153, Tokyo: University of Tokyo Press.

TREEDE, R.-D., MEYER, R.A., RAJA, S. & CAMBELL, J.N. (1992). Peripheral and central mechanisms of cutaneous hyperalgesia. *Progr. Neurobiol.*, **38**, 397–421.

UEDA, H., GE MING, HAZATO, T., KATAYAMA, T. & TAKAGI, H. (1985). Degradation of kyotorphin by a purified membrane-bound aminopeptidease from monkey brain: potentiation of kyotorphin-induced analgesia by a highly effective inhibitor bestatin. *Life Sci.*, **31**, 1865–1871.

UEDA, H., MATSUMOTO, S., YOSHIHARA, Y., FUKUSHIMA, N. & TAKAGI, H. (1986). Uptake and release of kyotorphin in rat brain synaptosomes. *Life Sci.*, **38**, 2405–2411.

UEDA, H., TATSUMI, K., SHIOMI, H. & TAKAGI, H. (1982). Analgesic dipeptide, kyotorphin (Tyr-Arg) is highly concentrated in the synaptosomal fraction of the rat brain. *Brain Res.*, **231**, 222–224.

UEDA, H., YOSHIHARA, Y., FUKUSHIMA, N., SHIOMI, H., NAKAMURA, A. & TAKAGI, H. (1987). Kyotorphin (tyrosyl-arginine) synthetase in rat brain synaptosomes. *J. Biol. Chem.*, **262**, 8165–8173.

UEDA, H., YOSHIHARA, Y., MISAWA, H., FUKUSHIMA, N., KATADA, T., UI, M., TAKAGI, H. & SATOH, H. (1989). The kyotorphin (tyrosyl-arginine) receptor and a selective reconstitution with purified Gi, measured with GTPase and phospholipase C assays. *J. Biol. Chem.*, **264**, 3732–3741.

YOSHIHARA, Y., UEDA, H., IMAJOH, S., TAKAGI, H. & SATOH, M. (1988). Calcium-activated neural proteases (CANP), a putative processing enzyme of the neuropeptide, kyotorphin, in the brain. *Biochem. Biophys. Res. Commun.*, **155**, 546–553.

(Received June 26, 1992
Revised August 5, 1992
Accepted August 10, 1992)

Br. J. Pharmacol. (1992), **107**, 1102–1107

Characterization of endothelium-dependent relaxations resistant to nitro-L-arginine in the porcine coronary artery

Tetsuhiko Nagao & [1]Paul M. Vanhoutte

Center for Experimental Therapeutics, Baylor College of Medicine, One-Baylor Plaza, Houston, Texas 77030, U.S.A.

1 Previous studies, demonstrated that endothelium-dependent relaxations which are resistant to nitro-L-arginine (an inhibitor of nitric oxide synthase) are accompanied by membrane hyperpolarization in the porcine coronary artery. The present experiments were designed to characterize further this type of endothelium-dependent relaxation in response to bradykinin by measuring isometric force in isolated rings of that artery. The experiments were performed in the presence of indomethacin to rule out vasoactive prostanoids.

2 Bradykinin induced comparable endothelium-dependent relaxations of proximal and distal rings of porcine coronary arteries contracted with prostaglandin $F_{2\alpha}$ in the presence of nitro-L-arginine.

3 Bradykinin and SIN 1 (a donor of nitric oxide) reduced contractions induced by prostaglandin $F_{2\alpha}$ in an additive fashion in the presence of nitro-L-arginine.

4 Bradykinin (in the presence of nitro-L-arginine) relaxed the tissues contracted with tetraethylammonium, prostaglandin $F_{2\alpha}$, phorbol 12, 13-diacetate or endothelin, with similar pD_2 values.

5 The time course of the relaxations induced by bradykinin (in the presence of nitro-L-arginine) and UK14304 (an α_2-adrenoceptor agonist, in the absence of the inhibitor of nitric oxide synthase) were comparable.

6 These results suggest that, in the porcine coronary artery, nitro-L-arginine-resistant relaxations (a) are distributed similarly in the proximal and distal parts of the artery, (b) contribute to inhibition of vascular smooth muscle with nitric oxide in an additive fashion, (c) occur during contractions induced by various contractile agents and (d) do not precede those mediated by nitric oxide.

Keywords: Endothelium-dependent hyperpolarization factor (EDHF); EDRF; hyperpolarization; K^+-channels; nitric oxide

Introduction

Endothelium-dependent relaxations of blood vessels by non-prostanoids can be explained by two major mechanisms; production of nitric oxide (or a related substance) by the endothelial cells and subsequent activation of soluble guanylate cyclase (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Furchgott, 1988; Myers *et al.*, 1989); and (b) endothelium-dependent hyperpolarization (Bolton *et al.*, 1984; Komori & Suzuki, 1987; Feletou & Vanhoutte, 1988; Komori *et al.*, 1988; Chen & Suzuki, 1989; Nagao & Vanhoutte, 1991; 1992). The latter appears to be caused by an unknown diffusible factor named endothelium-derived hyperpolarizing factor (EDHF; Bény & Brunet, 1988; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; Taylor & Weston, 1988) since the electrical event can be transferred from tissues with endothelium to those without endothelium (Feletou & Vanhoutte, 1988; Kauser *et al.*, 1989; Chen *et al.*, 1991).

In the porcine coronary artery, endothelium-dependent relaxations have two components which can be identified by their sensitivity to nitro-L-arginine, an inhibitor of nitric oxide synthase. For example, UK 14304 (an α_2-adrenoceptor agonist) induces endothelium-dependent relaxations which are nearly abolished by nitro-L-arginine. Therefore, nitric oxide (NO) appears to play a major role as a mediator in these relaxations. By contrast, a considerable part of the endothelium-dependent relaxation to bradykinin is resistant to nitro-L-arginine, oxyhaemoglobin (a scavenger of nitric oxide) or methylene blue (an inhibitor of soluble guanylate cyclase) and is most likely mediated by endothelium-dependent hyperpolarization (Flavahan *et al.*, 1989; Nishiye *et al.*, 1989; Nagao & Vanhoutte, 1992). In the porcine coronary artery, the contribution of nitric oxide and EDHF to the endothelium-dependent relaxations evoked by bradykinin varies with the concentration of the kinin tested. Thus, at 10^{-9} M, bradykinin, nitro-L-arginine inhibits approximately 50% of the response, while for concentrations higher than 10^{-8} M, the nitro-L-arginine part of the relaxation amounts to nearly 90% (Nagao & Vanhoutte, 1992). The present experiments were designed to characterize further the contribution of the nitro-L-arginine resistant component in endothelium-dependent relaxations of the porcine coronary artery. In particular, we addressed the following questions: (a) Do nitro-L-arginine-resistant relaxations to bradykinin differ in arteries of different diameter?; (b) do nitric oxide and EDHF act synergistically or in an additive fashion?; (c) do nitro-L-arginine resistant relaxations to bradykinin differ during contractions evoked by different vasoconstrictor substances?; and (d) does the time course of nitro-L-arginine-sensitive and -resistant relaxations differ?

Methods

Hearts of pigs were collected at a nearby slaughter house and were immersed immediately in ice-cold modified Krebs-Ringer bicarbonate solution (composition mM: NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, disodium calcium edetate (CaEDTA) 0.026, and glucose 11.1, aerated with 95% O_2 and 5% CO_2; control solution). Approximately 45 min later, the left anterior descending coronary arteries or their branches were excised. Arteries harvested from the area not more than 5 cm away from the coronary orifice of the aorta were termed 'proximal' (diameter 2.0 to 3.0 mm). 'Distal' arteries were collected from the apical area or from the diagonal branch (diameter 1.0 to 1.5 mm). The arteries were cut into rings (approximately 4 mm long) and

[1] Author for correspondence.

were suspended in organ chambers (25 ml) filled with control solution (37°C, pH 7.4) by means of two stainless steel stirrups. One of the stirrups was anchored to the bottom of the organ chamber and the other was connected to an isometric force transducer (Statham Universal UTC2, Statham Medical Instruments, Los Angeles, CA, U.S.A.). After 60 min of incubation, the arteries were stretched in a stepwise manner to the optimal point of their length-tension relationship (approximately 5 g for proximal and 1.5 g for distal arteries; determined in preliminary experiments with 30 mM KCl). Another incubation period of 60 min was allowed before the experimental procedure was continued. All the experiments were performed in the presence of indomethacin (10^{-5} M) to prevent the formation of endogenous prostanoids. Treatment with nitro-L-arginine was started 30 min before the measurement of tension. In some experiments, endothelial cells were removed by rolling the rings, through which a stainless steel wire or a tip of a watchmaker's forceps had been inserted, gently back and forth on a filter paper moistened with control solution. As a rule, the experiments were performed with proximal arteries, except when a comparison was made between proximal and distal arteries.

Drugs

The drugs used in the experiments were: bradykinin (Sigma, St Louis, MO, U.S.A.), human endothelin (endothelin-1, Peptide Institute INC. Osaka, Japan), indomethacin (Sigma), lemakalim (Beecham Pharmaceuticsl, U.K.), nitro-L-arginine (Aldrich, Milwaukee, WIS, U.S.A.), SIN 1 (3-morpholinosydnonimine, Cassella-Riedel, Frankfurt, Germany), phorbol 12, 13-diacetate (PDA), prostaglandin $F_{2\alpha}$, tetraethylammonium chloride (TEA; Sigma) and UK 14304 (5-bromo-N-(4,5 dihydro-1H-imidazol-2-yl)-6 quinoxalinamine; Pfizer, Central Research, Sandwich, Kent). Lemakalim and PDA were dissolved in ethanol and dimethylsulphoxide (DMSO), respectively. The final bath concentrations of the vehicles were less than 0.02 volume percent for the alcohol and 0.001 volume percent for DMSO. Stock solutions of indomethacin were prepared in an equal molar concentration of Na_2CO_3. All the other drugs were dissolved in distilled water.

Statistical analysis

The results are expressed as means ± s.e.mean. n represents the number of animals studied. Significance was tested with Student's t test (paired or unpaired, two-tailed), or with analysis of variance followed by Scheffe's F test. P values less than 0.05 were considered to be statistically significant.

Results

Diameter

Nitro-L-arginine (3×10^{-5} M) induced a slowly developing increase in tension, reaching less than 20% of the contraction induced by prostaglandin $F_{2\alpha}$ (5×10^{-6} M), in both proximal and distal rings. Bradykinin, applied cumulatively in the continuous presence of nitro-L-arginine, induced concentration-dependent relaxations, which were not significantly different in proximal and distal rings (Figure 1). Bradykinin did not induce relaxations either in proximal or distal rings without endothelium ($n = 6$, data not shown).

Interaction between NO and EDHF

The effect of bradykinin was compared in paired rings of the same arteries, in the absence and presence of SIN 1. Prostaglandin $F_{2\alpha}$ (5×10^{-6} M) contracted the rings of the control and the SIN 1 group to a comparable degree in the presence of nitro-L-arginine (3×10^{-5} M). SIN 1 (2 to 3×10^{-8} M), applied after the contractions induced by prostaglandin $F_{2\alpha}$ became stable, relaxed the tissues by 35 ± 7%. Bradykinin induced concentration-dependent relaxations in both groups (Figure 2a). The concentration-relaxation curves to bradykinin from the two groups were superimposable when the contractile level prior to the application of bradykinin was taken as 100% (Figure 2b).

The effect of UK14304 was compared in paired rings of

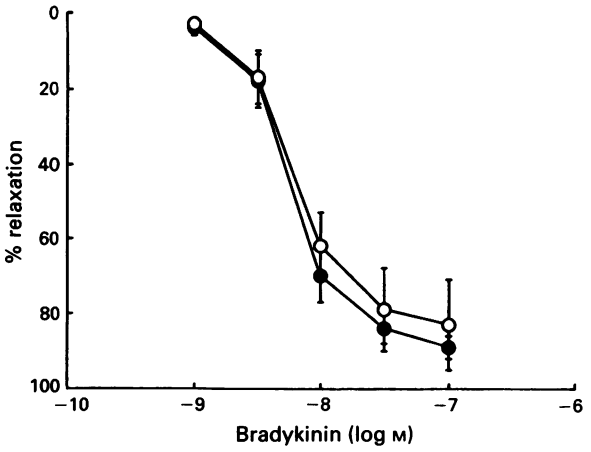

Figure 1 Relaxations induced by bradykinin in the presence of nitro-L-arginine in proximal (●) and distal (○) porcine coronary arteries. The tissues were incubated with nitro-L-arginine (3×10^{-5} M) for 30 min and then contracted with prostaglandin $F_{2\alpha}$ (5×10^{-6} M: proximal 7.7 ± 0.8 g, distal 1.9 ± 0.6 g). The tension developed to prostaglandin $F_{2\alpha}$ was taken as 100%. Bradykinin was applied in a cumulative fashion. Data are expressed as means ± s.e.mean, vertical bars ($n = 10$ for proximal and 8 for distal arteries, respectively).

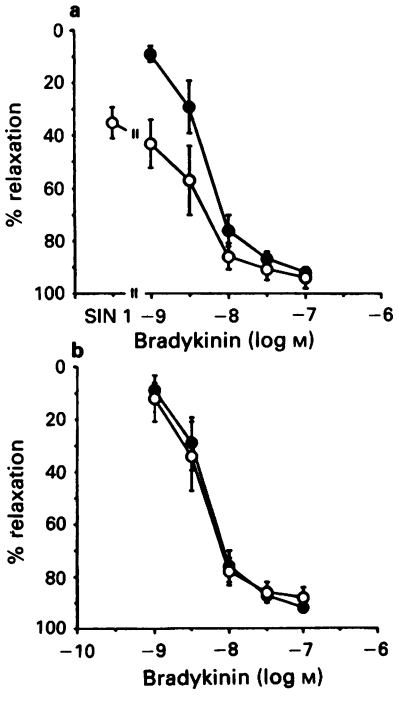

Figure 2 Interaction between 3-morpholinosydnonimine (SIN 1) and bradykinin in the presence of nitro-L-arginine (3×10^{-5} M) in porcine coronary arteries. The tissues were contracted with prostaglandin $F_{2\alpha}$ (5×10^{-6} M: control group 8.1 ± 0.1 g, SIN 1 group 8.5 ± 0.9 g). SIN 1 (2 to 3×10^{-8} M) relaxed the tissues by 35%. Bradykinin was applied in a cumulative manner in the absence (●) or presence (○) of SIN 1. The contractions induced by prostaglandin $F_{2\alpha}$ were taken as 100% in the control group. In the SIN 1 group, the contractions before (a) and after (b) application of SIN 1 were taken as 100%. Data are expressed as means ± s.e.mean (vertical bars) ($n = 5$).

the same arteries, in the absence and presence of lemakalim. Prostaglandin $F_{2\alpha}$ (5×10^{-6} M) contracted the rings of the control and the lemakalim group to a comparable degree. Lemakalim (1.0 to 1.7×10^{-7} M), applied after the contractions induced by prostaglandin $F_{2\alpha}$ became stable, relaxed the tissues by 29 ± 3%. UK 14304 induced concentration-dependent relaxations in the two groups (Figure 3a). The concentration-relaxation curves to UK 14304 from the two groups were superimposable when the contractile level prior to application of UK 14304 was taken as 100% (Figure 3b).

Nitro-L-arginine-resistant relaxations during contractions to various agonists

Porcine coronary arteries contracted in response to prostaglandin $F_{2\alpha}$ (5×10^{-6} M), TEA (10 mM), PDA (10^{-7} M) and endothelin (3×10^{-9} M) in the presence of nitro-L-arginine (3×10^{-5} M). The contractions induced by the concentrations selected for the various agents did not differ statistically in amplitude. Bradykinin induced concentration-dependent relaxations in all cases (Figure 4). The pD_2 values for the kinin did not differ significantly in the presence of the four contractile agents (Table 1). SIN 1, in the presence of nitro-L-arginine, relaxed the porcine coronary arteries contracted with the four agonists in a concentration-dependent manner (Figure 5). The pD_2 value for SIN 1 was not different among the tissues contracted with prostaglandin $F_{2\alpha}$, TEA, PDA and endothelin (Table 1). The ratios of pD_2 values between bradykinin and SIN 1 (pD_2[bradykinin]/pD_2[SIN 1), calculated for the individual tissues, were similar during contractions with the four agonists (Figure 6).

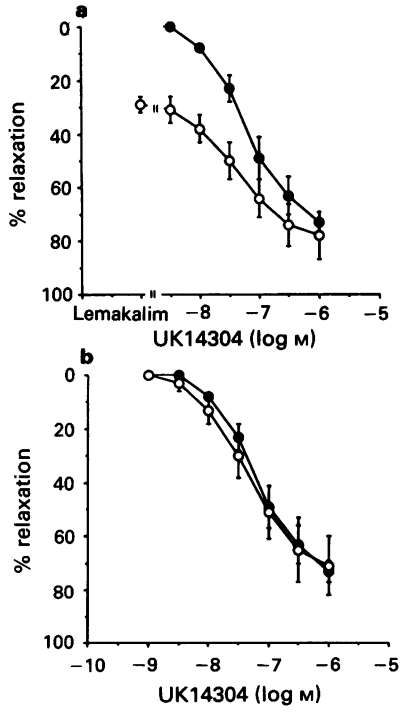

Figure 3 Interaction between lemakalim and UK14304 in porcine coronary arteries. The tissues were contracted with prostaglandin $F_{2\alpha}$ (5×10^{-6} M: control group 7.4 ± 1.3 g, lemakalim group 7.6 ± 2.0 g). Lemakalim (1.0 to 1.7×10^{-7} M) relaxed the tissues by 29%. UK 14304 was applied in a cumulative manner in the absence (●) or presence (○) of lemakalim. The contractions induced by prostaglandin $F_{2\alpha}$ were taken as 100% in the control group. In the lemakalim group, the contractions before (a) and after (b) application of lemakalim were taken as 100%. Data are expressed as means ± s.e.mean (vertical bars) ($n = 4$).

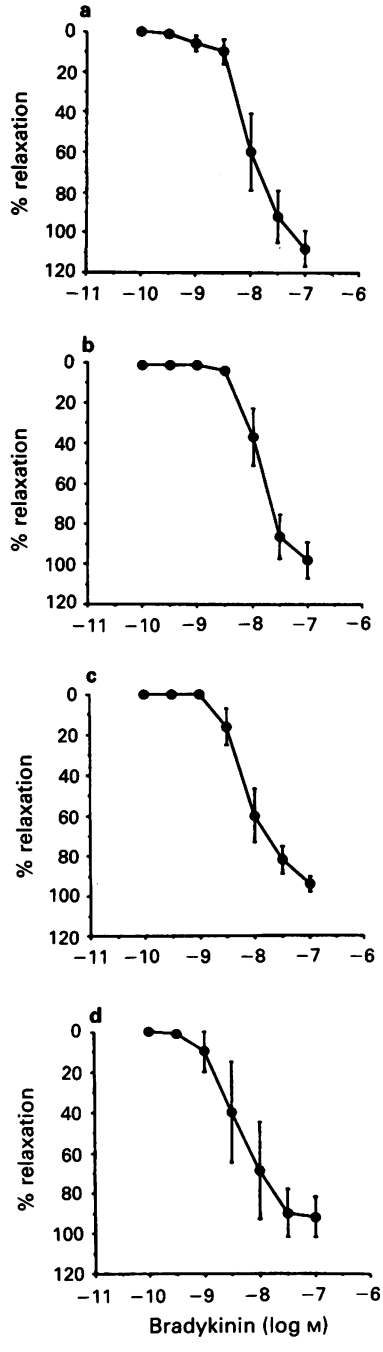

Figure 4 Effects of bradykinin on porcine coronary arteries contracted with (a) tetraethylammonium (10 mM, 3.5 ± 0.3 g), (b) prostaglandin $F_{2\alpha}$ (3×10^{-6} M, 6.9 ± 1.6 g), (c) phorbol 12, 13-diacetate (10^{-7} M, 10.0 ± 3.8 g) or (d) endothelin (3×10^{-9} M, 8.0 ± 1.4 g), in the presence of nitro-L-arginine (3×10^{-5} M). The contractions induced by each agent were taken as 100%. Bradykinin was applied in a cumulative manner. Data are expressed as means ± s.e.mean (vertical bars) ($n = 4$).

Time course

Paired rings of the same arteries were contracted with prostaglandin $F_{2\alpha}$. Prostaglandin $F_{2\alpha}$ (5×10^{-6} M) contracted the rings of the bradykinin group (in the presence of nitro-L-arginine, 3×10^{-5} M) and the UK 14304 group to a comparable level. UK 14304 (10^{-6} M) and bradykinin (10^{-8} M) induced 93 ± 4% and 99 ± 6% relaxations, respectively ($P > 0.05$). The time course of the relaxations induced by the two agents was comparable (Figure 7).

Table 1 pD_2 values for bradykinin and 3-morpholinosydnonimine (SIN 1) in tissues contracted with various agonists, in the presence of nitro-L-arginine (3×10^{-5} M)

	TEA	*$PGF_{2\alpha}$*	*PDA*	*Endothelin*
Bradykinin	8.02 ± 0.16	7.89 ± 0.13	8.09 ± 0.14	8.26 ± 0.26
SIN 1	7.33 ± 0.11	7.31 ± 0.03	7.09 ± 0.24	6.98 ± 0.22

TEA: tetraethylammonium, $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$, PDA: phorbol 12, 13-diacetate. Data shown as mean ± s.e.mean ($n = 4$).

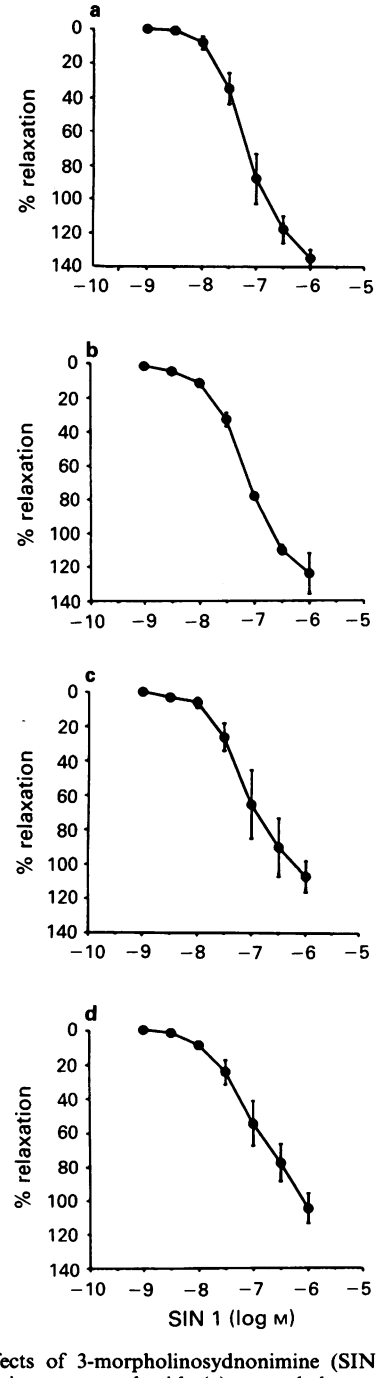

Figure 5 Effects of 3-morpholinosydnonimine (SIN 1) on porcine coronary arteries contracted with (a) tetraethylammonium (10 mM, 3.5 ± 0.3 g), (b) prostaglandin $F_{2\alpha}$ (3×10^{-6} M, 6.9 ± 1.6 g), (c) phorbol 12, 13-diacetate (10^{-7} M, 10.0 ± 3.8 g) or (d) endothelin (3×10^{-9} M, 8.0 ± 1.4 g), in the presence of nitro-L-arginine (3×10^{-5} M). The contractions induced by each agent were taken as 100%. SIN 1 was applied in a cumulative manner. Data are expressed as mean ± s.e.mean (vertical bars) ($n = 4$).

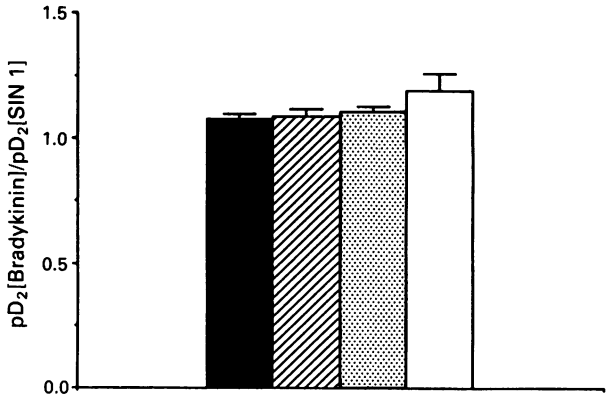

Figure 6 Ratios of the pD_2 for bradykinin and 3-morpholinosydnonimine (SIN 1) (pD_2[bradykinin]/pD_2[SIN 1]) in porcine coronary arteries contracted with tetraethylammonium (10 mM, solid column), prostaglandin $F_{2\alpha}$ (3×10^{-6} M, hatched column), phorbol 12, 13-diacetate (10^{-7} M, stippled column) or endothelin (3×10^{-9} M, open column), in the presence of nitro-L-arginine (3×10^{-5} M). The ratios were calculated in each tissue and averaged. Data are expressed as means ± s.e.mean (vertical bars) ($n = 4$).

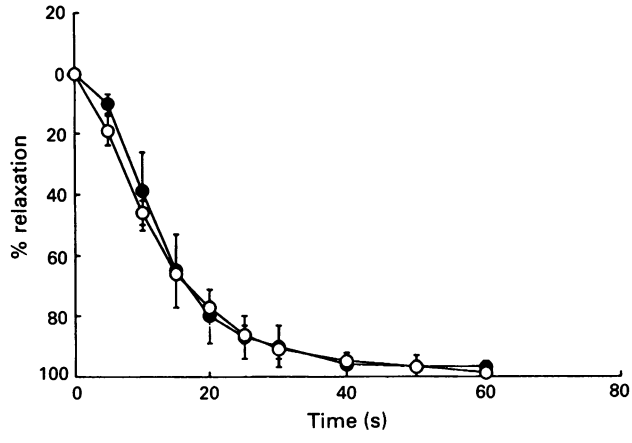

Figure 7 Time course of the relaxations induced by bradykinin [10^{-8} M, in the presence of nitro-L-arginine (3×10^{-5} M), O] and UK 14304 (10^{-6} M, ●) in porcine coronary arteries contracted with prostaglandin $F_{2\alpha}$ (3×10^{-6} M: UK 14304 group 3.5 ± 0.9 g, bradykinin group 3.4 ± 0.7 g). Data are expressed as mean ± s.e.mean (vertical bars) ($n = 4$).

Discussion

In the porcine coronary artery, UK 14304 and bradykinin cause endothelium-dependent relaxations (Flavahan *et al.*, 1989). Previous studies have shown that the relaxations induced by UK 14304 are due mainly to the release of nitric oxide, while those induced by bradykinin in the presence of

nitro-L-arginine are mediated in part by endothelium-dependent hyperpolarization (Flavahan *et al.*, 1989; Nagao & Vanhoutte, 1992). The present study also examined the effects of pharmacological agents that mimic the effects of nitric oxide or EDHF. SIN 1 is a donor of nitric oxice (Schrör *et al.*, 1989) and lemakalim ((−)-cromakalim, an opener of ATP-sensitive K^+-channels: Weir & Weston, 1986; Quast, 1988) hyperpolarizes vascular smooth muscle cells. Although it is still controversial whether or not endothelium-dependent hyperpolarization is generated by opening this type of K^+-channel (Bray & Quast, 1991; Chen *et al.*, 1991; Van de Voorde *et al.*, 1992), glibenclamide, a blocker of ATP-sensitive K^+-channels (Eltz, 1989), inhibits endothelium-dependent hyperpolarizations induced by acetylcholine in the middle cerebral artery of the rabbit (Standen *et al.*, 1989; Brayden, 1990). Whatever the subtype of K^+-channels involved in endothelium-dependent hyperpolarization of the porcine coronary artery may be, the mechanism of action underlying the response to EDHF and lemakalim is the same in that both hyperpolarize the coronary smooth muscle.

The distribution of nitro-L-arginine-resistant relaxations might vary according to the size of vessels. In fact, in the rat, the smaller the artery, the greater tendency for endothelium-dependent relaxations which are resistant to nitro-L-arginine to occur (Nagao *et al.*, 1992). However, the present experiments revealed no difference in nitro-L-arginine-resistant relaxations between proximal and distal coronary arteries of the pig. These observations suggest that, in this species, EDHF could play a role regardless of vascular diameter in the coronary vascular bed and, thus, that the size of the blood vessels is not the only determinant factor for the presence of nitro-L-arginine-resistant relaxations.

To judge from the interaction between SIN 1 and bradykinin (in the presence of nitro-L-arginine), or lemakalim and UK 14304, the two relaxing mechanisms (nitric oxide and EDHF) appear to operate in an additive fashion. However, the present observations do not completely rule out a synergism between the two mechanisms under more physiological conditions, when the arterial tone is determined by the balance of various endogenous constrictors, by myogenic mechanisms, and by dilator stimuli, such as the local blood flow or production of prostanoids.

Membrane hyperpolarization closes voltage-dependent Ca^{2+}-channels and reduced Ca^{2+}-influx through that class of channels. Accordingly, the contractions induced by Ca^{2+}-influx through voltage-dependent Ca^{2+}-channels should be more sensitive to EDHF (Taylor & Weston 1988). By contrast, if a contraction is induced mainly by receptor-operated mechanisms such as Ca^{2+}-release from intracellular store sites or receptor-mediated Ca^{2+}-influx, EDHF may be less effective. Thus, the effect of bradykinin (in the presence of nitro-L-arginine) may be variable during contractions induced by different agonists. The agonists used in the present experiments were TEA (voltage-dependent Ca^{2+}-influx through depolarization of vascular smooth muscle by blocking K^+-channels: Karashima & Kuriyama, 1982), endothelin (Ca^{2+}-release from cellular stores: Wagner-Mann & Sturek, 1991) and voltage-dependent Ca^{2+}-influx (Goto *et al.*, 1989), prostaglandin $F_{2\alpha}$, (receptor-operated mechanism without or with moderate depolarization: Feletou & Vanhoutte 1988; Komori *et al.*, 1988) and PDA (activation of protein kinase C: Castagna *et al.*, 1982). The present results showed that the pD_2 values for SIN 1 and bradykinin (in the presence of nitro-L-arginine) did not differ among the agonists tested. It seemed reasonable to compare the potency of bradykinin (in the presence of nitro-L-arginine) to that of SIN 1, by calculating the ratio of pD_2 values for the two relaxing agents in each tissue. Again, the contractions induced by TEA, prostaglandin $F_{2\alpha}$, endothelin and PDA were equally sensitive to bradykinin based on their sensitivity to SIN 1. The contractions induced by PDA were inhibited by bradykinin through the nitro-L-arginine resistant mechanism as effectively as those to the other agonists, despite the fact that a phorbol ester (phorbol 12, 13-dibutyrate) affects little the intracellular Ca^{2+} level in the porcine coronary artery (Mori *et al.*, 1990). Thus, the inhibitory effect of membrane hyperpolarization is not prominent during contractions induced by membrane depolarization, but can be observed during various types of contractions. In support of these observations, lemakalim reduces the breakdown of inositol triphosphate and inhibits noradrenaline-induced contractions in Ca^{2+}-free solution, possibly through a mechanism dependent on membrane hyperpolarization (Ito *et al.*, 1991).

It is unlikely that TEA affects the K^+-channels involved in the generation of endothelium-dependent hyperpolarization in the porcine coronary artery, since the threshold concentration of bradykinin to evoke hyperpolarization (Nagao & Vanhoutte, 1992) and nitro-L-arginine-resistant relaxations (present study) during contraction to that agent are similar. Although TEA inhibits endothelium-dependent hyperpolarizations to acetylcholine in certain tissues (Kauser *et al.*, 1989; Chen *et al.*, 1991), the inhibition may result from the antimuscarinic property of the K^+-channel blocker (Bolduni *et al.*, 1990).

Endothelium-dependent hyperpolarization has a transient nature in many instances (Komori & Suzuki, 1987; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988). A possible explanation for this characteristic of the hyperpolarization could be that the relaxations induced by hyperpolarization precede those induced by nitric oxide until the latter has reached its peak (Taylor & Weston, 1988). This appears to be unlikely, at least in the porcine coronary artery, because the endothelium-dependent relaxations induced by nitro-L-arginine-sensitive (nitric oxide) and insensitive (EDHF) mechanism had the same time course. In some tissues such as the aorta of the rat, acetylcholine induces more sustained hyperpolarization (over 10 min: Chen & Suzuki, 1989), suggesting that hyperpolarization contributes to endothelium-dependent relaxations more than by simply causing an early relaxation until the nitric oxide system reaches full activation.

In conclusion, endothelium-dependent hyperpolarization, when assessed by the action of nitro-L-arginine-resistant relaxations to bradykinin and lemakalim, is an effective mechanism to counteract in cooperation with nitric oxide not only the contractions induced by membrane depolarizing agents but also those induced by various other mechanisms.

The authors are grateful to Mr Barnabas Desta and Mr Gregory Green for their technical assistance. Lemakalim and SIN 1 were kindly provided by Beecham and Cassella-Riedel Pharmaceutical Company, respectively. Supported in part by NIH grant HL31183.

References

BÉNY, J.-L. & BRUNET, P.C. (1988). Neither nitric oxide nor nitroglycerin accounts for all the characteristics of endothelially mediated vasodilatation of pig coronary arteries. *Blood Vessels*, **25**, 308–311.

BOLDUNI, W., COSTA, L.G. & MURPHY, S.D. (1990). Potassium ions potentiate the muscarinic receptor-stimulated phosphoinositide metabolism in cerebral cortex slices: a comparison of neonatal and adult rat. *Neurochem. Res.*, **15**, 33–39.

BOLTON, T.B., LANG, R.J. & TAKEWAKI, T. (1984). Mechanism of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. *J. Physiol.*, **351**, 549–572.

BRAY, K. & QUAST, U. (1991). Differences in the K^+-channel opened by cromakalim, acetylcholine and substance P in rat aorta and porcine coronary artery. *Br. J. Pharmacol.*, **102**, 585–594.

BRAYDEN, J.E. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilatation. *Am. J. Physiol.*, **259**, H668–H673.

CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters. *J. Biol. Chem.*, **257**, 7847–7851.

CHEN, G. & SUZUKI, H. (1989). Some electrical responses of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. *J. Physiol.*, **410**, 91–106.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br. J. Pharmacol.*, **95**, 1165–1174.

CHEN, G., YAMAMOTO, Y., MIWA, K. & SUZUKI, H. (1991). Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. *Am. J. Physiol.*, **260**, H1888–H1892.

ELTZE, M. (1989). Glibenclamide is a competitive antagonist of cromakalim, pinacidil and RP 49356 in guinea-pig pulmonary artery. *Eur. J. Pharmacol.*, **165**, 231–239.

FELETOU, M. & VANHOUTTE, P.M. (1988) Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93**, 515–524.

FLAVAHAN, N.A., SHIMOKAWA, H. & VANHOUTTE, P.M. (1989). Pertussis toxin inhibits endothelium-dependent relaxations to certain agonists in porcine coronary arteries. *J. Physiol.*, **408**, 549–560.

FURCHGOTT, R.F. (1988). Studies on relaxation of rabbit aorta by sodium nitrate: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrate and the endothelium-derived relaxing factor is nitric oxide. In *Mechanism of Vasodilatation*, Vol. 4. ed. Vanhoutte, P.M., pp. 401–414. New York, U.S.A.: Raven.

GOTO, K., KASUYA, Y., MATSUKI, N., TAKUWA, Y., KURIHARA, H., ISHIKAWA, T., KIMURA, S., YANAGISAWA, M. & MASAKI, T. (1989). Endothelin activates the dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3915–3918.

IGNARRO, L.J., BYRNS, R.E., BUGA, G.M. & WOOD, K.S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ. Res.*, **61**, 866–879.

ITO, S., KAJIKURI, J., ITOH, T. & KURIYAMA, H. (1991). Effects of lemakalim on changes in Ca^{2+} concentration and mechanical activity induced by noradrenaline in the rabbit mesenteric artery. *Br. J. Pharmacol.*, **104**, 227–233.

KARASHIMA, T. & KURIYAMA, H. (1981). Electrical properties of smooth muscle cell membrane and neuromuscular transmission in the guinea-pig basilar artery. *Br. J. Pharmacol.*, **74**, 495–504.

KAUSER, K.W., STEKIEL, W.J., RUBANYI, G. & HARDER, D.R. (1989). Mechanism of action of EDRF on pressurized arteries: Effect on K^+ conductance. *Circ. Res.*, **65**, 199–204.

KOMORI, K., LORENZ, R.R. & VANHOUTTE, P.M. (1988). Nitric oxide, ACh, and electrical and mechanical properties of canine arterial smooth muscle. *Am. J. Physiol.*, **255**, H207–H212.

KOMORI, K. & SUZUKI, H. (1987). Electrical responses of smooth muscle cells during cholinergic vasodilatation in the rabbit saphenous artery. *Circ. Res.*, **61**, 586–593.

MORI, T., YANAGISAWA, T. & TAIRA, N. (1990). Phorbol 12, 13-dibutyrate increases vascular tone but has a dual action on intracellular calcium levels in porcine coronary arteries. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 251–255.

MYERS, P.R., MINOR, R.L.Jr., GUERRA, R.Jr., BATES, J.N. & HARRISON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocystein than nitric oxide. *Nature*, **345**, 161–163.

NAGAO, T., ILLIANO, S. & VANHOUTTE, P.M. (1992). Heterogenous distribution of endothelium-dependent relaxations resistant to nitro-L-arginine in the arterial tree of the rat. *Am. J. Physiol.*, (in press).

NAGAO, T. & VANHOUTTE, P.M. (1991). Membrane hyperpolarization contributes to endothelium-dependent relaxations induced by acetylcholine in the femoral vein of the rat. *Am. J. Physiol.*, **261**, H1034–H1038.

NAGAO, T. & VANHOUTTE, P.M. (1992). Hyperpolarization as a mechanism for endothelium-dependent relaxations in the porcine coronary artery. *J. Physiol.*, **445**, 355–367.

NISHIYE, E., NAKAO, K., ITOH, T. & KURIYAMA, H. (1989). Factors inducing endothelium-dependent relaxation in the guinea-pig basilar artery as estimated from the actions of haemoglobin. *Br. J. Pharmacol.*, **96**, 645–655.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

QUAST, U. (1988). Inhibition of the effects of K^+ channel stimulator cromakalim (BRL 34915) by glibenclamide and forskolin. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**(suppl.), R72.

SCHRÖR, K., FORSTER, S., WODITSCH, I. & SCHRÖDER, H. (1989). Generation of NO from molsidomine (SIN 1) in vitro and its relationship to changes in coronary vessel tone. *J. Cardiovasc. Pharmacol.*, **14**(suppl. 11), S29–S34.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends. Pharmacol. Sci.*, **9**, 72–74.

VAN DE VOORDE, J., VANHEEL, B. & LEUSEN, I. (1992). Endothelium-dependent relaxation and hyperpolarization in aorta from control and renal hypertensive rats. *Circ. Res.*, **70**, 1–8.

WAGNER-MANN, C. & STUREK, M. (1991). Endothelin mediates Ca influx and release in porcine coronary smooth muscle cells. *Am. J. Physiol.*, **260**, C771–C777.

WEIR, S. & WESTON, A.H. (1986). The effects of BRL 34915 and nicorandil on electrical and mechanical activity and on ^{86}Rb efflux in rat blood vessels. *Br. J. Pharmacol.*, **88**, 121–128.

(Received June 30, 1992
Revised August 3, 1992
Accepted August 11, 1992)

Br. J. Pharmacol. (1992), **107**, 1108–1115

Effect of a 5-lipoxygenase inhibitor and leukotriene antagonist (PF 5901) on PAF-induced airway responses in neonatally immunized rabbits

Caroline M. Herd, *Donna Donigi-Gale, *T. Scott Shoupe & [1]Clive P. Page

Department of Pharmacology, King's College London, Manresa Road, London SW3 6LX and *International Molecular Discovery, The Purdue Frederick Company, Norwalk, Connecticut, U.S.A.

1 Aerosol administration of platelet activating factor (PAF) (80 $\mu g\,ml^{-1}$ for 60 min) to neonatally immunized rabbits caused bronchoconstriction which was far in excess of that produced by a comparable aerosol of bovine serum albumin (BSA), the carrier molecule for PAF. Bronchoconstriction of a similar magnitude was elicited by PAF in immunized, sham-immunized and normal rabbits.

2 Aerosol administration of PAF to immunized rabbits induced enhanced airway responsiveness to inhaled histamine in all animals tested, 24 h and 72 h after exposure. In not all cases had airways responsiveness returned to basal levels at 1 week following PAF challenge. In contrast, following exposure of immunized rabbits to BSA, no significant changes in airway responsiveness to histamine were evident at any of the measured time points.

3 A significant increase in the total number of inflammatory cells recovered in bronchoalveolar lavage (BAL) fluid was determined 24 h and 72 h following PAF exposure in immunized rabbits. This was associated with a significant increase in the number of neutrophils and eosinophils. Similar changes were observed following exposure of PAF to normal and sham-immunized rabbits. No change in the total number of inflammatory cells was obtained in BAL after BSA challenge to immunized rabbits; however, neutrophil numbers were significantly increased.

4 PF 5901, a specific inhibitor of the 5-lipoxygenase pathway of arachidonic acid metabolism and a leukotriene D_4 antagonist, at a dose of 10 mg (direct intratracheal administration) significantly inhibited the airway resistance (R_L) component of the bronchoconstriction induced by PAF in neonatally-immunized rabbits. Doses of 10 mg, 3 mg and 1 mg PF 5901 (direct intratracheal administration) were sufficient to inhibit significantly the PAF-induced increase in airways responsiveness to inhaled histamine in immunized rabbits. PF 5901 however, failed to alter the pulmonary cell infiltration induced by PAF, as assessed by BAL.

5 We suggest from the results of the present study that PAF induces consistent and long-lasting increases in airways responsiveness to histamine in immunized rabbits, which is mediated, at least in part, by products of the 5-lipoxygenase metabolic pathway. Furthermore, the inability of PF5901 to inhibit the influx of inflammatory cells into the airway lumen following PAF challenge may suggest that bronchial hyperresponsiveness and cellular infiltration are not strictly associated events.

Keywords: PAF; leukotrienes; rabbit airways; inflammation; airway hyperresponsiveness

Introduction

Bronchial asthma is characterized by bronchoconstriction, mucosal oedema, mucus hypersecretion, bronchial hyperresponsiveness and a recruitment of leucocytes, in particular eosinophils, into the airways. It has been suggested that platelet activating factor (PAF) is a mediator of this disease as it can reproduce many of these features, both in experimental animals and in man (Page, 1988). Consequently, much research has been focussed on the elucidation of the precise role of this family of phospholipids in allergy and inflammation.

PAF has been shown to induce both acute bronchoconstriction and a longer-lasting bronchial hyperresponsiveness in a variety of laboratory animals (reviewed in Page, 1988). However, the response to PAF in man is variable (Cuss *et al.*, 1986; Rubin *et al.*, 1987; Chung & Barnes, 1989; Chung *et al.*, 1989; Kaye & Smith, 1990; Lai *et al.*, 1990; Spencer *et al.*, 1990b), the basis of which is not known. Similarly, the response of normal rabbits to PAF has also been shown to be variable, where bronchial hyperresponsiveness was induced in some, but not all normal animals studied (Coyle *et al.*, 1990).

The mechanisms by which PAF produces bronchoconstriction and airway hyperresponsiveness has yet to be determined. PAF has been shown to enhance the release of leukotrienes from isolated perfused lung of the rabbit (Voelkel *et al.*, 1982) and from chopped rat lung (Beaubien *et al.*, 1984). A role for the lipoxygenase products of arachidonic acid, particularly leukotrienes formed by the 5-lipoxygenase pathway, has been suggested to account for a number of PAF-induced effects including bronchoconstriction (Bonnet *et al.*, 1983), vascular permeability, and in the acute increase in airway responsiveness to histamine following PAF exposure (Anderson & Fennessy, 1988). Similar findings have been obtained *in vitro*, where hyperresponsiveness to PAF in lungs from actively sensitized guinea-pigs is partly related to the generation of lipoxygenase products (Pretolani *et al.*, 1989).

The breakdown of arachidonic acid leads to the formation of the sulphidopeptide leukotrienes C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4) which induce bronchoconstriction (Drazen *et al.*, 1980), airway hyperresponsiveness (O'Hickey *et al.*, 1991), mucus secretion (Marom *et al.*, 1982) and increased vascular permeability (Hua *et al.*, 1985), and leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE). LTB_4 and 5-HETE are potent stimulators of leukocyte functions, including the chemotaxis, chemokinesis and aggregation of

[1] Author for correspondence.

polymorphonuclear leukocytes (Ford-Hutchinson *et al.*, 1981; Ford-Hutchinson, 1991).

In the present study, the ability of a specific and selective leukotriene synthesis inhibitor and LTD_4 antagonist, PF 5901 (2-[3-(1-hydroxyhexyl)phenoxymethyl]quinoline hydrochloride) (Van inwegen *et al.*, 1987; Evans *et al.*, 1991) to influence bronchoconstriction, pulmonary cell infiltration and airway hyperresponsiveness induced by PAF in spontaneously breathing rabbits was investigated. Preliminary findings of this work have been presented to the American Thoracic Society (Herd & Page, 1991) and to the British Pharmacological Society (Herd *et al.*, 1991).

Methods

Animals

New Zealand White (NZW) rabbits (Froxfield Farms, Petersfield, Hants) of either sex were used throughout the study. Rabbits used were allergen-immunized, sham-immunized or normal. The immunization of neonatal rabbits was performed by a previously described method (McKenniff *et al.*, 1991). Briefly, rabbits were injected intraperitoneally (0.5 ml) within 24 h of birth with *Alternaria tenuis* extract (1 mg ml^{-1}) in aluminium hydroxide ($Al(OH)_3$) moist gel. As a concurrent sham-immunized control, littermates were injected with $Al(OH)_3$ alone (30 mg in 0.5 ml saline). Antigen and/or adjuvant administration was repeated weekly for the first month and then biweekly for the next two months in immunized and sham-immunized rabbits, respectively. In other experiments, naive animals were kept for 3 months. The methodology described in this study was subject to Home Office approval and performed under the Animals (Scientific Procedures) Act 1986.

At 3 months of age, the animals were transferred from the breeding unit to our laboratory, where they were skin tested to determine their sensitivity to the antigen.

Pulmonary function measurements

Rabbits (1.8–3.7 kg) were pre-medicated with diazepam (5 mg ml^{-1}, 5 mg kg^{-1} i.p.) and subsequently anaesthetized with Hypnorm (0.4 ml kg^{-1}, i.m.; a mixture of fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}), a regime which produces neuroleptanalgesia and is recommended for recovery anaesthesia in laboratory rabbits (Flecknall, 1987). Animals were placed in a supine position and intubated with a 3.0 mm endotracheal tube (Mallinckrodt Laboratories, Athlone, Ireland). The cuff was then inflated and the tube attached to a heated Fleisch pneumotachograph (size 00). Flow was measured with a Validyne differential pressure transducer (model MP 45-14-871; Validyne Engineering Corp., Northridge, C.A.). Pleural pressure was estimated by placing an oesophageal balloon in the lower third of the oesophagus to obtain the maximum expiratory pressure. Transpulmonary pressure, the difference betwen thoracic and pleural pressure, was measured with another Validyne differential pressure transducer (model MP 45-24-871) connected between the outflow of the endotracheal tube and the oesophageal balloon. The flow was integrated to obtain a continuous recording of tidal volume. Measurements of total lung resistance (R_L) and dynamic compliance (C_{dyn}) were calculated by an online respiratory analyser (PMS Version 4.0, Mumed Ltd., London) as previously described (Spina *et al.*, 1991). Neuroleptanalgesia was maintained throughout the course of the experiment by administration of Hypnorm i.m. approximately every 30 min (Flecknall, 1987).

Measurement of airway responsiveness to histamine

After measurement of baseline lung function, rabbits were exposed to an aerosol of sterile saline for 2 min and lung function parameters recorded. Airway responsiveness was determined by exposing animals to cumulative concentrations of aerosolized histamine (1.25–80 mg ml^{-1}; 2 min per concentration) administered directly to the lungs via an endotracheal tube. Pulmonary function was recorded following each 2 min exposure.

Aerosols were generated by an ultrasonic nebulizer (Devilbiss Health Care, UK Ltd., Hounslow, Middlesex) which has previously been demonstrated to generate particles of which the majority are in the 0.5–5 μm diameter range.

The provocation concentration (PC) of histamine that produced a 50% increase in R_L (PC_{50}) and 35% decrease in C_{dyn} (PC_{35}) was determined for each animal and used as indices of airway responsiveness.

PAF challenge and drug administration

On day 2, animals were re-anaesthetized and challenged with either PAF or 0.25% bovine serum albumin (BSA) (the carrier molecule for PAF). After exposure to an aerosol of BSA for 2 min, rabbits were exposed to PAF (80 μg ml^{-1}) or BSA over a 1 h period, after which time respiratory parameters were then recorded. Control animals received a 2 min aerosol of BSA followed by a 60 min aerosol of BSA containing the same concentration of ethanol as that received by the PAF-treated animals (approximately 1%).

On days 3, 5 and 9, increasing concentrations of histamine were administered to the anaesthetized rabbits as on day 1 and the PC_{50} (R_L) and the PC_{35} (C_{dyn}) values determined.

Drug studies

A suspension of PF 5901 (0.5 mg, 1 mg, 3 mg or 10 mg in a volume of 0.5 ml) or vehicle was instilled directly into the lung via a cannula passed into the airways, to the point of the bifurcation, via the endotracheal tube, 1 h before the start of the PAF aerosol. Similarly, a suspension of PF 5901 (10 mg) was instilled into the airways 1 h before a corresponding aerosol of 0.25% BSA. In these experiments respiratory parameters were recorded prior to, and 1, 15, 30 and 60 min following the drug administration, then as previously described for the BSA challenge.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed immediately after completion of airway responsiveness to histamine. The airways were lavaged by use of a polyethylene catheter inserted into the lung via the endotracheal tube. Five ml of 0.9% sterile saline was injected into the lungs then immediately aspirated, with 50% recovery of BAL fluid. Total cell counts were determined under light microscopy using an improved Neubauer haemocytometer. For differential cell counts, 25 μl aliquots were used for centrifugation (Shandon cytospin 2; Shandon Southern Instruments, Sewickley, PA, U.S.A.) and the cells were stained with Lendrum's stain (haematoxylin and chromotrope 2R) to facilitate the discrimination of eosinophils. A total of 200 cells were counted differentially and classified as either neutrophils, eosinophils or mononuclear cells based on standard morphological criteria. All cytospins were counted 'blind'.

Analysis of results

Results of the lung function studies are expressed as mean ± s.e.mean. *In vivo* histamine potency values were derived from measurements of airway resistance (R_L) (PC_{50}) and dynamic compliance (C_{dyn}) (PC_{35}) and are expressed as the geometric means together with upper and lower values for s.e.mean. For statistical purposes PC_{50} and PC_{35} values were $\log_{10}$ transformed.

One-way analyses of variance were used to analyse the bronchoconstriction data (R_L and C_{dyn}) (expressed as percen-

tage change). Two-way analayses of variance for repeated measures were employed to analyse the histamine potency data before and at the 3 time points after PAF administration. The total cell counts and mononuclear cell counts obtained from BAL before and 24 h, 72 h and 1 week after PAF challenge were subjected to the same analysis. Eosinophil and neutrophil counts before and after PAF challenge were analyzed by a Freidman two-way analysis of variance by ranks (Chi-squared determination) as the variances were found to be non-homogenous. A requirement of this analysis is equal group sizes therefore missing values were estimated (Winer, 1971). Where appropriate, Tukey's HSD test or distribution-free multiple comparisons were used to determine differences in means when multiple comparisons were made. Results were considered significant if $P<0.05$.

Drugs

Alternaria tenuis extract was obtained from Greer Laboratories Inc. Lenoir, N.C., U.S.A. (40,000 PNU ml^{-1}, 1 mg ml^{-1}) and aluminium hydroxide ($Al(OH)_3$) moist gel from FSA Laboratory supplies, Loughborough, U.K. Histamine diphosphate and bovine serum albumin (low endotoxin) were obtained from Sigma (Poole, Dorset); PAF was purchased from Novabiochem (Nottingham, U.K.); diazepam (Valium, Roche, U.K.); fentanyl citrate (Hypnorm, Janssen Pharmaceutical Ltd., U.K.); PF 5901 (2-[3-(1-hydroxyhexyl)phenoxymethyl]quinoline hydrochloride) (The Purdue Frederick Company, Norwalk, Connecticut, U.S.A.) (100 mg) was dissolved in 2 ml polyethylene glycol 400 and diluted with 0.9% saline as required.

Results

Airway responsiveness in vivo

No significant difference was observed between airway responsiveness (airway resistance R_L or dynamic compliance C_{dyn}) to inhaled histamine at 3 months in rabbits neonatally immunized with the antigen *Alternaria tenuis* and those rabbits sham-immunized only (Tables 1A and B). The increased responsiveness of both immunized and sham-immunized rabbits to inhaled histamine compared with naive rabbits did not reach statistical significance (Tables 1A and B).

Effect of PAF

Acute bronchospasm In the 3 animal groups i.e. allergen-immunized, sham-immunized and naive, a 1 h aerosol of PAF caused an increase in airway resistance and a decrease in dynamic compliance to a similar extent (Figure 1). This bronchoconstrictor response to PAF had resolved by the time of the next lung function measurement i.e. by 24 h (data not shown). The administration of an aerosol of 0.25% BSA to immunized rabbits caused changes in R_L and C_{dyn} that were significantly smaller than those changes elicited in the other 3 animal groups (Figure 1).

Airway hyperresponsiveness At 24 h following PAF administration in immunized rabbits, airway responsiveness to inhaled histamine (R_L and C_{dyn}) was significantly increased compared to pre-PAF levels (Tables 1A and B). This PAF-induced hyperrresponsiveness was still evident 72 h after PAF administration (Tables 1A and B). Both the R_L and C_{dyn} components had returned to pre-PAF levels 1 week following PAF challenge (Tables 1A and B). In the sham-immunized and normal groups, PAF did not significantly alter airway responsiveness to inhaled histamine at 24 h, 72 h or 1 week following challenge when considering either the R_L or C_{dyn} component (Tables 1A and B). The individual responses (histamine PC_{50} and PC_{35}) of immunized, sham-immunized and normal rabbits before and 24 h after PAF challenge are

Table 1 Airway responsiveness to histamine prior to, and 24 h, 72 h and 1 week following PAF challenge (80 $\mu g ml^{-1}$) in immunized, sham-immunized and normal rabbits: the effect of 0.25% bovine serum albumin (vehicle for PAF) on airway responsiveness to histamine in immunized rabbits is also represented.

A		*Histamine* PC_{50} (mg ml^{-1})			
		Pre	24 h	72 h	1 week
Immunized/PAF	*n*	10	10	7	6
	mean	11.54	3.44*	3.88*	14.39
	s.e.mean	1.20	1.26	1.47	1.91
Immunized/BSA	*n*	4	4	3	3
	mean	11.69	10.33	12.30	8.71
	s.e.mean	1.95	1.56	1.50	1.61
Sham/PAF	*n*	10	10	10	8
	mean	13.43	16.48	10.81	7.12
	s.e.mean	1.42	1.53	1.32	1.39
Normal/PAF	*n*	10	10	10	9
	mean	16.71	10.94	10.79	12.33
	s.e.mean	1.37	1.42	1.33	1.46
B		*Histamine* PC_{35} (mg ml^{-1})			
		Pre	24 h	72 h	1 week
Immunized/PAF	*n*	10	10	7	6
	mean	14.87	4.58*	6.32*	9.61
	s.e.mean	1.29	1.02	1.50	1.70
Immunized/BSA	*n*	4	4	3	3
	mean	11.17	6.59	7.05	4.90
	s.e.mean	1.85	1.66	1.12	1.30
Sham/PAF	*n*	10	10	10	8
	mean	9.14	5.35	7.35	8.23
	s.e.mean	1.27	1.31	1.45	1.38
Normal/PAF	*n*	10	10	10	9
	mean	25.76	18.79	13.18	10.74
	s.e.mean	1.59	1.46	1.36	1.59

(a) Histamine PC_{50} is the concentration of histamine (aerosol) (mg ml^{-1}) required to cause a 50% increase in airway resistance (R_L); (b) Histamine PC_{35} is the concentration of histamine (aerosol) (mg ml^{-1}) required to cause a 35% fall in dynamic compliance (C_{dyn}).
PAF treatment significantly increased airway responsiveness to histamine at 24 h and 72 h following challenge in immunized rabbits (*$P<0.05$).

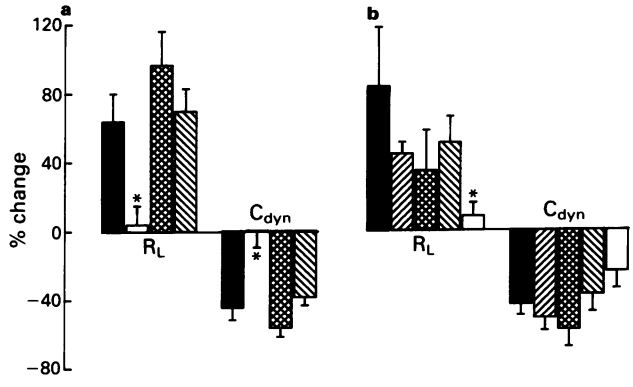

Figure 1 (a) Percentage change in airway resistance (R_L) and dynamic compliance (C_{dyn}) following PAF aerosol (80 $\mu g ml^{-1}$) in immunized (solid columns), sham-immunized (cross-hatched columns) and normal (hatched columns) rabbits. Also shown is R_L and C_{dyn} following BSA aerosol in immunized rabbits (open columns). *$P<0.05$ compared with PAF aerosol. (b) Percentage change in airway resistance (R_L) and dynamic compliance (C_{dyn}) following PAF aerosol (80 $\mu g ml^{-1}$) in immunized rabbits pretreated with PF 5901 vehicle (solid columns), 0.5 mg (right diagonal columns), 1 mg (cross-hatched columns), 3 mg (left diagonal columns) and 10 mg (open columns).
*$P<0.05$ compared with vehicle control.

presented in Figures 2a and b. In contrast to the sham-immunized and normal rabbits, every immunized rabbit developed airway hyperresponsiveness to histamine 24 h after PAF. Exposure of 0.25% BSA (1 h aerosol) to immunized rabbits did not alter airway responsiveness to histamine at any of the measured time points (Tables 1A and B).

Effect of PF 5901 in allergen-immunized rabbits

Airway responsiveness Baseline airway responsiveness (both R_L and C_{dyn}) to inhaled histamine in immunized rabbits was not significantly different in groups that received either vehicle only, PF 5901 0.5 mg, 1 mg, 3 mg or 10 mg (Tables 2A and B). The combined mean values were R_L: $PC_{50} = 11.6 \pm 1.2$ mg ml^{-1} ($n = 30$); $C_{dyn} = 10.0 \pm 1.1$ mg ml^{-1} ($n = 30$).

Baseline lung function Single doses of vehicle or PF 5901 10 mg instilled directly into the lungs of immunized rabbits had no significant effect on baseline lung function (R_L or C_{dyn}) measured 1, 15, 30 or 60 min following administration (data not shown; $n = 4$).

PAF-induced bronchoconstriction Direct intratracheal instillation of the vehicle for PF 5901 did not affect PAF-induced bronchoconstriction compared with untreated rabbits (R_L + 83.6 ± 34.2% ($n = 5$) and + 63.8 ± 16.1% ($n = 10$), respectively; C_{dyn} − 43.0 ± 6.5% ($n = 5$) and − 44.5 ± 7.5% ($n = 10$), respectively). Bronchoconstriction induced by PAF was not signficantly different in groups of allergen-immunized rabbits pretreatment with vehicle or with PF 5901 at doses of 0.5 mg, 1.0 mg and 3 mg directly administered into the airway (Figure 1b). PF 5901 10 mg however, significantly inhibited the R_L component of the PAF-induced bronchoconstriction ($P<0.05$) without exhibiting a significant inhibitory effect on C_{dyn} (Figure 1b).

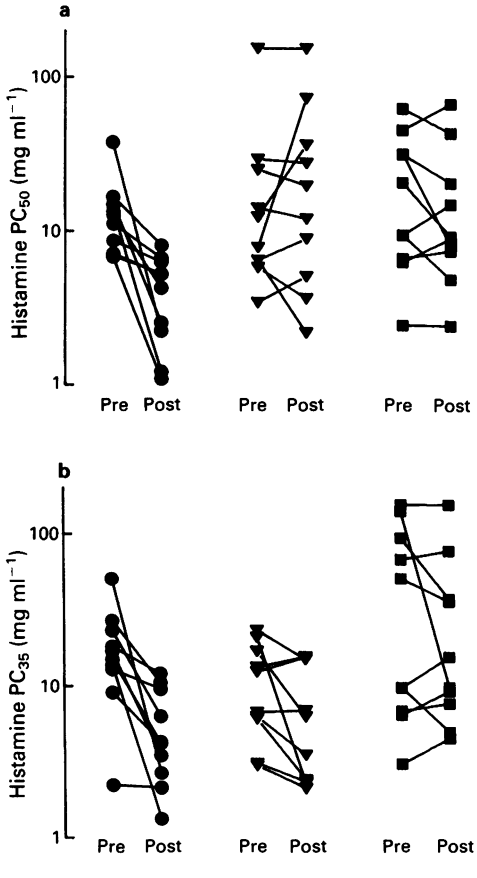

Figure 2 Individual histamine (a) PC_{50} (R_L) and (b) PC_{35} (C_{dyn}) values before and 24 h after aerosol exposure of PAF (80 μg ml^{-1}) in immunized (●), sham-immunized (▼) and normal (■) rabbits.

Table 2 The effect of PF 5901 (vehicle, 0.5 mg, 1 mg, 3 mg and 10 mg) on airway responsiveness to histamine prior to, and 24 h, 72 h and 1 week following PAF challenge (80 μg ml^{-1}) in immunized rabbits

A PF 5901		*Histamine PC_{50}* (mg ml^{-1}) Pre	24 h	72 h	1 week
Vehicle	*n*	6	6	6	6
	mean	12.76	4.06*	5.82*	7.79
	s.e.mean	1.52	1.41	1.59	1.63
0.5 mg	*n*	6	6	6	5
	mean	14.85	6.22*	5.66*	7.15
	s.e.mean	1.02	1.58	1.43	1.40
1 mg	*n*	6	6	4	4
	mean	9.04	10.74	6.84	10.89
	s.e.mean	1.46	1.56	1.27	1.38
3 mg	*n*	6	6	6	6
	mean	10.74	14.52	25.76	12.19
	s.e.mean	1.32	1.64	1.84	1.41
10 mg	*n*	6	6	6	6
	mean	11.54	11.51	11.72	8.09
	s.e.mean	1.56	1.67	1.02	1.43

B PF 5901		*Histamine PC_{35}* (mg ml^{-1}) Pre	24 h	72 h	1 week
Vehicle	*n*	6	6	6	6
	mean	11.25	3.59*	6.71	5.31
	s.e.mean	1.02	1.37	1.56	1.42
0.5 mg	*n*	6	6	6	5
	mean	13.93	5.83*	8.61	7.78
	s.e.mean	1.36	1.60	1.36	1.02
1 mg	*n*	6	6	4	4
	mean	7.24	7.80	5.02	8.07
	s.e.mean	1.46	1.46	1.10	1.37
3 mg	*n*	6	6	6	6
	mean	10.91	13.27	16.63	8.87
	s.e.mean	1.30	1.51	1.51	1.44
10 mg	*n*	6	6	6	6
	mean	7.83	8.57	8.17	5.11
	s.e.mean	1.33	1.70	1.37	1.42

(a) Histamine PC_{50} is the concentration of histamine (aerosol) (mg ml^{-1}) required to cause a 50% increase in airway resistance (R_L); (b) Histamine PC_{35} is the concentration of histamine (aerosol) (mg ml^{-1}) required to cause a 35% fall in dynamic compliance (C_{dyn}).
*$P<0.05$ compared with Pre value

Effect of PF 5901 treatment on PAF-induced airway hyperresponsiveness

Airway responses to inhaled histamine (PC_{50} or PC_{35}) were not significantly different in animals treated with PF 5901 10 mg 1 h before BSA aerosol at 24 h, 72 h or 1 week following challenge, compared with pre-BSA values (data not shown). Single doses of 1 mg, 3 mg and 10 mg PF 5901 directly instilled into the lungs of allergen-immunized rabbits were sufficient to inhibit significantly PAF-induced airway hyperresponsiveness at 24 h following PAF challenge for both R_L and C_{dyn}, compared with the vehicle-treated control group ($P<0.05$) (Tables 2A and B, Figures 3a and b).

Brochoalveolar lavage

Effect of PAF on cell counts in bronchoalveolar lavage Before PAF exposure, mononuclear cells comprised more than 95% of the resident cell population with a small (less than 5%) number of neutrophils (Table 3). The percentage of neutrophils, eosinophils and mononuclear cells recovered

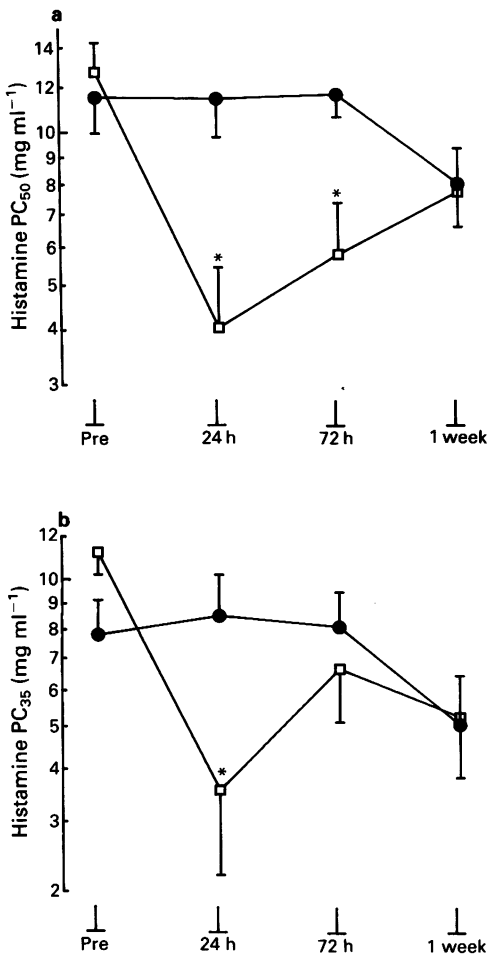

Figure 3 Effect of PF 5901 vehicle (□) ($n = 6$) and PF 5901 10 mg (●) ($n = 6$) on PAF-induced airway hyperresponsiveness in immunized rabbits: (a) histamine PC_{50} is the concentration of histamine required to cause a 50% increase in airway resistance (R_L); (b) histamine PC_{35} is the concentration of histamine required to cause a 35% decrease in dynamic compliance (C_{dyn}). *$P<0.05$ compared with pre-PAF control.

Table 3 Percentage of neutrophils, eosinophils and mononuclear cells recovered from bronchoalveolar lavage (BAL) in immunized, sham-immunized and normal rabbits

	% cells		
	Neutrophils	*Eosinophils*	*Mononuclear cells*
Immunized ($n = 7$)	3.21 ± 0.65	0.93 ± 0.34	95.71 ± 0.77
Sham-immunized ($n = 9$)	2.83 ± 0.82	0.59 ± 0.24	96.67 ± 0.88
Normal ($n = 10$)	4.30 ± 0.97	0.10 ± 0.10	95.60 ± 1.01

Values are mean ± s.e.mean.

from BAL prior to PAF exposure in immunized, sham-immunized and normal rabbits is shown in Table 3.

The mean pretreatment cell counts from the 3 groups of rabbits i.e. immunized, sham-immunized and normals were not significantly different (Table 4). Total leukocyte counts were significantly elevated in the airways as assessed by BAL 24 h after PAF exposure in immunized, sham-immunized and normal rabbits ($P<0.05$) (Table 4). Total cell counts remained elevated at 72 h following PAF in the immunized and normal rabbit groups ($P<0.05$) (Table 4). By 1 week, counts had returned to pre-PAF levels (Table 4) in all groups.

Although there was an increase in the number of mononuclear cells in BAL 24 h and/or 72 h after PAF challenge in all 3 animal groups, this was not statistically significant. In contrast, a significant increase in the number of BAL neutrophils and eosinophils was observed at 24 h and 72 h following PAF in immunized, sham-immunized and normal rabbits ($P<0.05$).

Exposure to immunized rabbits of an aerosol of 0.25% BSA induced a small but insignificant cellular infiltrate into the airways. However, there was a significant increase in the number of neutrophils present in the airways 24 h following BSA ($P<0.05$) (Table 5).

Effect of PF 5901 treatment on PAF-induced changes in cell counts in BAL

The PAF-induced cellular infiltration profile for both total and differential cell counts observed in control rabbits treated with vehicle was not significantly different from that observed in rabbits treated with PF 5901 at any dose (Table 6).

Table 4 Number of total cells and differential cell counts recovered from bronchoalveolar lavage (BAL) before (Pre) and 24 h, 72 h and 1 week following exposure to PAF (80 μg ml^{-1}) in immunized, sham-immunized and normal rabbits

	× 10^5 cells ml^{-1}			
	Total	*Neutrophils*	*Eosinophils*	*Mononuclear cells*
Immunized				
Pre ($n = 7$)	1.03 ± 0.28	0.031 ± 0.009	0.006 ± 0.003	0.99 ± 0.28
24 h ($n = 7$)	4.19 ± 0.57*	1.120 ± 0.330*	0.241 ± 0.106*	2.34 ± 0.56
72 h ($n = 7$)	2.66 ± 0.59*	0.760 ± 0.230*	0.090 ± 0.027*	1.74 ± 0.42
1 week ($n = 5$)	1.17 ± 0.42	0.213 ± 0.056	0.014 ± 0.006	0.94 ± 0.38
Sham-immunized				
Pre ($n = 8$)	1.04 ± 0.39	0.024 ± 0.014	0.008 ± 0.004	1.01 ± 0.38
24 h ($n = 8$)	3.63 ± 0.87*	1.630 ± 0.520*	0.187 ± 0.044*	1.82 ± 0.45
72 h ($n = 6$)	2.13 ± 0.61	0.860 ± 0.273*	0.173 ± 0.087*	1.09 ± 0.31
1 week ($n = 6$)	2.02 ± 0.55	0.439 ± 0.157	0.138 ± 0.067*	1.44 ± 0.40
Normal				
Pre ($n = 10$)	1.28 ± 0.23	0.056 ± 0.153	0.001 ± 0.001	1.22 ± 0.23
24 h ($n = 10$)	3.09 ± 0.52*	1.880 ± 0.441*	0.160 ± 0.038*	1.04 ± 0.16
72 h ($n = 10$)	4.43 ± 1.35*	1.847 ± 0.594*	0.302 ± 0.189*	2.27 ± 0.69
1 week ($n = 8$)	3.09 ± 1.12	0.961 ± 0.302*	0.260 ± 0.159*	1.87 ± 0.69

Results are expressed as mean ± s.e.mean.
PAF exposure significantly increased the total number of cells and the number of neutrophils and eosinophils recovered in BAL cf. Pre PAF levels in all 3 rabbit groups (*$P<0.05$).

Table 5 Number of total cells and differential cell counts recovered from bronchoalveolar lavage (BAL) in immunized rabbits before (Pre) and 24 h, 72 h and 1 week following exposure to bovine serum albumin (BSA 0.25%)

	× 10^5 cells ml^{-1}			
	Total	*Neutrophils*	*Eosinophils*	*Mononuclear cells*
Pre ($n = 4$)	1.13 ± 0.48	0.22 ± 0.16	0.003 ± 0.002	0.90 ± 0.33
24 h ($n = 4$)	2.38 ± 0.95	1.17 ± 0.67*	0.031 ± 0.009	1.17 ± 0.33
72 h ($n = 3$)	1.38 ± 0.29	0.61 ± 0.16	0.050 ± 0.016	0.72 ± 0.19
1 week ($n = 3$)	1.20 ± 0.40	0.23 ± 0.51	0.040 ± 0.029	0.93 ± 0.34

Results are expressed as mean ± s.e.mean.
BSA exposure significantly increased the total number of neutrophils recovered in BAL cf. Pre BSA levels (*$P < 0.05$).

Table 6 Number of total cells and differential cell counts recovered from bronchoalveolar lavage (BAL) before (Pre) and 24 h, 72 h and 1 week following exposure to PAF (80 μg ml^{-1}) in immunized rabbits pretreated with PF 5901 0 mg, 0.5 mg, 1 mg, 3 mg and 10 mg

		× 10^5 cells ml^{-1}			
		Total	*Neutrophils*	*Eosinophils*	*Mononuclear cells*
0 mg (vehicle)	Pre ($n = 6$)	0.99 ± 0.18	0.076 ± 0.017	0.003 ± 0.002	0.91 ± 0.18
	24 h ($n = 6$)	2.39 ± 0.46*	0.982 ± 0.131*	0.235 ± 0.118*	1.17 ± 0.31
	72 h ($n = 6$)	2.98 ± 0.86	1.740 ± 0.770*	0.151 ± 0.037*	1.07 ± 0.19
	1 week ($n = 6$)	1.60 ± 0.32	0.383 ± 0.065*	0.154 ± 0.465*	1.06 ± 0.26
0.5 mg	Pre ($n = 6$)	1.03 ± 0.23	0.121 ± 0.073	0.002 ± 0.002	0.98 ± 0.22
	24 h ($n = 6$)	5.27 ± 1.42*	3.368 ± 0.960*	0.340 ± 0.120*	1.50 ± 0.55
	72 h ($n = 6$)	3.37 ± 1.69	2.404 ± 1.699*	0.161 ± 0.070*	0.99 ± 0.28
	1 week ($n = 4$)	1.81 ± 0.74	0.695 ± 0.230*	0.087 ± 0.027*	1.03 ± 0.50
1 mg	Pre ($n = 6$)	0.98 ± 0.20	0.059 ± 0.036	0.012 ± 0.004	0.88 ± 0.14
	24 h ($n = 5$)	3.52 ± 0.84*	2.194 ± 0.598*	0.358 ± 0.178*	0.97 ± 0.22
	72 h ($n = 4$)	1.26 ± 0.19	0.412 ± 0.103*	0.049 ± 0.014*	0.80 ± 0.19
	1 week ($n = 3$)	1.15 ± 0.24	0.270 ± 0.066	0.005 ± 0.005	0.88 ± 0.18
3 mg	Pre ($n = 5$)	1.16 ± 0.39	0.045 ± 0.010	0.013 ± 0.010	1.10 ± 0.37
	24 h ($n = 4$)	4.43 ± 1.32*	3.153 ± 0.941*	0.179 ± 0.102*	1.09 ± 0.30
	72 h ($n = 5$)	1.76 ± 0.59	0.688 ± 0.331*	0.081 ± 0.016*	0.92 ± 0.26
	1 week ($n = 4$)	1.54 ± 0.48	0.407 ± 0.135*	0.152 ± 0.092*	0.98 ± 0.33
10 mg	Pre ($n = 4$)	1.03 ± 0.17	0.015 ± 0.005	0.003 ± 0.010	0.82 ± 0.26
	24 h ($n = 4$)	3.53 ± 0.33*	1.084 ± 0.190*	0.445 ± 0.024*	1.20 ± 0.42
	72 h ($n = 4$)	1.15 ± 0.31	0.323 ± 0.161*	0.090 ± 0.046*	0.74 ± 0.15
	1 week ($n = 4$)	0.60 ± 0.15	0.034 ± 0.012	0.021 ± 0.005*	0.55 ± 0.16

Results are expressed as mean ± s.e.mean.
PAF exposure significantly increased the total number of cells and the number of neutrophils and eosinophils recovered in BAL cf. Pre PAF levels in rabbits pretreated with vehicle or with PF 5901 (any dose) (*$P < 0.05$).

Discussion

The current study has demonstrated that aerosolized PAF will induce acute bronchospasm in normal rabbits and rabbits that have been neonatally immunized with antigen. It is of interest that the degree of acute bronchoconstriction induced by PAF in the immunized rabbits is not significantly different from that induced in the sham-immunized or normal rabbits. This agrees with studies in man, where subjects with bronchial asthma are known to be hyperresponsive to inhaled histamine when compared with normals, yet asthmatics appear not to be hyperresponsive to inhaled PAF (Cuss *et al.*, 1986; Rubin *et al.*, 1987; Chung & Barnes, 1989). Furthermore, PAF-induced bronchoconstriction in the rabbit appears to be partly mediated via the release of 5-lipoxygenase products since it can be significantly reduced by pretreatment with PF 5901. The significant inhibition of the resistance component of the PAF-induced bronchoconstriction only may suggest that the contribution of lipoxygenase products to the alterations induced by PAF in the large airways is greater than that in the smaller airways. These results support observations in man that cysteinyl-leukotriene antagonists inhibit not only bronchoconstriction induced by PAF (Spencer *et al.*, 1990a; Kidney *et al.*, 1991), but also that induced by antigen (Taylor *et al.*, 1991) and by cold air (Israel *et al.*, 1991). The cellular source of these lipoxygenase products remains to be determined, but is unlikely to be airway muscle itself since PAF does not contract human bronchial smooth muscle directly (Schellenberg *et al.*, 1983), and there appears to be only a low concentration of PAF receptors located on human airway smooth muscle (Goldie *et al.*, 1990).

A significant difference in airway responsiveness to histamine between immunized and sham-immunized rabbits has been previously reported (McKenniff *et al.*, 1991). This finding is based on large numbers of animals as there is considerable overlap between the groups. However, in the present experiments no significant difference in airways responsiveness was observed between immunized and sham-immunized animals, suggesting that the adjuvant alone may induce airway hyperresponsiveness. This lack of significant difference between the groups may be due to the relatively small numbers of animals used.

The present study has shown that aerosolised PAF will induce bronchial hyperresponsiveness to inhaled histamine in some, but not all normal rabbits, supporting previous studies (Coyle *et al.*, 1990). However, interestingly, PAF induced bronchial hyperresponsiveness to inhaled histamine in all neonatally immunized rabbits to some degree. PAF-induced hyperresponsiveness lasted for 72 h but had returned to baseline in the majority of animals by one week. The mechanisms(s) underlying the ability of PAF to induce bron-

chial hyperresponsiveness to a greater degree in immunized rather than normal or sham-immunized rabbits is unknown, but PAF has previously been reported to elicit eosinophil infiltration in the skin of allergic subjects but not in normal subjects (Henocq & Vargaftig, 1988; Fadel *et al.*, 1990), and has been reported to elicit a greater degree of eosinophils infiltration into the lungs of actively immunized guinea-pigs when compared with normal guinea-pigs (Sanjar *et al.*, 1989). However, in our study there was no significant difference observed between the number of inflammatory cells recruited into bronchoalveolar lavage fluid from normal, sham-immunized and immunized rabbits following exposure to PAF. Nonetheless, the inflammatory cells resident in the airway of the neonatally immunized rabbit may be primed as described in other situations (Frigas & Gleich, 1986) and therefore more readily release their stored and preformed cytotoxic mediators in response to PAF stimulation.

The ability of PAF to induce bronchial hyperresponsiveness in the neonatally immunized rabbit was significantly attenuated in a dose-related manner by prior treatment with PF 5901. As this drug had no effect on the airway responsiveness to histamine following BSA challenge, this inhibitory action of PF 5901 is not attributable to histamine H_1 antagonism or via some non-specific effect on airway hyperresponsiveness. PF 5901 was originally described as a 5-lipoxygenase inhibitor (Van Inwegen *et al.*, 1987) and is now recognized as being a 5-lipoxygenase-activating protein (FLAP) inhibitor similar to the prototype FLAP inhibitor MK 886 (Evans *et al.*, 1991). Following cell stimulation, leucocyte 5-lipoxygenase is translocated from its cytosolic location to a cell membrane site (Rouzer & Kargman, 1988) where it is fully activated by FLAP, an 18,000-D membrane protein. Full expression of 5-lipoxygenase and FLAP is required in cells to catalyze the C-5 oxygenation of arachidonic acid to generate 5-HPETE, the key intermediate in the formation of leukotrienes (Samuelsson *et al.*, 1987). PF 5901 is also a moderately potent antagonist of LTD_4 (Van Inwegen *et al.*, 1987).

Of particular interest is the recent finding that PF 5901 will also inhibit PAF synthesis in rodent mast cells (Hogaboam *et al.*, 1992). In recent studies, LTC_4, LTD_4 and LTE_4 have been shown to increase airway responsiveness to histamine in asthmatic but not normal subjects (Arm *et al.*, 1988; O'Hickey *et al.*, 1991). Furthermore, in atopic subjects the LTD_4 receptor antagonist ICI 204.219 was found to inhibit significantly antigen-induced airway hyperresponsiveness in response to histamine (Taylor *et al.*, 1991). Which of the actions of PF 5901 is responsible for the inhibition of PAF-induced bronchial hyperresponsiveness has not yet been determined, but it is perhaps of relevance that we have previously reported that capsaicin will also inhibit PAF-induced bronchial hyperresponsiveness in the rabbit, through a mechanism unrelated to its ability to deplete sensory neuropeptides (Spina *et al.*, 1991) and that capsaicin has been reported to inhibit 5-lipoxygenase activity in human neutrophils (Flynn *et al.*, 1986).

In agreement with previous studies, PAF was found to induce an influx of inflammatory cells (predominantly neutrophils and eosinophils) into the airways as assessed by bronchoalveolar lavage (BAL) (Coyle *et al.*, 1990; Spina *et al.*, 1991). The accumulation and activation of inflammatory cells within the airways has been suggested to lead to epithelial damage causing the exposure of nerve endings in the bronchial lumen, which are thought to initiate an increase in airway responsiveness (Barnes, 1986). In the present study we have shown that this effect of PAF on cellular infiltration is inducible in normal, sham-immunized and immunized rabbits to a similar extent. In general, the recruitment of these cells persists within the lung for at least 3 days following PAF exposure, with baseline levels returning by one week.

However, PF 5901 did not inhibit PAF-induced cellular infiltration into the airways as assessed by BAL, despite inhibiting the associated bronchial hyperresponsiveness. This finding is supportive of other experiments performed in this model where pretreatment of neonatally immunized rabbits with capsaicin inhibits bronchial hyperresponsiveness but not the pulmonary eosinophil infiltration induced by PAF (Spina *et al.*, 1991). Furthermore, in guinea-pigs, capsaicin will inhibit antigen-induced bronchial hyperresponsiveness without modifying eosinophil infiltration (Ladenius & Biggs, 1989; Matsuse *et al.*, 1991) and cytokines can cause pulmonary eosinophilia without bronchial hyperresponsiveness (Kings *et al.*, 1990). Low dose antigen in guinea-pigs has been reported to induce eosinophil accumulation in BAL in the absence of any alteration in airways responsiveness, and at a higher dose of antigen, ketotifen, AH 21132, dexamethasone and aminophylline were shown to inhibit the pulmonary eosinophilia and not the associated airway hyperresponsiveness (Sanjar *et al.*, 1990). Together these results suggest that the presence of eosinophils within the airway lumen may not be a prerequisite for the development of bronchial hyperresponsiveness. Such an interpretation is consistent with recent clinical observations that bronchial hyperresponsiveness may be present in asthmatics without an observed eosinophil infiltrate (Lundgren *et al.*, 1988) and conversely, chronic eosinophilic bronchitis is not always associated with airway hyperresponsiveness (Gibson *et al.*, 1989). However, in the absence of data on the activation state of the eosinophils present in the vehicle- and PF 5901-treated groups, caution must be exercised in ruling out the participation of eosinophils at this time.

In conclusion therefore, PAF induces long-lasting bronchial hyperresponsiveness in neonatally immunized rabbits which can be inhibited by PF 5901, an effect that may not be related to the associated pulmonary infiltration of inflammatory cells.

References

ANDERSON, G.P. & FENNESSY, M.R. (1988). Lipoxygenase metabolites mediate increased airways responsiveness to histamine after acute platelet activating factor exposure in the guinea pig. *Agents Actions*, **24**, 8–19.

ARM, J.P., SPUR, B.W. & LEE, T.H. (1988). The effects of inhaled leukotriene E4 on the airway responsiveness to histamine in subjects with asthma and normal subjects. *J. Allergy Clin. Immunol.*, **82**, 654–660.

BARNES, P.J. (1986). Asthma as an axon reflex. *Lancet*, **i**, 242–245.

BEAUBIEN, B.B., TIPPINS, J.R. & MORRIS, H.R. (1984). Platelet-activating factor stimulation of peptidoleukotriene release: inhibition by vasoactive polypeptide. *Biochem. Biophys. Res. Commun.*, **125**, 105–108.

BONNET, J., THIBAUDEAU, D. & BESSIN, P. (1983). Dependency of the PAF-acether induced bronchospasm on the lipoxygenase pathway in the guinea pig. *Prostaglandins*, **26**, 457–466.

CHUNG, K.F. & BARNES, P.J. (1989). Effects of platelet activating factor on airway calibre, airway responsiveness, and circulating cells in asthmatic subjects. *Thorax*, **44**, 108–115.

CHUNG, K.F., DENT, G. & BARNES, P.J. (1989). Effects of salbutamol on bronchoconstriction, bronchial hyperresponsiveness, and leukocyte responses induced by platelet activating factor in man. *Thorax*, **44**, 102–107.

COYLE, A.J., SPINA, D. & PAGE, C.P. (1990). PAF-induced bronchial hyperresponsiveness in the rabbit: contribution of platelets and airway smooth muscle. *Br. J. Pharmacol.*, **101**, 31–38.

CUSS, F.M., DIXON, C.M.S. & BARNES, P.J. (1986). Effects of inhaled platelet activating factor on pulmonary function and bronchial responsiveness in man. *Lancet*, **ii**, 189–192.

DRAZEN, J.M., AUSTEN, K.F., LEWIS, R.A., CLARK, D.A., GOTO, G., MARFAT, A. & COREY, E.J. (1980). Comparative airway and vascular activities of leukotrienes C-1 and D in vivo and in vitro. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 4354–4358.

EVANS, J.F., LEVEILLE, C., MANCINI, J.A., PRASIT, P., THERIEN, T., ZAMBONI, R., GAUTHIER, J.Y., FORTIN, R., CHARLESON, P., MACINTYRE, D.E., LUELL, S., BACH, T.J., MEURER, R., GUAY, J., VICKERS, P.J., ROUZER, C.A., GILLARD, J.W. & MILLER, D.K. (1991). 5-lipoxygenase-activating protein is the target of a quinoline class of leukotriene synthesis inhibitors. *Mol. Pharmacol.*, **40**, 22–27.

FADEL, R., DAVID, B., HERPIN-RICHARD, N., BORGNON, A., RASSEMONT, R. & RIHOUX, J-P. (1990). In vivo effects of cetirizine on cutaneous reactivity and eosinophil migration induced by platelet activating factor (Paf-acether) in man. *J. Allergy Clin. Immunol.*, **86**, 314–320.

FLECKNALL, P.A. (1987). *Laboratory Animal Anaesthesia: An Introduction for Research Workers and Technicians.* pp. 98–100. London: Academic Press.

FLYNN, D.L., RAFFERTY, M.F. & BOCTOR, A.M. (1986). Inhibition of human neutrophil 5-lipoxygenase activity by gingerdione, shogaol, capsaicin and related pungent compounds. *Prostaglandins Leuk. Med.*, **24**, 195–198.

FORD-HUTCHINSON, A.W. (1991). Inhibition of leukotriene biosynthesis. *Ann. N.Y. Acad. Sci.*, **629**, 133–142.

FORD-HUTCHINSON, A.W., BRAY, M.A., DOIG, M.U., SHIPLEY, M.E. & SMITH, M.J.H. (1981). Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature*, **286**, 264–265.

FRIGAS, E. & GLEICH, G.J. (1986). The eosinophil and the pathophysiology of asthma. *J. Allergy Clin. Immunol.*, **77**, 527–537.

GIBSON, P.G., DOLOVICH, J., DENBERG, J., RAMSDALE, E.H. & HARGREAVE, F.E. (1989). Chronic cough: Eosinophilic bronchitis without asthma. *Lancet*, **i**, 1346–1348.

GOLDIE, R.G., PEDERSEN, K.E., SELF, G.J., RIGBY, P.J. & PATERSON, J.W. (1990). Autoradiographic distribution of specific binding sites for the PAF receptor antagonist [^{3}H]-WEB 2086 in human non-diseased and asthmatic bronchi and periferal lung. *Am. Rev. Respir. Dis.*, **141**, A725.

HENOCQ, E. & VARGAFTIG, B.B. (1988). Skin eosinophilia in atopic diseases. *J. Allergy Clin. Immunol.*, **81**, 691–695.

HERD, C.M. & PAGE, C.P. (1991). Effect of platelet activating factor on airways hyperresponsiveness in neonatally immunised rabbits. *Am. Rev. Respir. Dis.*, **143**, A153.

HERD, C.M., SHOUPE, T.S. & PAGE, C.P. (1991). Effect of PF 5901 on PAF-induced airway responses in neonatally immunised rabbits. *Br. J. Pharmacol.*, **104**, 170P.

HOGABOAM, C.M., DONIGI-GALE, D., SHOUPE, T.S., BISSONNETTE, E.Y., BEFUS, A.D. & WALLACE, J.L. (1992). Platelet-activating factor synthesis by peritoneal mast cells and its inhibition by two quinoline-based compounds. *Br. J. Pharmacol.*, **105**, 87–92.

HUA, X.-Y., DAHLEN, S.E., LUNDBERG, J.M. & HEDQVIST, P. (1985). Leukotrienes C4, D4 and E4 cause widespread and extensive plasma extravasation in the guinea-pig. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **330**, 136–141.

ISRAEL, E., DERMARKARIAN, R., ROSENBERG, M., SPERLING, R., TAYLOR, G., RUBIN, P. & DRAZEN, J.M. (1991). The effects of a 5-lipoxygenase inhibitor on asthma induced by cold, dry air. *N. Engl. J. Med.*, **323**, 1740–1744.

KAYE, M.G. & SMITH, L.J. (1990). Effects of inhaled leukotriene D_4 and platelet-activating factor on airway reactivity in normal subjects. *Am. Rev. Respir. Dis.*, **141**, 993–997.

KIDNEY, J.C., RIDGE, S., CHUNG, K.F. & BARNES, P.J. (1991). Inhibition of PAF-induced bronchoconstriction by the oral leukotriene D4 receptor antagonist, ICI 204,219 in normal subjects. *Am. Rev. Respir. Dis.*, **143**, A811.

KINGS, M.A., CHAPMAN, I.D., KRISTERSSON, A., SANJAR, S. & MORLEY, J. (1990). Human recombinant lymphokines and cytokines induce pulmonary eosinophilia in the guinea pig which is inhibited by ketotifen and AH 21-132. *Int. Arch. Allergy Appl. Immunol.*, **91**, 354–361.

LADENIUS, A.R.C. & BIGGS, D.F. (1989). Capsaicin prevents the induction of airway hyperresponsiveness in a guinea-pig model of asthma. *Am. Rev. Respir. Dis.*, **139**, A232.

LAI, C.K.W., JENKINS, J.R., POLOSA, R. & HOLGATE, S.T. (1990). Inhaled PAF fails to induce airway hyperresponsiveness to methacholine in normal human subjects. *J. Appl. Physiol.*, **68**, 919–926.

LUNDGREN, R., SODERBERG, M., HORSTEDT, P. & STENLING, R. (1988). Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids. *Eur. Respir. J.*, **1**, 883–889.

MAROM, Z.M., SHELHAMER, J.H., BACH, M.K., MORTON, D.R. & KALINER, M.A. (1982). Slow reacting substances, leukotrienes C4 and D4, increase the release of mucus from human airways in vitro. *Am. Rev. Respir. Dis.*, **126**, 449–451.

MATSUSE, T., THOMSON, R.J., CHEN, X.-R., SALARI, H. & SCHELLENBERG, R.R. (1991). Capsaicin inhibits airway hyperresponsiveness but not lipoxygenase activity or eosinophilia after repeated aerosolized antigen in guinea pigs. *Am. Rev. Respir. Dis.*, **144**, 368–372.

MCKENNIFF, M.G., RICCIO, M.M., SEEDS, E.A.M. & PAGE, C.P. (1991). Neonatal immunisation of rabbits induces long-term airways hyperresponsiveness. *Am. Rev. Respir. Dis.*, **143**, A39.

O'HICKEY, S.P., HAWKSWORTH, R.J., FONG, C.Y., ARM, J.P., SPUR, B.W. & LEE, T.H. (1991). Leukotrienes C4, D4, and E4 enhance histamine responsiveness in asthmatic airways. *Am. Rev. Respir. Dis.*, **144**, 1053–1057.

PAGE, C.P. (1988). The role of platelet activating factor in asthma. *J. Allergy Clin. Immunol.*, **81**, 144–152.

PRETOLANI, M., LEFORT, J., DUMAREY, C. & VARGAFTIG, B.B. (1989). Role of lipoxygenase metabolites for the hyperresponsiveness to platelet activating factor of lungs from actively sensitized guinea pigs. *J. Pharmacol. Exp. Ther.*, **248**, 3535–3539.

ROUZER, C.A. & KARGMAN, S. (1988). Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J. Biol. Chem.*, **263**, 10980–10988.

RUBIN, A.-H.E., SMITH, L.J. & PATTERSON, R. (1987). The bronchoconstrictor properties of platelet activating factor in humans. *Am. Rev. Respir. Dis.*, **136**, 1145–1151.

SAMUELSSON, B., DAHLEN, S.-E. & LINDGREN, J.A. (1987). Leukotrienes and lipoxins: structures, biosynthesis and biological effects. *Science*, **237**, 1171–1176.

SANJAR, S., AOKI, S., KINGS, M. & MORLEY, J. (1989). Sensitisation to antigen or treatment with rh-GMCSF intensifies the eosinophilic response to PAF in the guinea-pig. *Am. Rev. Respir. Dis.*, **139**, 371A.

SANJAR, S., AOKI, S., KRISTERSSON, A., SMITH, D. & MORLEY, J. (1990). Antigen challenge induces pulmonary airway eosinophil accumulation and airway hyperreactivity in sensitised guinea pigs. *Br. J Pharmacol.*, **99**, 679–686.

SCHELLENBERG, R.R., WALKER, B. & SNYDER, F. (1983). Platelet-dependent contraction of human bronchus by platelet activating factor. *J. Allergy Clin. Immunol.*, **71**, 145.

SPENCER, D.A., EVANS, J.M., GREEN, S.E., PIPER, P.J. & COSTELLO, J.F. (1990a). Bronchospasm induced by platelet activating factor is reduced by a selective cysteinyl-leukotriene antagonist in normal man. *Am. Rev. Respir. Dis.*, **141**, A218.

SPENCER, D.A., GREEN, S.E., EVANS, J.M., PIPER, P.J. & COSTELLO, J.F. (1990b). Platelet activating factor does not cause a reproducible increase in bronchial responsiveness in normal man. *Clin. Exp. Allergy*, **20**, 525–532.

SPINA, D., MCKENNIFF, M.G., COYLE, A.J., SEEDS, E.A.M., TRAMONTANA, M., PERRETTI, F., MANZINI, S. & PAGE, C.P. (1991). Effect of capsaicin on PAF-induced bronchial hyperresponsiveness and pulmonary cell accumulation in the rabbit. *Br. J. Pharmacol.*, **103**, 1268–1274.

TAYLOR, I.K., O'SHAUGHNESSY, K.M., FULLER, R.W. & DOLLERY, C.T. (1991). Effect of cysteinyl-leukotriene receptor antagonist ICI 204.219 on allergen-induced bronchoconstriction and airway hyperreactivity in atopic subjects. *Lancet*, **337**, 690–694.

VAN INWEGEN, R.G., KHANDWALA, A., GORDON, R., SONNINO, P., COUTTS, S. & JOLLY, S. (1987). REV-5901: an orally effective peptidoleukotriene antagonist, detailed biochemical/pharmacological profile. *J. Pharmacol. Exp. Ther.*, **241**, 118–124.

VOELKEL, N.F., WORTHEN, S., REEVES, J.T., HENSON, P.M. & MURPHY, R.C. (1982). Nonimmunological production of leukotrienes induced by platelet activating factor. *Science*, **218**, 286–288.

WINER, B.J. (1971). *Statistical Principles in Experimental Design.* pp. 487–490. New York: McGraw-Hill.

(Received June 30, 1992
Revised July 23, 1992
Accepted August 11, 1992)

Br. J. Pharmacol. (1992), **107**, 1116–1120

Potassium channel openers, NIP-121 and cromakalim, enhance the relaxation induced by sodium nitroprusside in the guinea-pig isolated trachea

Ken-ichi Shikada & Sakuya Tanaka

Shiraoka Research Station of Biological Science, Nissan Chemical Industries Ltd., Shiraoka, Saitama 349-02, Japan

1 The effect of the potassium channel openers, NIP-121 and cromakalim, on agonist-induced relaxation of the guinea-pig isolated trachea was investigated and the results were compared with those in the epithelium-denuded trachea.

2 Tracheal strips were incubated with a potassium channel opener or vehicle for 30 min in the presence of 5 μM indomethacin and then contracted with 30 nM leukotriene D_4 (LTD_4). Relaxant agents were added to the organ bath after the LTD_4-elicited contraction had reached a plateau.

3 In epithelium-intact trachea, NIP-121 0.1 μM and cromakalim 1 μM, which did not modify the LTD_4 (30 nM)-induced contraction, significantly enhanced the sodium nitroprusside (SNP)-induced relaxation. This enhancement of relaxation was not seen in the case of relaxation induced by the cyclic AMP-dependent bronchodilators isoprenaline, vasoactive intestinal peptide or prostaglandin E_2. The enhancement of SNP-induced relaxation by NIP-121 and cromakalim was abolished in the presence of the ATP-sensitive potassium channel blocker, glibenclamide (1 μM). NIP-121 and cromakalim did not produce any significant changes in the relaxation induced by 8-bromoguanosine-cyclic monophosphate (8-Br-cyclic GMP), a cyclic GMP analogue.

4 In epithelium-denuded trachea, SNP-induced relaxation alone was significantly enhanced but that induced by 8-Br-cyclic GMP was not changed. Neither NIP-121 nor cromakalim enhanced SNP-induced relaxation in denuded trachea.

5 These results suggest that in the presence of an intact epithelium the enhancement by NIP-121 and cromakalim of the relaxation of guinea-pig tracheal smooth muscle induced by SNP may be associated with the opening of glibenclamide-sensitive potassium channels.

Keywords: Potassium channel opener; NIP-121; cromakalim; sodium nitroprusside; isoprenaline; vasoactive intestinal peptide; prostaglandin E_2; leukotriene D_4; epithelium; guinea-pig trachea

Introduction

The potassium channel opener, cromakalim, relaxes guinea-pig (Allen *et al.*, 1986; Arch *et al.*, 1988), bovine (Gater, 1989; Longmore *et al.*, 1991) and human (Taylor *et al.*, 1988; Black *et al.*, 1990) airway smooth muscle as well as vascular smooth muscle (Cox, 1990). Cromakalim is effective not only in preventing histamine-induced collapse in unanaesthetized guinea-pigs (Arch *et al.*, 1988) but also cholinergic- and noncholinergic-mediated bronchoconstriction in guinea-pig isolated airways (McCaig & De Jonckheere, 1989; Ichinose & Barnes, 1990). Cromakalim stimulates the efflux of potassium ions from tracheal smooth muscle (Allen *et al.*, 1986; Gater, 1989; Longmore *et al.*, 1991) but does not affect intracellular cyclic nucleotide levels (Murray *et al.*, 1990). Since the pharmacological profile of cromakalim is different from clinically used bronchodilators, β-adrenoceptor agonists or xanthines, other potassium channel openers are being developed as bronchodilators (Paciorek *et al.*, 1990; Raeburn & Brown, 1991). NIP-121 (Figure 1) is a potent newly synthesized potassium channel opener (Masuda *et al.*, 1991), and it has about 10–20 times greater potency in relaxing guinea-pig isolated tracheal contraction than cromakalim (Shikada *et al.*, 1991b).

Bronchial epithelium has been reported to play an important role in modulating the responsiveness of airway smooth muscle to drugs. Mechanical removal of the epithelium from the isolated trachea increases the responsiveness to various bronchoconstrictors; histamine (Braunstein *et al.*, 1988), acetylcholine (Holroyde, 1986), leukotrienes (Hay *et al.*, 1987), adenosine (Advenier *et al.*, 1988), substance P (Devillier *et al.*, 1988) and endothelin (Hay, 1990), and also enhances the responsiveness to the bronchodilators isoprenaline (Lennart Lundblad & Persson, 1988) and sodium nitroprusside (SNP) (Farmer *et al.*, 1986). These results indicate that the presence or the absence of epithelium is an important factor to be taken into account in investigating the responsiveness of guinea-pig isolated trachea to bronchoactive substances.

In the present study we investigated the effect of the potassium channel openers, NIP-121 and cromakalim, on the responsiveness of guinea-pig isolated trachea to a range of bronchodilators and the results were compared with those obtained with epithelium-denuded trachea.

Methods

Tracheae were removed from male Hartley guinea-pigs (250–400 g) stunned by a blow to the head. Each trachea

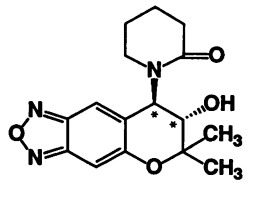

Figure 1 Chemical structure of NIP-121.

was cut spirally and divided into two or three segments (one was the control). In some experiments, the epithelium was removed mechanically by gently rubbing the luminal surface with a cotton-tipped applicator. Individual tissues were suspended under an applied load of 1 g in a 10 ml organ bath containing 8 ml of modified Tyrode solution at 37°C and gassed continuously with 95% O_2 + 5% CO_2. The composition of the modified Tyrode solution was (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHPO_4$ 0.3, $NaHCO_3$ 20 and dextrose 11. The contractility was measured isotonically (Type TD-112 S, Nihon Kohden). After the tissues had equilibrated for 50–60 min, maximal response to histamine (100 μM) was obtained. Subsequent contractile responses were expressed as percentages of the maximal response to histamine. The tissues were washed several times for 30 min to re-establish baseline tension and were then incubated for an additional 30 min with a potassium channel opener (either NIP-121, 0.1 μM or cromakalim, 1 μM), its blocker (glibenclamide 1 μM) or with the combination of either NIP-121 or cromakalim and glibenclamide in the presence of 5 μM indomethacin. Leukotriene D_4 (LTD_4) at a concentration of 30 nM was added to the organ bath. Contractile responses induced by LTD_4 were not attenuated by either NIP-121 or cromakalim (at the above concentrations) in either epithelium-intact or epithelium-denuded tracheas. After the contractile response elicited by LTD_4 had reached a plateau, one concentration-relaxation curve was obtained for a relaxant in each tissue. The relaxant reponse was expressed as a percentage of the maximum relaxation obtained with 1 mM aminophylline added to the organ bath at the end of the experiment. The negative logarithms of EC_{50} values (concentration producing 50% of the maximal relaxant response) were calculated by linear regression analysis applied to the linear portion of each concentration-response curve. All results were expressed as means ± s.e.mean, and statistical significance ($P<0.05$) was determined by Student's one-tailed t test for paired observations.

Drugs

The following agents were used: LTD_4 (Ultrafine Chemicals, England); vasoactive intestinal peptide (VIP), (−)-isoprenaline hydrochloride, aminophylline, indomethacin, 8-bromoguanosine 3':5'-cyclic monophosphate (8-Br-cyclic GMP), glibenclamide, pyrilamine maleate, nordihydroguaiaretic acid (NDGA) (Sigma Chemical Co., USA); histamine dihydrochloride, SNP (Wako Pure Chemical Industries, Japan); atropine sulphate (E. Merck, Germany); prostaglandin E_2 (PGE_2) (Nacalai Tesque, Japan); phosphoramidon (Peptide Institute, Japan); NIP-121 and cromakalim were synthesized by the Nissan Chemical Industries Ltd. (Central Research Laboratory, Japan).

Indomethacin was dissolved in 100% ethanol, glibenclamide, NDGA, PGE_2, NIP-121 and cromakalim were dissolved in 100% dimethylsulphoxide. Other drugs were dissolved in distilled water. Drug vehicles did not cause any change in the tracheal response.

Results

Effect of NIP-121 and cromakalim on agonist-induced relaxation of the guinea-pig isolated trachea contracted with 30 nM leukotriene D_4

We previously showed that in guinea-pig isolated trachea the relaxant effect of a bronchodilator was greater when the magnitude of LTD_4-induced contraction was smaller (Shikada *et al.*, 1991a). For this reason, concentrations of NIP-121 and cromakalim that did not affect the LTD_4-induced contraction were used in the present study. At these concentrations NIP-121, 0.1 μM and cromakalim, 1 μM significantly enhanced SNP-induced relaxation as compared with paired controls (Figure 2, a,b) seen as a leftward shift in the respective concentration-response curves for SNP. Neither NIP-121 nor cromakalim affected either the isoprenaline-induced relaxation (Figure 2, c,d) or that induced by VIP or PGE_2 (Table 1). SNP, isoprenaline and VIP caused complete relaxation while PGE_2 produced partial relaxation (76 ± 4%). Neither NIP-121 nor cromakalim caused a significant change in the maximal relaxation induced by PGE_2 (NIP-121, 73 ± 8%; cromakalim, 77 ± 6%, $P>0.05$). Higher concentrations of NIP-121 (>0.1 μM) and cromakalim (>1 μM) were not used in the present study because they strongly inhibited the 30 nM LTD_4-induced contraction (data not shown).

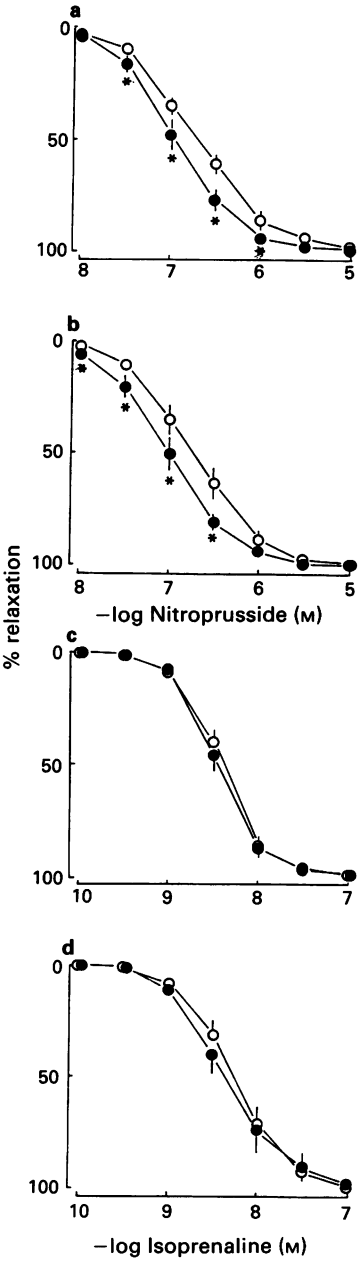

Figure 2 Effect of NIP-121, 0.1 μM (●; a,c) and cromakalim, 1 μM (●; b,d) on dose-response curve to sodium nitroprusside (a,b) or isoprenaline (c,d) in the guinea-pig isolated trachea contracted with leukotriene D_4 (30 nM). Each point represents the mean of 5–7 preparations in paired control tissues and in test tissues; (± s.e.mean) shown by vertical lines. *Indicates a significant difference from the corresponding values in the paired control (○).

Table 1 Effect of NIP-121 (0.1 μM) and cromakalim (1 μM) on agonist- induced relaxation of the isolated guinea-pig trachea contracted with leukotriene D_4 (LTD_4, 30 nM)

	NIP-121 (0.1 μM)		*Cromakalim* (1 μM)	
Condition	*% contraction*	*−log EC_{50}*	*% contraction*	*−log EC_{50}*
Sodium nitroprusside				
Control	74 ± 3	6.72 ± 0.08	69 ± 4	6.74 ± 0.10
Test	74 ± 3	6.96 ± 0.08*	64 ± 3	7.02 ± 0.10*
Isoprenaline				
Control	75 ± 3	8.42 ± 0.06	71 ± 4	8.28 ± 0.11
Test	78 ± 1	8.46 ± 0.08	72 ± 3	8.35 ± 0.14
VIP				
Control	75 ± 4	8.25 ± 0.02	75 ± 3	8.44 ± 0.05
Test	78 ± 2	8.24 ± 0.01	70 ± 6	8.48 ± 0.05
PGE_2				
Control	80 ± 3	6.05 ± 0.06	86 ± 4	6.23 ± 0.12
Test	78 ± 3	6.09 ± 0.12	80 ± 7	6.18 ± 0.17

Data are the means ± s.e.mean of 4–7 preparations in paired control tissues and in test tissues. % contraction refers to the % of that induced by histamine. VIP: vasoactive intestinal peptide; PGE_2: prostaglandin E_2. *Indicates a significant difference from the corresponding values in the paired control.

Table 2 shows that the ATP-sensitive potassium channel blocker, glibenclamide (1 μM), had little effect on SNP-induced relaxation but it prevented the enhancement of SNP relaxation induced by NIP-121 or cromakalim. This result suggests that the enhancement of SNP-induced relaxation by NIP-121 and cromakalim is dependent upon the activation of ATP-sensitive potassium channels.

SNP is known to be a guanylate cyclase stimulator and to increase intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels. 8-Br-cyclic GMP, which is an analogue of cyclic GMP, produced concentration-dependent and maximum relaxation of the LTD_4 contraction. Neither NIP-121 nor cromakalim caused any significant change in 8-Br-cyclic GMP-induced relaxation (data not shown).

Effect of NIP-121 and cromakalim on sodium nitroprusside-induced relaxation in epithelium-denuded trachea

When the tracheal lumen was gently rubbed with cotton, the LTD_4 concentration-response curve was significantly shifted to the left as compared with the paired control (−log EC_{50}; rubbed trachea 8.49 ± 0.09; control 8.26 ± 0.10, $P<0.05$) without affecting the maximum response at a concentration of 30 nM LTD_4. The relaxation induced by SNP in rubbed trachea elicited by 30 nM LTD_4 was significantly greater than that in the paired control, but that induced by 8-Br-cyclic GMP was not changed (Table 3). Neither NIP-121 nor cromakalim caused a significant leftward shift of the concentration relaxation curve in response to SNP in the rubbed trachea (Figure 3). The maximal relaxation was unchanged by these compounds (data not shown).

Effect of various inhibitors on sodium nitroprusside-induced relaxation of intact tracheal preparation

Farmer *et al.*, (1986) suggested that SNP may cause the tracheal epithelium to release a smooth muscle excitatory factor. It could therefore be hypothesized that NIP-121 and cromakalim might either modulate the release of, or antagonize the action of, such a factor. In an attempt to verify this supposition we examined whether SNP-induced relaxation was influenced by either selective antagonists or inhibitors of putative candidates for such a factor, namely acetylcholine, substance P, histamine and peptide leukotrienes. SNP-induced relaxation of the intact trachea contracted with 30 nM LTD_4 was not influenced by the anticholinoceptor agent, atropine at 1 μM, the neutral endopeptidase inhibitor, phosphoramidon at 10 μM, the antihistamine,

Table 2 Effect of NIP-121 (0.1 μM) and cromakalim (1 μM) in the presence of glibenclamide (GBC, 1 μM) on sodium nitroprusside-induced relaxation of the guinea-pig isolated trachea contracted with leukotriene D_4 (30 nM)

Condition	*% contraction*	*−log EC_{50}*
Control	75 ± 3	6.87 ± 0.08
GBC	78 ± 3	6.80 ± 0.05
Control	78 ± 4	6.72 ± 0.11
NIP-121 + GBC	80 ± 3	6.76 ± 0.08
Control	77 ± 3	6.63 ± 0.08
Cromakalim + GBC	80 ± 3	6.55 ± 0.08

Data are the means ± s.e.mean of 5 preparations in paired control tissues and in test tissues. % contraction refers to the % of that induced by histamine.

Table 3 Effect of epithelium removal on sodium nitroprusside- and 8-Br-cyclic GMP-induced relaxation of the guinea-pig isolated trachea contracted with leukotriene D_4 (30 nM)

Condition	*% contraction*	*−log EC_{50}*
Sodium nitroprusside		
Control	81 ± 3	6.73 ± 0.09
Rubbed	82 ± 4	7.01 ± 0.08*
8-Br-cyclic GMP		
Control	81 ± 4	4.96 ± 0.03
Rubbed	83 ± 3	4.92 ± 0.06

Data are the means ± s.e.mean of 5 preparations in paired control tissues and in tested tissues. % contraction refers to the % of that induced by histamine.
*Indicates a significant difference from the corresponding values in the paired control.

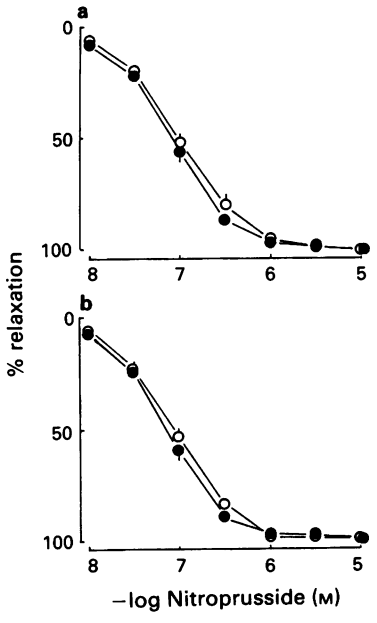

Figure 3 Lack of effect of NIP-121, 0.1 μM (●, a) and cromakalim, 1 μM (●, b) on dose-response curve to sodium nitroprusside in epithelium-rubbed guinea-pig trachea contracted with leukotriene D_4 (30 nM). Each point represents the mean of 5 preparations in paired control tissues (○) and in test tissues (●); (± s.e.mean) shown by vertical lines.

pyrilamine at 1 μM, or the lipoxygenase inhibitor, NDGA at 10 μM (Table 4).

Discussion

NIP-121 at 0.1 μM and cromakalim at 1 μM did not inhibit the LTD_4 (30 nM)-induced contraction but significantly enhanced the SNP-induced relaxation to the same extent in the guinea-pig isolated trachea. We previously showed that the relaxant effect of a bronchodilator in the guinea-pig isolated trachea increased as the magnitude of the contractile response decreased (Shikada *et al.*, 1991a). Because higher concentrations of NIP-121 (>0.1 μM) and cromakalim (>1 μM) markedly inhibited LTD_4 (30 nM)-induced contraction, we used 0.1 μM NIP-121 and 1 μM cromakalim, which did not affect the LTD_4-induced contraction, for investigating the effect of these potassium channel openers on agonist-induced relaxation. Since these concentrations of NIP-121 and cromakalim caused a marked and similar reduction in the spontaneous tone in the guinea-pig isolated trachea (Shikada *et al.*, 1991b), it seems reasonable to assume that they are potassium channel opening concentrations, although sub-maximal in this respect. The enhancement of SNP-induced relaxation by NIP-121 or cromakalim was abolished when the ATP-sensitive potassium channel blocker, glibenclamide, was present with NIP-121 or cromakalim. These results suggest that both NIP-121 and cromakalim enhance SNP-induced relaxation by stimulating ATP-sensitive potassium channels.

Table 4 Effect of modulators affecting bronchoactive substances on sodium nitroprusside-induced relaxation of the guinea-pig trachea contracted with leukotriene D_4 (30 nM)

Condition	*% contraction*	*−log EC_{50}*
Control	77 ± 3	6.73 ± 0.14
Atropine, 1 μM	77 ± 6	6.66 ± 0.19
Phosphoramidon, 10 μM	68 ± 3	6.64 ± 0.10
Control	81 ± 5	6.67 ± 0.05
Pyrilamine, 1 μM	85 ± 5	6.57 ± 0.05
NDGA, 10 μM	88 ± 8	6.56 ± 0.13

Data are the means ± s.e.mean of 4–5 preparations in paired control tissues and in test tissues. % contraction refers to the % of that induced by histamine.

NIP-121 and cromakalim enhanced SNP-induced relaxation but did not cause a significant change in the relaxation induced by the adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent bronchodilators isoprenaline, VIP and PGE_2. Since SNP has been used as a guanylate cyclase stimulator and to increase intracellular cyclic GMP levels, a possible interpretation is that NIP-121 and cromakalim selectively enhance cyclic GMP-dependent relaxation in the guinea-pig trachea. However, neither NIP-121 nor cromakalim enhanced the relaxation induced by 8-Br-cyclic GMP, a cyclic GMP analogue. This result suggests that both NIP-121 and cromakalim enhance SNP-induced relaxation without affecting cyclic GMP-induced relaxation of the tracheal smooth muscle.

In epithelium-rubbed trachea, NIP-121 and cromakalim failed to enhance SNP-induced relaxation. These results suggest that NIP-121 and cromakalim enhance SNP-induced relaxation in the tracheal smooth muscle by stimulating ATP-sensitive potassium channels present in the epithelium. Farmer *et al.*, (1986) stated that nitroprusside may cause the epithelium to release a smooth muscle excitatory factor. In this study the dose-relaxation curve in response to SNP in epithelium-rubbed trachea was significantly shifted to the left, but that in response to 8-Br-cyclic GMP was not changed. If putative smooth muscle excitatory factors are released from the epithelium during SNP-induced relaxation, an alternative interpretation is that NIP-121 and cromakalim counteract the release of these factors from the epithelium or inhibit the action of these factors on smooth muscle. However, the anti-cholinoceptor agent, atropine, the anti-histamine, pyrilamine, the 5-lipoxygenase-inhibitor, NDGA and the neutral endopeptidase inhibitor, phosphoramidon had no effect on SNP-induced relaxation of epithelium-intact trachea. These results suggest that acetylcholine, histamine, peptide-leukotrienes and substance P are not involved in SNP-induced relaxation. We previously found that NIP-121 and cromakalin strongly relax prostanoid-induced contraction of the isolated trachea (Shikada *et al.*, 1991b). It is extremely unlikely that prostaglandins are released and modulate SNP-induced relaxation, because our experiments were carried out in the presence of the cyclo-oxygenase inhibitor, indomethacin.

Another possible interpretation is that both NIP-121 and cromakalim act as selective inhibitors of cyclic GMP-dependent phosphodiesterase and cause a synergistic effect on SNP-induced relaxation. Since NIP-121 and cromakalim failed to enhance SNP-induced relaxation in the epithelium-rubbed trachea, neither compound may be able to act as an inhibitor of cyclic GMP-dependent phosphodiesterase in the tracheal smooth muscle. However, it may still be possible for NIP-121 and cromakalim to act as inhibitors of cyclic GMP-dependent phosphodiesterase in the guinea-pig tracheal epithelium.

In conclusion, the present results suggest that in the presence of an intact epithelium, the enhancement by NIP-121 and cromakalim of the relaxation of the guinea-pig trachea induced by SNP may be associated with the opening of glibenclamide-sensitive potassium channels. The present findings indicate that potassium channel openers may regulate the airway responsiveness via an indirect, epithelium-dependent pathway, in addition to providing direct bronchodilatation. The reason for the inability of either NIP-121 or cromakalim to enhance the relaxant effects of the other bronchodilators used in this study is unclear. This finding may be related to these bronchodilators possibly

already opening potassium channels to the extent that the sub-maximal potassium channel-opening concentrations of NIP-121 and cromakalim used had no additional relaxant effect.

References

ADVENIER, C., DEVILLIER, P., MATRAN, R. & NALINE, E. (1988). Influence of epithelium on the responsiveness of guinea-pig isolated trachea to adenosine. *Br. J. Pharmacol.*, **93**, 295–302.

ALLEN, S.L., BOYLE, J.P., CORTIJO, J., FOSTER, R.W., MORGAN, G.P. & SMALL, R.C. (1986). Electrical and mechanical effects of BRL34915 in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **89**, 395–405.

ARCH, J.R.S., BUCKLE, D.R., BUMSTEAD, J., CLARKE, G.D., TAYLOR, J.F. & TAYLOR, S.G. (1988). Evaluation of the potassium channel activator cromakalim (BRL34915) as a bronchodilator in the guinea pig: comparison with nifedipine. *Br. J. Pharmacol.*, **95**, 763–770.

BLACK, J.L., ARMOUR, C.L., JOHNSON, P.R.A., ALOUAN, L.A. & BARNES, P.J. (1990). The action of a potassium channel activator, BRL38227 (Lemakalim), on human airway smooth muscle. *Am. Rev. Respir. Dis.*, **142**, 1384–1389.

BRAUNSTEIN, G., LABAT, C., BRUNELLESCHI, S., BENVENISTE, J., MARSAC, J. & BRINK, C. (1988). Evidence that the histamine sensitivity and responsiveness of guinea-pig isolated trachea are modulated by epithelial prostaglandin E_2 production. *Br. J. Pharmacol.*, **95**, 300–308.

COX, R.H. (1990). Effects of putative K^+ channel activator BRL-34915 on arterial contraction and ^{86}Rb efflux. *J. Pharmacol. Exp. Ther.*, **252**, 51–59.

DEVILLIER, P., ADVENIER, C., DRAPEAU, G., MARSAC, J. & REGOLI, D. (1988). Comparison of the effects of epithelium removal and of an enkephalinase inhibitor on the neurokinin-induced contractions of guinea-pig isolated trachea. *Br. J. Pharmacol.*, **94**, 675–684.

FARMER, S.G., FEDAN, J.S., HAY, D.W.P. & RAEBURN, D. (1986). The effects of epithelium removal on the sensitivity of guinea-pig isolated trachealis to bronchodilator drugs. *Br. J. Pharmacol.*, **89**, 407–414.

GATER. P.R. (1989). Effects of K^+-channel openers on bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **98**, 660P.

HAY, D.W.P. (1990). Mechanism of endothelin-induced contraction in guinea-pig trachea: comparison with rat aorta. *Br. J. Pharmacol.*, **100**, 383–392.

HAY, D.W.P., FARMER, S.G., RAEBURN, D., MUCCITELLI, R.M., WILSON, K.A. & FEDAN, J.S. (1987). Differential effects of epithelium removal on the responsiveness of guinea-pig tracheal smooth muscle to bronchoconstrictors. *Br. J. Pharmacol.*, **92**, 381–388.

HOLROYDE, M.C. (1986). The influence of epithelium on the responsiveness of guinea-pig isolated trachea. *Br. J. Pharmacol.*, **87**, 501–507.

ICHINOSE, M. & BARNES, P.J. (1990). A potassium channel activator modulates both excitatory noncholinergic and cholinergic neurotransmission in guinea pig airways. *J. Pharmacol. Exp. Ther.*, **252**, 1207–1212.

LENNART LUNDBLAD, K.A. & PERSSON, C.G.A. (1988). The epithelium and the pharmacology of guinea-pig tracheal tone *in vitro*. *Br. J. Pharmacol.*, **93**, 909–917.

LONGMORE, J., BRAY, K.M. & WESTON, A.H. (1991). The contribution of Rb-permeable potassium channels to the relaxant and membrane hyperpolarizing actions of cromakalim, RP49356 and diazoxide in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **102**, 979–985.

MASUDA, Y., ARAKAWA, C., YAMASHITA, T., MIYAJIMA, M., SHIGENOBU, K., KASUYA, Y. & TANAKA, S. (1991). Potassium channel opening properties of a novel compound, NIP-121, cromakalim and nicorandil in rat aorta and portal vein. *Eur. J. Pharmacol.*, **195**, 323–331.

MCCAIG, D.J. & DE JONCKHEERE, B. (1989). Effect of cromakalim on bronchoconstriction evoked by cholinergic nerve stimulation in guinea-pig isolated trachea. *Br. J. Pharmacol.*, **98**, 662–668.

MURRAY, M.A., FOSTER, R.W. & SMALL, R.C. (1990). Effects of the K^+ channel openers cromakalim and RP 49356 on the cyclic nucleotide content of guinea-pig isolated trachealis muscle. *Br. J. Pharmacol.*, **100**, 367P.

PACIOREK, P.M., COWLRICK, I.S., PERKINS, R.S., TAYLOR, J.C., WILKINSON, G.F. & WATERFALL, J.F. (1990). Evaluation of the bronchodilator properties of Ro 31-6930, a novel potassium channel opener, in the guinea-pig. *Br. J. Pharmacol.*, **100**, 289–294.

RAEBURN, D. & BROWN, T.J. (1991). RP 49356 and cromakalim relax airway smooth muscle in vitro by opening a sulphonylurea-sensitive K^+ channel: a comparison with nifedipine. *J. Pharmacol. Exp. Ther.*, **256**, 480–485.

SHIKADA, K., YAMAMOTO, A. & TANAKA, S. (1991a). Effects of phosphodiesterase inhibitors on vasoactive intestinal peptide-induced relaxation of isolated guinea-pig trachea. *Eur. J. Pharmacol.*, **195**, 389–394.

SHIKADA, K., YAMAMOTO, A. & TANAKA, S. (1991b). NIP-121 and cromakalim, potassium channel openers, preferentially suppress protanoid-induced contraction of the guinea-pig isolated trachea. *Eur. J. Pharmacol.*, **209**, 69–73.

TAYLOR, S.G., BUMSTEAD, J., MORRIS, J.E.J., SHAW, D.J. & TAYLOR, J.F. (1988). Cromakalim inhibits cholinergic-mediated responses in human isolated bronchioles but not in guinea-pig airways. *Br. J. Pharmacol.*, **92**, 795P.

(Received May 18, 1992
Revised July 20, 1992
Accepted August 11, 1992)

Br. J. Pharmacol. (1992), **107**, 1121–1128

Attenuation of vasoconstriction by endogenous nitric oxide in rat caudal artery

[1]Phuong A. Vo, Julianne J. Reid & Michael J. Rand

Department of Pharmacology, University of Melbourne, Victoria, Australia

1 The effects of N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl ester (L-NAME), haemoglobin and methylene blue have been examined on vascular reactivity in the rat isolated caudal artery. The effects of L-NNA and sodium nitroprusside were also investigated on the stimulation-induced (S-I) efflux of noradrenaline in the rat caudal artery.

2 L-NNA (10 μM) and L-NAME (10 μM) significantly attenuated the vasodilator responses to acetylcholine (1 nM–1 μM), but had no effect on vasodilator responses to papaverine (1–100 μM).

3 Vasoconstrictor responses to sympathetic nerve stimulation (3 Hz, 10 s), noradrenaline (0.01–1 μM), methoxamine (1–10 μM), 5-hydroxytryptamine (0.01–0.3 μM), phenylephrine (0.1–10 μM), endothelin-1 (10 nM) and KCl (40 mM) were significantly enhanced by 10 μM L-NNA. L-NAME (10 μM) caused a significant enhancement of vasoconstrictor responses to noradrenaline and sympathetic nerve stimulation in endothelium-intact, but not in endothelium-denuded tissues.

4 Haemoglobin and methylene blue (both 10 μM) enhanced the vasoconstrictor responses to sympathetic nerve stimulation and noradrenaline. The enhancements were absent in endothelium-denuded arterial segments.

5 In endothelium-denuded arterial segments precontracted with phenylephrine, the vasodilator responses to the nitric oxide donor, sodium nitroprusside (0.1–300 nM), were decreased by increasing the level of precontraction.

6 L-NNA (10 μM) had no effect on the S-I efflux of radioactivity from arteries in which transmitter stores had been labelled with [^{3}H]-noradrenaline.

7 These results suggest that endothelial nitric oxide attenuates vasoconstrictor responses in the rat caudal artery through activation of soluble guanylate cyclase to decrease smooth muscle contractility. Therefore, the findings provide evidence that nitric oxide acts as a functional antagonist to oppose vasoconstriction.

Keywords: Haemoglobin; methylene blue; nitric oxide; N^G-nitro-L-arginine; N^G-nitro-L-arginine methyl ester; rat caudal artery; sodium nitroprusside; sympathetic nerve stimulation; vasoconstriction; vasodilatation

Introduction

The vascular endothelium synthesizes and releases endothelium-derived relaxing factor (EDRF) (Furchgott, 1984; Moncada *et al.*, 1991a). EDRF mediates the vasodilator effect of acetycholine and other endothelium-dependent vasodilators on blood vessels (Furchgott, 1984) through the activation of soluble guanylate cyclase in the smooth muscle (Waldman & Murad, 1987). Nitric oxide has been reported to account for the biological activity of EDRF (Palmer *et al.*, 1987) and is formed from the conversion of L-arginine to L-citrulline (Palmer *et al.*, 1988). This synthetic pathway is catalysed by the enzyme, nitric oxide synthase, which can be inhibited by the L-arginine analogues, N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA) and N^G-nitro-L-arginine methyl ester (L-NAME) (Sakuma *et al.*, 1988; Moore *et al.*, 1990; Rees *et al.*, 1990).

Intravenous administration of L-NMMA or L-NAME induces an increase in mean arterial blood pressure and inhibits the hypotension induced by acetylcholine and bradykinin in the anaesthetized rat (Rees *et al.*, 1989; 1990). Similar observations have been reported in man, where infusion of L-NMMA into the brachial artery reduces blood flow in the forearm (Vallance *et al.*, 1989). These observations suggest that nitric oxide is important in the regulation of blood flow and pressure.

It has also been suggested that nitric oxide has a functional role in the modulation of smooth muscle reactivity. Inhibition of nitric oxide synthesis with L-arginine analogues enhances contractile responses in a number of preparations, including the rat anococcygeus (Li & Rand, 1989), the dog mesenteric artery (Toda & Okamura, 1990), and the rat aorta and pulmonary artery (Crawley *et al.*, 1990; Moncada *et al.*, 1991b; Topouzis *et al.*, 1991). We have recently shown that inhibition of nitric oxide synthesis with L-NNA enhances vasoconstrictor responses to sympathetic nerve stimulation and noradrenaline in the rat caudal artery (Reid *et al.*, 1991; Vo *et al.*, 1991). The enhancement was prevented by L-arginine and was endothelium-dependent.

In the present study, we have extended these observations in the rat caudal artery in order to investigate further the influence of nitric oxide on vascular reactivity. The aims of the study were 3 fold. Firstly, since the specificity of L-arginine analogues as inhibitors of nitric oxide synthesis has been questioned by some investigators (e.g., Thomas *et al.*, 1989; Cocks & Angus, 1991), the effect of a range of inhibitors of nitric oxide-mediated responses has been investigated on vasoconstrictor responses to noradrenaline and sympathetic nerve stimulation, namely: L-NAME, another inhibitor of nitric oxide synthesis; haemoglobin, which inactivates nitric oxide; and methylene blue, an inhibitor of soluble guanylate cyclase, the biological 'receptor' for nitric oxide. Secondly, the effect of L-NNA on vasoconstrictor responses to agonists other than noradrenaline has been examined to determine whether or not nitric oxide-mediated attenuation of vasoconstriction is specific to noradrenaline.

[1] Author for correspondence.

Thirdly, the effect of L-NNA and the nitric oxide donor sodium nitroprusside has been investigated on the stimulation-induced (S-I) release of noradrenaline from the rat caudal artery, to determine whether nitric oxide-mediated modulation of vasoconstrictor responses to sympathetic nerve stimulation involves prejunctional mechanisms.

Methods

Sprague-Dawley rats (250–350 g) of either sex were pretreated with heparin (1000 u kg^{-1}, i.p.) 30 min before they were killed by stunning with a blow to the head and exsanguination. Two segments of the central caudal artery (2 cm) were carefully dissected free from surrounding connective tissue. Each segment was cannulated at the proximal end with SP 31 polyethylene tubing; the distal end was ligated and an incision was made in the wall of the vessel below the tie. The segment was mounted vertically with the distal end uppermost, under 0.5 g tension. Physiological salt solution (PSS) was perfused through the lumen at 37°C with a Gilson Minipuls 3 peristaltic pump at a rate of 1.5 ml min^{-1} and the perfusate was allowed to superfuse the adventitial surface of the vessel. The PSS was continuously bubbled with 95% O_2/5% CO_2 and had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.14. The perfusion pressure was measured with a Cobe CDX-III pressure transducer connected to a Grass polygraph recorder. Increases in perfusion pressure were taken as indices of vasoconstriction. This method of perfusion/superfusion has been used extensively to assess vascular reactivity (e.g., Medgett & Langer, 1984; 1986; Xiao & Rand, 1989; Vo *et al.*, 1991).

Periarterial nerves were stimulated with 0.1 ms monophasic square pulses at supramaximal voltage (60 V) delivered from a Grass S88 stimulator through circular bipolar platinum electrodes placed around the proximal end of the artery. An equilibration period of 45 min was allowed before experimental observations were made.

Vasoconstrictor responses

Vasoconstrictor responses to sympathetic nerve stimulation, or to cumulative concentrations of noradrenaline, phenylephrine, methoxamine or 5-hydroxytryptamine, or to 40 mM KCl, were obtained before and 30 min after exposure to L-NNA or L-NAME, or 15 min after exposure to haemoglobin or methylene blue. The effects of L-NAME, haemoglobin and methylene blue on vasoconstrictor responses to sympathetic nerve stimulation and noradrenaline were also examined in endothelium-denuded preparations. In these experiments, the vascular endothelium was removed by perfusing arterial segments with a stream of 95% O_2/5% CO_2 at a pressure of 60–70 mmHg for 90 s and then re-perfusing with PSS (Spokas & Folco, 1984; Vo *et al.*, 1991). In time-control experiments, L-NNA, L-NAME, haemoglobin and methylene blue were absent from the perfusate. In experiments where endothelin-1 was used as the vasoconstrictor agent, control responses and responses in the presence of L-NNA were obtained from two different segments of artery taken from the same animal.

Vasodilator responses

In all experiments, the presence of functional endothelium was determined by the vasodilator action of acetylcholine (1 μM) in arteries precontracted with phenylephrine (3 μM); endothelium-intact arterial segments relaxed by at least 50% of the precontraction and endothelium-denuded segments failed to relax in response to acetylcholine. In separate experiments in which the endothelium was intact, cumulative concentration-response curves to acetylcholine and papaverine were obtained in arteries that were precontracted with phenylephrine (3 μM) before and 30 min after exposure to L-NNA or L-NAME. In endothelium-denuded arterial segments, vasodilator responses to sodium nitroprusside were obtained at different levels of precontraction with phenylephrine.

S–I release of noradrenaline

The S–I release of noradrenaline was investigated by the method of Rajanayagam *et al.* (1989). Briefly, segments of rat caudal artery (3 cm in length) were incubated in [^{3}H]-noradrenaline (0.23 μM, 10 μCi ml^{-1} for 30 min) to label transmitter stores. After washing for 90 min, the arteries were given two periods of stimulation ($Stim_1$ and $Stim_2$, 40 min apart) with 0.1 ms monophasic square pulses at supramaximal voltage (60 V) and a frequency of 5–20 Hz for 10 or 60 s. L-NNA or sodium nitroprusside was introduced 30 or 10 min before $Stim_2$, respectively. Idazoxan was added to the perfusate 20 min before either $Stim_1$ or $Stim_2$, as indicated. The vascular endothelium was removed in experiments in which sodium nitroprusside was used.

The perfusate was collected in 2-min samples and analysed for radioactivity by liquid scintillation counting. Counting efficiency, determined by automatic external standardization, was approximately 25%, and all measurements were recorded as disintegrations per min (d.p.m.). The resting efflux of radioactivity was taken as the mean of the radioactive content in the three 2-min samples taken immediately before each stimulation period. The S–I efflux of radioactivity was calculated as the difference between five times the resting efflux and the sum of the radioactive content in the five 2-min samples from the start of stimulation, and was expressed as a fraction (FR) of the radioactivity in the tissue at the commencement of stimulation. The S–I efflux of radioactivity was used as an index of the S–I release of transmitter noradrenaline. The fractional S–I efflux evoked by $Stim_2$ is expressed as a percentage of that evoked by $Stim_1$ (FR_2/FR_1).

Drugs and solutions

The following drugs were used: acetylcholine perchlorate (Sigma), haemoglobin (Sigma), idazoxan hydrochloride (Reckitt & Colman), indomethacin (Merck Sharp & Dohme), methoxamine hydrochloride (Burroughs Wellcome), methylene blue (Sigma), N^G-nitro-L-arginine (L-NNA, Sigma), N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), noradrenaline bitartrate (Sigma), (−)-[ring-2,5,6-^{3}H]-noradrenaline (specific activity 43.7 Ci $mmol^{-1}$; New England Nuclear), papaverine hydrochloride (Sigma), phenylephrine hydrochloride (Sigma), prazosin hydrochloride (Sigma), 5-hydroxytryptamine creatinine sulphate (Sigma), sodium nitroprusside (Sigma) and tetrodotoxin (Sigma).

Drugs were dissolved in distilled water to give 10 mM stock solutions, except indomethacin which was dissolved in 0.1 M Na_2CO_3 and prazosin which was dissolved in 2% glycerol and 30% dextrose.

Depolarization-induced contractions to KCl were elicited by replacing normal PSS with a solution in which the equivalent amount of NaCl was replaced by 40 mM KCl.

Statistical analyses

Results are expressed as mean and s.e.mean. Differences between means were assessed by Student's *t* test (one-tailed), or by one- or by two-way analysis of variance (ANOVA), followed by planned comparisons, as indicated. Analyses were carried out with the software package CSS (Statsoft). Values of $P<0.05$ were taken to indicate statistical significance.

Results

Effect of L-NNA and L-NAME on vasoconstrictor responses

Stimulation of sympathetic nerves (3 Hz, 10 s) produced a submaximal increase in perfusion pressure of 41.3 ± 4.5 mmHg ($n = 24$) in the rat caudal artery; the response was consistent throughout the experiment (Table 1) and was abolished by 0.3 μM tetrodotoxin or 10 nM prazosin (data not shown). Exposure of the rat caudal artery to 10 μM L-NNA or 10 μM L-NAME for 30 min had no effect on the resting perfusion pressure of 8.2 ± 0.5 mmHg ($n = 18$), but significantly enhanced the vasoconstrictor response to sympathetic nerve stimulation (Table 1, $P<0.05$, unpaired Student's t test). The enhancement caused by 10 μM L-NAME was absent in endothelium-denuded segments (Table 1). The α_2-adrenoceptor antagonist, idazoxan (0.1 μM) did not affect the enhancing effect of L-NNA on vasoconstrictor responses to sympathetic nerve stimulation ($n = 4$, data not shown).

In time-control experiments, increases in perfusion pressure produced by cumulative concentrations of noradrenaline ($n = 5$), phenylephrine ($n = 4$), methoxamine ($n = 5$) and 5-hydroxytryptamine ($n = 5$) did not alter during the course of the experiment (data not shown). However, 10 μM L-NNA significantly enhanced the vasoconstrictor responses produced by noradrenaline, methoxamine, phenylephrine and 5-hydroxytryptamine (Figures 1 and 2, $P<0.05$, two-way ANOVA). L-NAME (10 μM) caused a significant enhancement of the vasoconstrictor responses to noradrenaline (Figure 3, $P<0.05$, two-way ANOVA) and the enhancement was absent in endothelium-denuded arterial segments (Figure 3).

Endothelin-1 (10 nM) caused slowly developing increases in perfusion pressure in the rat caudal artery. The perfusion pressure returned to baseline 60 min after the removal of endothelin-1, however, subsequent exposure to 10 nM endothelin-1 did not elicit a further response. Therefore, the effect of endothelin-1 in the absence and presence of L-NNA was determined in two different arterial segments taken from the same animal. Pretreatment of arterial segments with 10 μM L-NNA for 30 min produced significantly greater responses ($P<0.05$, unpaired Student's t test) to 10 nM endothelin-1 (21.4 ± 3.2 mmHg, $n = 7$) than in the absence of L-NNA (12.7 ± 2.1 mmHg, $n = 6$). The enhancing effect of L-NNA on responses to endothelin-1 might be underestimated because of the intrinsic property of endothelin-1 of inducing the release of nitric oxide (Warner *et al.*, 1989).

Table 1 The vasoconstrictor responses to nerve stimulation (3 Hz, 10 s) after a 30-min exposure to 10 μM N^G-nitro-L-arginine (L-NNA) or 10 μM N^G-nitro-L-arginine methyl ester (L-NAME), and after a 15-min exposure to 10 μM haemoglobin or 10 μM methylene blue in endothelium-intact or -denuded segments of rat caudal artery

	% of initial response[a]	
Treatment	*Endothelium-intact*	*Endothelium-denuded*
Time-control	107 ± 4% (4)	101 ± 5% (5)
L-NNA	203 ± 9% (6)*	Not done
L-NAME	149 ± 6% (6)*	100 ± 2% (4)
Haemoglobin	140 ± 5% (4)*	102 ± 5% (4)
Methylene blue	149 ± 4% (4)*	104 ± 2% (3)

[a]Results are expressed as a percentage of the initial response before the addition of inhibitors. Values are mean ± s.e.mean from the number of experiments indicated in parentheses.
*Significantly different from the time-control ($P<0.05$, unpaired Student's t test).

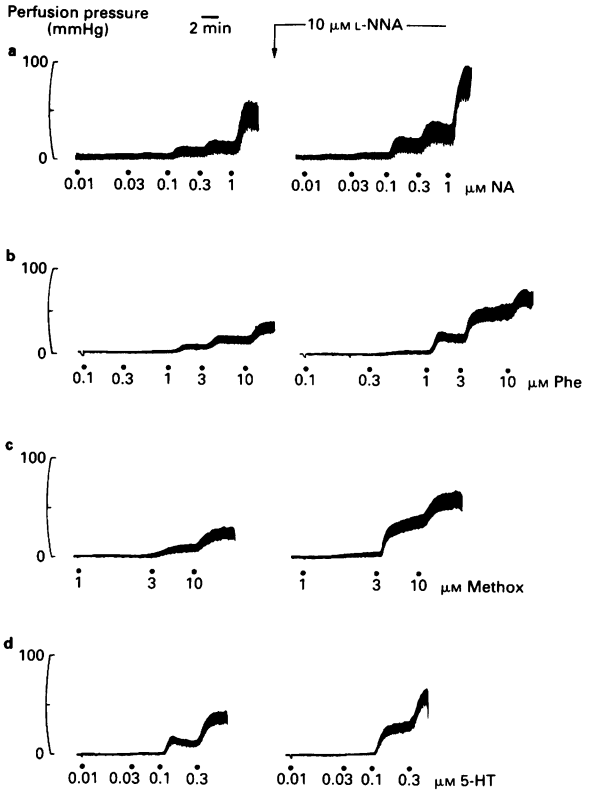

Figure 1 Recordings showing the enhancing effect of 10 μM N^G-nitro-L-arginine (L-NNA) on increases in perfusion pressure produced by cumulative concentrations of (a) noradrenaline (NA), (b) phenylephrine (Phe), (c) methoxamine (Methox) and (d) 5-hydroxytryptamine (5-HT) in endothelium-intact segments of rat caudal artery. L-NNA was added to the perfusate 30 min before the second set of responses was obtained.

The vasoconstrictor response to 40 mM KCl was consistent over a 60-min period (initial response: 79.2 ± 6.0 mmHg; response after 60 min: 84.8 ± 7.2 mmHg; $n = 5$), but was significantly enhanced after a 30-min exposure to 10 μM L-NNA from 74.7 ± 9.3 mmHg to 137 ± 12 mmHg ($P<0.05$, paired Student's t test, $n = 3$).

Effect of haemoglobin and methylene blue on vasoconstrictor responses

Haemoglobin and meythlene blue (both 10 μM) had no effect on the resting perfusion pressure, but caused a significant enhancement of vasoconstrictor responses to sympathetic nerve stimulation (Table 1, $P<0.05$, unpaired Student's t test) and to noradrenaline (Figure 4, $P<0.05$, two-way ANOVA) in the rat caudal artery. The enhancements caused by haemoglobin and methylene blue were absent in endothelium-denuded arterial segments (Table 1, Figure 4).

Effect of L-NNA and L-NAME on vasodilator responses

Acetylcholine and papaverine caused concentration-dependent relaxations of arterial segments precontracted with 3 μM phenylephrine (Figures 5 and 6). L-NNA (10 or 100 μM) and L-NAME (10 μM) had no effect on the resting perfusion pressure, but caused an increase in the sensitivity of the arterial segments to phenylephrine. Therefore, the concentration of phenylephrine was adjusted from 3 μM to 0.3–1 μM in order to achieve an increase in perfusion pressure equivalent to that obtained before the addition of L-NNA or L-NAME. L-NNA (10 or 100 μM) and L-NAME (10 μM) significantly attenuated the vasodilator responses to acetylcholine (Figure

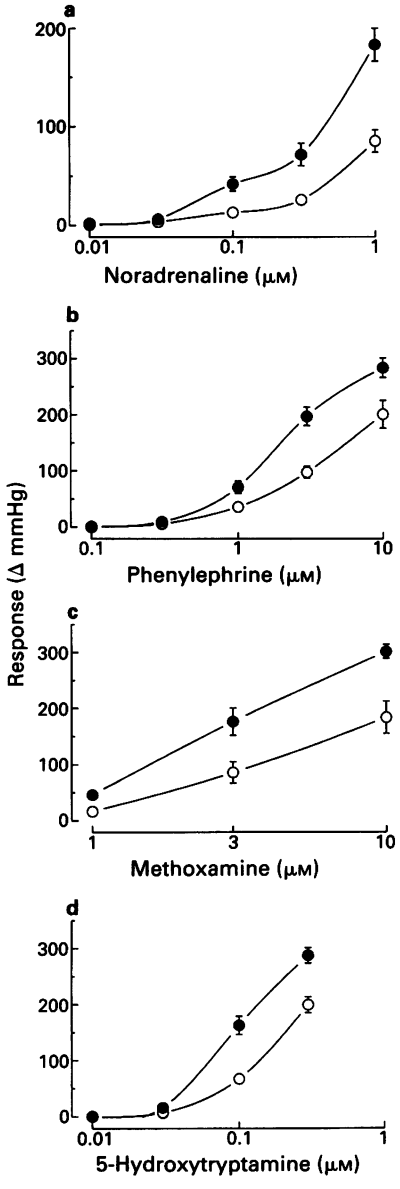

Figure 2 Effect of N^G-nitro-L-arginine (L-NNA) on vasoconstrictor responses to (a) noradrenaline, (b) phenylephrine, (c) methoxamine and (d) 5-hydroxytryptamine in endothelium-intact segments of rat caudal artery. Vasoconstrictor responses before (○) and 30 min after (●) exposure to 10 μM L-NNA are shown. Results are expressed as mean and s.e.mean (vertical bars) from four to six experiments.

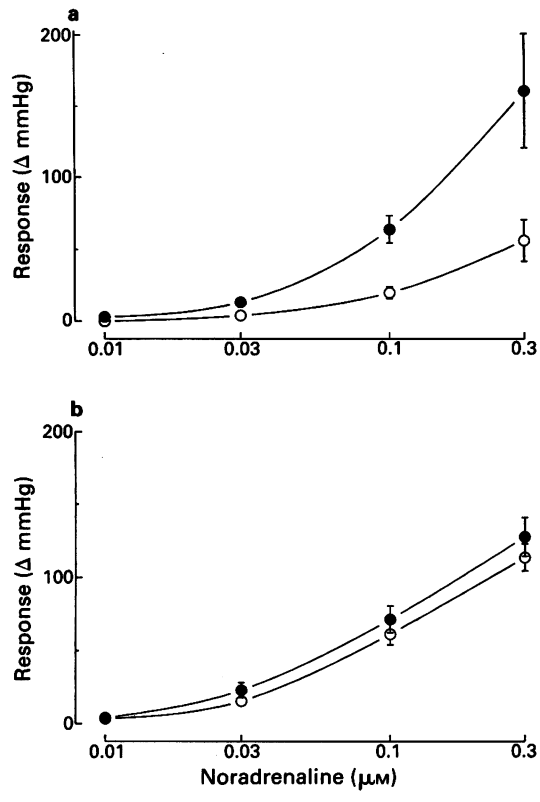

Figure 3 Vasoconstrictor responses to noradrenaline in (a) endothelium-intact and (b) endothelium-denuded segments of the rat caudal artery before (○) and 30 min after (●) exposure to 10 μM N^G-nitro-L-arginine methyl ester. Results are expressed as mean and s.e.mean (vertical bars) from four experiments.

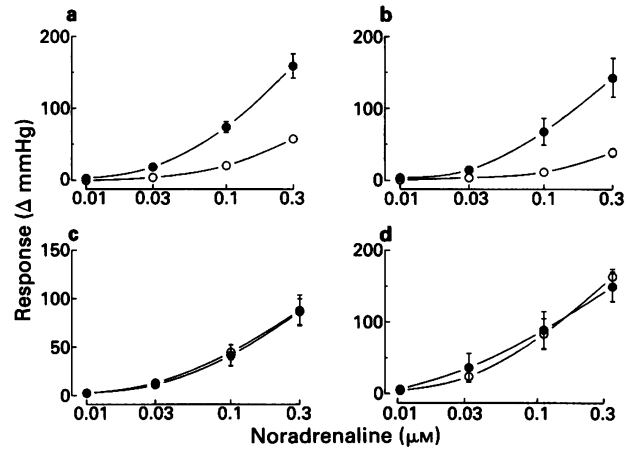

Figure 4 Vasoconstrictor responses to noradrenaline in (a,b) endothelium-intact and (c,d) endothelium-denuded segments of the rat caudal artery. Vasoconstrictor responses before (○) and after (●) 15 min exposure to (a,c) 10 μM methylene blue or (b,d) 10 μM haemoglobin. Results are expressed as mean and s.e.mean (vertical bars) from four to six experiments.

5, $P<0.05$, two-way ANOVA). In contrast, the vasodilator responses to papaverine were not significantly affected by 10 μM L-NNA or 10 μM L-NAME (Figure 6, $P>0.05$, two-way ANOVA). In time-control experiments, vasodilator responses to acetylcholine and papaverine did not change significantly over the time course of the experiment (Figures 5 and 6, $P>0.05$, two-way ANOVA).

Effect of level of precontraction on sensitivity to sodium nitroprusside

To test whether the sensitivity to nitric oxide was changed by the size of the vasoconstrictor response, vasodilator responses to the nitric oxide donor, sodium nitroprusside, were obtained in endothelium-denuded arteries which had been precontracted to different levels. Sodium nitroprusside caused concentration-dependent relaxations of arterial segments precontracted with phenylephrine (1 or 2 μM) by 51 ± 4 mmHg ($n = 5$, Figure 7). At a higher level of precontraction (96 ± 7 mmHg, $n = 5$) obtained with 3 or 10 μM phenylephrine, the relaxant responses to sodium nitroprusside were significantly attenuated (Figure 7, $P>0.05$, two-way ANOVA).

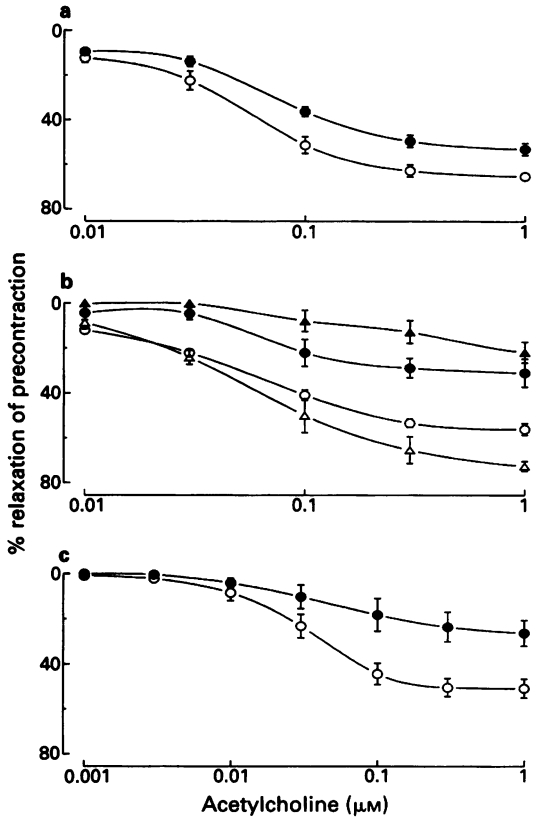

Figure 5 Effect of N^G-nitro-L-arginine (L-NNA) and N^G-nitro-L-arginine methyl ester (L-NAME) on vasodilator responses to acetylcholine in endothelium-intact arteries precontracted with phenylephrine (0.3–3 μM). (a) Time-control experiments in which responses were obtained 30 min apart without the addition of L-NNA or L-NAME (first curve: ○; second curve: ●). (b) Vasodilator responses before (open symbols) and 30 min after (closed symbols) exposure to 10 μM (circles) and 100 μM (triangles) L-NNA. (c) Vasodilator responses before (○) and 30 min after (●) exposure to 10 μM L-NAME. Results are expressed as mean and s.e.mean (vertical bars) from three to five experiments.

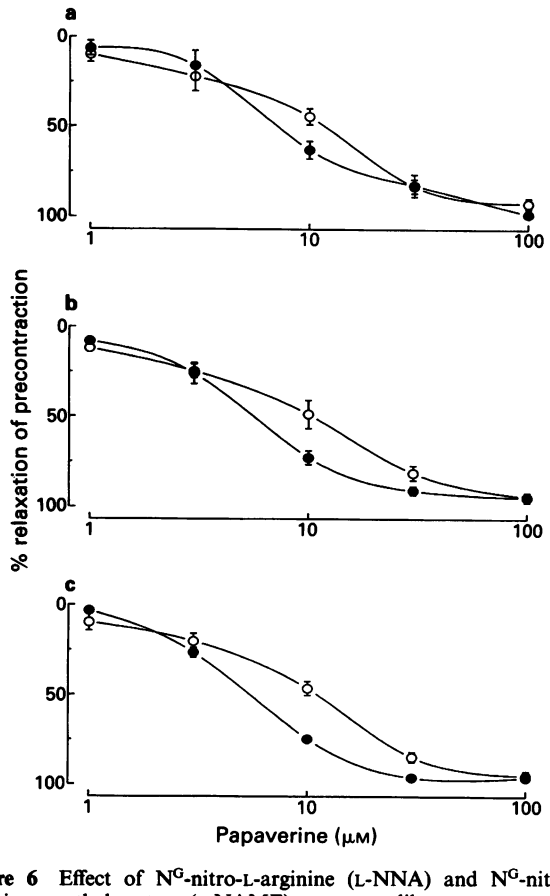

Figure 6 Effect of N^G-nitro-L-arginine (L-NNA) and N^G-nitro-L-arginine methyl ester (L-NAME) on vasodilator responses to papaverine in endothelium-intact arteries precontracted with phenylephrine (0.3–3 μM). (a) Time-control experiments in which responses were obtained 30 min apart without the addition of L-NNA or L-NAME (first curve: ○; second curve: ●). (b) Vasodilator responses before (○) and 30 min after (●) exposure to 10 μM L-NNA. (c) Vasodilator responses before (○) and 30 min after (●) exposure to 10 μM L-NAME. Results are expressed as mean and s.e.mean (vertical bars) from four experiments.

Effect of L-NNA and sodium nitroprusside on S–I efflux of noradrenaline

The resting radioactive efflux from arteries previously incubated in [^{3}H]-noradrenaline fell expontentially for the first 35 min after incubation and after 90 min reached a plateau of 4542 ± 459 d.p.m. ($n = 4$). Electrical stimulation (5 Hz, 60 s, $Stim_1$) caused an increase in the efflux of radioactivity of 6299 ± 303 d.p.m. ($n = 4$) above resting levels. L-NNA (10 and 100 μM) had no effect on the resting efflux of radioactivity. When 10 μM L-NNA was added to the perfusate 30 min before $Stim_2$, the S–I efflux of radioactivity (FR_2/FR_1) was 93 ± 2% ($n = 3$) and was not significantly different from control values (91 ± 2%, $n = 4$).

In order to check that an effect of L-NNA was not being masked by the α_2-adrenoceptor inhibitory feedback system, experiments were carried out in the presence of the α_2-adrenoceptor antagonist, idazoxan. Idazoxan (0.1 μM) introduced 20 min before $Stim_2$ (20 Hz, 10 s), enhanced FR_2/FR_1 from 99 ± 2% ($n = 3$) to 225 ± 13% ($n = 4$). When idazoxan (0.1 μM) was present throughout perfusion, the addition of either 10 or 100 μM L-NNA 30 min before $Stim_2$ caused small and inconsistent changes in the S–I radioactive efflux (Table 2). In addition, with idazoxan (0.1 μM) present throughout the experiment, the nitric oxide donor sodium nitroprusside slightly enhanced the S–I efflux of radioactivity from endothelium-denuded preparations (Table 2).

Discussion

The present study confirms and extends our earlier observations (Reid *et al.*, 1991; Vo *et al.*, 1991) that inhibition of nitric oxide synthesis (with either L-NNA or L-NAME) enhances the vasoconstrictor responses to sympathetic nerve stimulation and noradrenaline in the rat caudal artery. In addition, suppression of nitric oxide-mediated responses through inhibition of soluble guanylate cyclase with methylene blue, and binding of nitric oxide to the Fe-containing haem group of haemoglobin (Martin *et al.*, 1985; Ignarro, 1989) also caused an enhancement of the vasoconstrictor responses to sympathetic nerve stimulation and noradrenaline in this preparation. The enhancements were absent in endothelium-denuded arterial segments. Thus, the effects of the nitric oxide synthase inhibitors, L-NNA and L-NAME, appear to be specific, since similar effects on vasoconstrictor responses were observed when nitric oxide-mediated responses were inhibited by other mechanisms. Previous reports have shown that L-NMMA or L-NNA enhanced vasoconstrictor responses to nerve stimulation and noradrenaline in a number of different vascular preparations, including the rat mesenteric arterial bed (Way *et al.*, 1991) and renal vascular bed (Reid & Rand, 1992), the dog coronary artery (Berkenboom *et al.*, 1991; Toda & Okamura, 1990) and mesenteric artery (Toda & Okamura, 1990), the guinea-pig pulmonary artery (Liu *et al.*, 1991), and the

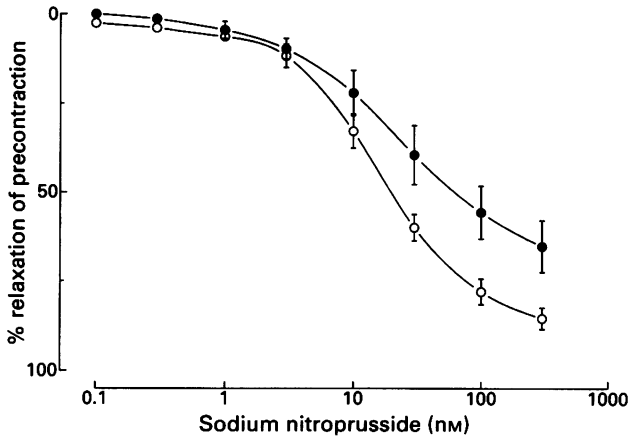

Figure 7 Vasodilator responses to sodium nitroprusside in endothelium-denuded arteries precontracted with 1 or 2 μM (○) and 3 or 10 μM (●) phenylephrine which produced an increase in perfusion pressure of 51 ± 4 mmHg ($n = 5$) and 96 ± 7 mmHg ($n = 5$), respectively. Results are expressed as mean and s.e.mean (vertical bars) from five experiments.

Table 2 The effect of N^G-nitro-L-arginine (L-NNA) and sodium nitroprusside on the stimulation-induced (S–I) efflux of radioactivity from endothelium-intact and endothelium-denuded segments of rat caudal artery, respectively. The arteries were subjected to two periods of stimulation ($Stim_1$ and $Stim_2$) 40 min apart.

	S–I efflux of radioactivity (FR_2/FR_1)[a]	
	10 Hz, 10 s	20 Hz, 10 s
Endothelium-intact arteries		
Control	99.3 ± 2.2% (4)	104.2 ± 5.9% (4)
L-NNA (μM)		
10	115.6 ± 7.2% (3)*	107.4 ± 5.9% (3)
100	95.3 ± 3.3% (4)	85.7 ± 3.4% (4)*
Endothelium-denuded arteries		
Control	88.0 ± 1.0% (4)	
Sodium nitroprusside (μM)		
0.1	101.6 ± 2.9% (4)*	
1	122.9 ± 3.9% (4)*	
10	113.0 ± 5.1% (3)*	

Idazoxan (0.1 μM) was present throughout the experiment and L-NNA or sodium nitroprusside was introduced 30 or 10 min before $Stim_2$ respectively.
[a]The S–I efflux of radioactivity evoked by $Stim_2$ is expressed as a percentage of that evoked by $Stim_1$ (FR_2/FR_1). Values are mean ± s.e.mean from the number of experiments indicated in parentheses. *Significant difference from the appropriate control ($P<0.05$, one-way ANOVA followed by planned comparisons).

human coronary artery (Berkenboom *et al.*, 1991). Methylene blue has also been shown to potentiate noradrenaline-mediated contractions in rat mesenteric and femoral arteries (Urabe *et al.*, 1991), and in dog and human coronary arteries (Berkenboom *et al.*, 1991). Furthermore, haemoglobin has been reported to cause endothelium-dependent potentiation of the contraction to phenylephrine in bovine pulmonary artery and vein (Gold *et al.*, 1990), in accord with the present study. Together these findings suggest that nitric oxide is released from endothelial cells to oppose vasoconstriction, and that the phenomenon is not restricted to either specific vascular beds or species. In addition, the results strengthen the proposal that endothelium-derived nitric oxide is a modulator of smooth muscle contractility.

The inhibitory effect of L-NNA and L-NAME on the vasodilator responses to acetylcholine observed in this study are in agreement with reports by Moore *et al.* (1990) and Rees *et al.* (1990). In contrast, vasodilator responses to the direct smooth muscle relaxant, papaverine, were not affected by either L-NNA or L-NAME. Therefore, the synthesis inhibitors suppressed endothelium-dependent, but not endothelium-independent vasodilatation. These results provide further evidence supporting the specific inhibition of nitric oxide synthesis by L-NNA and L-NAME in the rat caudal artery.

It is possible that the enhancing effect of L-NNA on vasoconstrictor responses to sympathetic nerve stimulation might involve the modulation by L-NNA of the release of noradrenaline from sympathetic nerve terminals. However, this explanation is unlikely because L-NNA (10 μM) had no effect on the S–I efflux of noradrenaline from the rat caudal artery. The absence of an effect of L-NNA on the release of noradrenaline could not be due to a lack of responsiveness of the preparation to prejunctional modulation; the S–I release of noradrenaline could be enhanced by idazoxan, which is consistent with prejunctional modulation by an α_2-adrenoceptor autoinhibitory feedback system in this tissue (Starke, 1987).

The S–I release of radioactivity from endothelium-denuded arterial segments was enhanced by the nitric oxide donor, sodium nitroprusside, but this effect was small and not concentration-dependent. These findings are in contrast to those of Greenberg *et al.* (1990), who reported that sodium nitroprusside decreased the S–I efflux of noradrenaline from segments of dog mesenteric artery, but only at low frequencies (4 Hz) of stimulation. The discrepancy may be partly related to differences between vascular preparations and/or to frequency of stimulation. In our study, frequencies below 5 Hz were not used, because the release of radioactivity was below the limit of detection.

The enhancement of vasoconstriction produced by inhibition of nitric oxide synthesis was not specific to noradrenaline-mediated responses; L-NNA was found to enhance the vasoconstrictor responses induced by methoxamine, 5-hydroxytryptamine, phenylephrine and endothelin-1, as well as depolarization with a high concentration of potassium. Nitric oxide attenuated both receptor- and nonreceptor-mediated vasoconstriction. Other investigators have also reported that nitric oxide synthase inhibitors enhance the contraction in isolated vascular preparations to substances other than noradrenaline, including phenylephrine (Crawley *et al.*, 1990; Gold *et al.*, 1990; Liu *et al.*, 1991), endothelin-1 (Ito *et al.*, 1991) and prostaglandin $F_{2\alpha}$ (Liu *et al.*, 1991).

Although it is apparent that endogenous nitric oxide is released to attenuate contractile responses in the vasculature, the precise mechanism behind the stimulation of nitric oxide release is not clear. Noradrenaline and 5-hydroxytryptamine have been shown to produce endothelium-dependent relaxation through activation of α_2-adrenoceptors and 5-HT receptors, respectively (Cocks & Angus, 1983; Berkenboom *et al.*, 1990). However, it is unlikely that α_2-adrenoceptors are responsible for the stimulation of nitric oxide production in the present study, since the α_2-adrenoceptor antagonist idazoxan did not influence the enhancing effect of L-NNA on vasoconstrictor responses to sympathetic nerve stimulation.

Alternatively, the enhancement of vasoconstrictor responses observed in this study may result from an inhibition of 'basal' release of nitric oxide (for review, see Moncada *et al.*, 1991a) which provides continuous antagonism to contractile responses. This possibility is unlikely in the rat caudal artery since L-NNA, L-NAME, haemoglobin or methylene blue do not affect resting smooth muscle tone. On the other hand, the low resting tone under our experimental conditions might render the caudal artery insensitive to the vasodilator action of 'basal' nitric oxide; the vasodilatation would then

only be detectable after an increase in arterial tone as a result of vasoconstriction. If inhibition of a 'basal' production of nitric oxide could explain the enhancement of vasoconstrictor responses by nitric oxide synthase inhibitors, the same amount of basally released nitric oxide would be available to antagonize vasoconstriction irrespective of the magnitude of contraction. However, this is not the case: there is a positive linear correlation between increases in perfusion pressure caused by sympathetic nerve stimulation and vasoconstrictor agents in the presence of L-NNA and increases obtained in the absence of L-NNA (Figure 8). The regression line has a slope of approximately 2, indicating that the perfusion pressure is doubled when nitric oxide synthesis is inhibited. Furthermore, it was demonstrated that the sensitivity to nitric oxide does not increase with increasing levels of tone, because the vasodilator response to the nitric oxide donor, sodium nitroprusside, did not increase when the level of precontraction was increased; in fact, responses were slightly attenuated by increasing precontraction. Since an altered sensitivity to nitric oxide cannot explain the correlation between level of tone and magnitude of enhancement, the amount of nitric oxide released must be proportional to the level of contraction. Thus a 'basal' release of nitric oxide alone could not fully account for the antagonism of vasoconstriction.

Fluid shear stress produced by a change in perfusate velocity has been demonstrated to stimulate the release of nitric oxide from endothelial cells (Rubanyi *et al.*, 1986; Buga *et al.*, 1991). In the present study, endothelial shear stress caused by increases in vascular tone may provide the stimulus for nitric oxide release. In further support of this suggestion, Lamontagne *et al.* (1992) reported that an increase in shear stress caused by vasoconstriction at constant flow was partially responsible for the release of EDRF from the coronary circulation of the rabbit isolated heart. Additional evidence for a direct relationship between nitric oxide release and tension can be acquired from a study by Ohno *et al.* (1990) using rabbit aorta, where tissue cyclic guanosine monophosphate levels were elevated with increases in resting tension or stretch in endothelium-intact rings but not in endothelium-denuded rings. Alternatively, shear stress may cause the production of a mediator such as endothelin-1, which could stimulate nitric oxide release from endothelial cells (Yanagisawa & Masaki, 1989).

In conclusion, vasoconstrictor responses to sympathetic nerve stimulation and a range of vasoconstrictor agonists were enhanced in the rat isolated caudal artery by nitric oxide synthesis inhibition (with L-NNA and L-NAME), inactivation of nitric oxide (with haemoglobin), and inhibition of guanylate cyclase (with methylene blue). The enhancements were absent in endothelium-denuded arterial segments. These observations indicate that endogenous nitric oxide activates the soluble guanylate cyclase in vascular smooth muscle to induce relaxation, thus opposing vasoconstriction. The release of nitric oxide, probably in response to either shear stress itself or to an intermediate stimulator caused by shear stress, acts as a functional antagonist possibly to limit blood vessel damage during vasoconstriction.

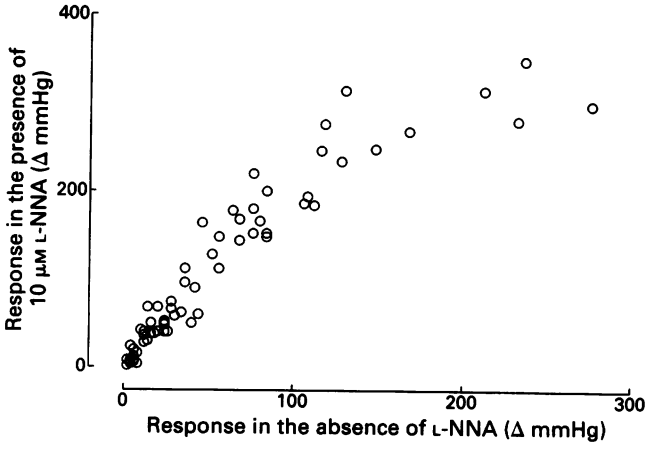

Figure 8 Relationship between the increases in perfusion pressure caused by sympathetic nerve stimulation and vasoconstrictor agonists in the absence and in the presence of 10 μM N^G-nitro-L-arginine (L-NNA). Each point represents the responses from an individual experiment. The points with an abscissa of less than 200 mmHg were fitted to a linear regression, which had a correlation coefficient of 0.955 and a slope of 1.9. The regression line starts to plateau when the abscissa rises above 200 mmHg, probably reflecting the physical limitation of the arterial preparation.

This work was supported by a Programme Grant from the National Health and Medical Research Council of Australia. P.A.V. is in receipt of a National Heart Foundation of Australia Postgraduate Research Scholarship.

References

BERKENBOOM, G., UNGER, P., FANG, Z.Y. & FONTAINE, J. (1991). Endothelium-derived relaxing factor and protection against contraction to norepinephrine in isolated canine and human coronary arteries. *J. Cardiovasc. Pharmacol.*, **17(S3)**, S127–S132.

BUGA, G.M., GOLD, M.E., FUKUTO, J.M. & IGNARRO, L.J. (1991). Shear stress-induced release of nitric oxide from endothelial cells grown on beads. *Hypertension*, **17**, 187–193.

COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. *Nature*, **305**, 627–630.

COCKS, T.M. & ANGUS, J.A. (1991). Evidence that contractions of isolated arteries by L-NMMA and NOLA are not due to inhibition of basal EDRF release. *J. Cardiovasc. Pharmacol.*, **17(S3)**, S159–S164.

CRAWLEY, D.E., LIU, S.F., EVANS, T.W. & BARNES, P.J. (1990). Inhibitory role of endothelium-derived relaxing factor in rat and human pulmonary arteries. *Br. J. Pharmacol.*, **101**, 166–170.

FURCHGOTT, R.F. (1984). The role of endothelium in the responses of vascular smooth muscle to drugs. *Annu. Rev. Pharmacol. Toxicol.*, **24**, 175–197.

GOLD, M.E., WOOD, K.S., BYRNS, R.E., FUKUTO, J. & IGNARRO, L.J. (1990). N^G-methyl-L-arginine causes endothelium-dependent contraction and inhibition of cyclic GMP formation in artery and vein. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 4430–4434.

GREENBERG, S.S., DIECKE, F.P.J., CANTOR, E., PEEVY, K. & TANAKA, T.P. (1990). Inhibition of sympathetic neurotransmitter release by modulators of cyclic GMP in canine vascular smooth muscle. *Eur. J. Pharmacol.*, **187**, 409–423.

IGNARRO, L.J. (1989). Biological actions and properties of endothelium-derived nitric oxide formed and release from artery and vein. *Circ. Res.*, **65**, 1–21.

ITO, S., JUNCOS, L.A., NUSHIRO, N., JOHNSON, C.S. & CARRETERO, O.A. (1991). Endothelium-derived relaxing factor modulates endothelin action in afferent arterioles. *Hypertension*, **17**, 1052–1056.

LAMONTAGNE, D., POHL, U. & BUSSE, R. (1992). Mechanical deformation of vessel wall and shear stress determine the basal release of endothelium-derived relaxing factor in the intact rabbit coronary vascular bed. *Circ. Res.*, **70**, 123–130.

LI, C.G. & RAND, M.J. (1989). Evidence for a role of nitric oxide in the neurotransmitter system mediating relaxation of the rat anococcygeus muscle. *Clin. Exp. Pharmacol. Physiol.*, **16**, 933–938.

LIU, S.F., CRAWLEY, D.E., EVANS, T.W. & BARNES, P.J. (1991). Endogenous nitric oxide modulates adrenergic neural vasoconstriction in guinea-pig pulmonary artery. *Br. J. Pharmacol.*, **104**, 565–569.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium dependent and glyceryl trinitrate induced relaxation by hemoglobin and methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MEDGETT, I.C. & LANGER, S.Z. (1984). Heterogeneity of smooth muscle alpha-adrenoceptors in the rat tail artery *in vitro*. *J. Pharmacol. Exp. Ther.*, **229**, 823–830.

MEDGETT, I.C. & LANGER S.Z. (1986). Influence of neuronal uptake on the contribution of smooth muscle α_2-adrenoceptors to vasoconstrictor responses to noradrenaline in SHR and WKY isolated tail arteries. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **332**, 43–49.

MONCADA, S., PALMER, M.J. & HIGGS, E.A. (1991a). Nitric oxide: physiology, pathology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MONCADA, S., REES, D.D., SCHULZ, R. & PALMER, R.M.J. (1991b). Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 2166–2170.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.S.W., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro-arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

OHNO, M., OCHIAI, M., TAGUCHI, J., HARA, K., AKASUKA, N. & KUROKAWA, K. (1990). Stretch may enhance the release of endothelium-derived relaxing factor in rabbit aorta. *Biochem. Biophys. Res. Commun.*, **173**, 1038–1042.

PALMER, R.M.J., FERRIGO, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

RAJANAYAGAM, M.A.S., MUSGRAVE, I.F., RAND, M.J. & MAJEWSKI, H. (1989). Facilitation of noradrenaline release by isoprenaline is not mediated by angiotensin II in mouse atria and rat tail artery. *Arch. Int. Pharmacodyn.*, **299**, 185–199.

REES, D.D., PALMER, M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

REES, D.D., PALMER, M.J., SCHULZ, P.J., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

REID, J.J., VO, P.A., LIEU, A.T., WONG-DUSTING, H.K. & RAND, M.J. (1991). Modulation of norepinephrine-induced vasoconstriction by endothelin-1 and nitric oxide in rat tail artery. *J. Cardiovasc. Pharmacol.*, **17(S7)**, S272–S275.

REID, J.J. & RAND, M.J. (1992). Renal vasoconstriction is modulated by nitric oxide. *Clin. Exp. Pharmacol. Physiol.*, **19**, 376–379.

RUBANYI, G.M., ROMERO, J.C. & VANHOUTTE, P.M. (1986). Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.*, **250**, H1145–H1149.

SAKUMA, I., STUEHR, D.J., GROSS, S.S., NATHAN, C. & LEVI, R. (1988). Identification of arginine as a precursor of endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8664–8667.

SPOKAS, E.G. & FOLCO, G.C. (1984). Intima-related vasodilatation of the perfused rat caudal artery. *Eur. J. Pharmacol.*, **100**, 211–217.

STARKE, K. (1987). Presynaptic α-autoreceptors. *Rev. Physiol. Biochem. Pharmacol.*, **107**, 73–146.

THOMAS, G., COLE, E.A. & RAMWELL, P.W. (1989). N^G-Monomethyl L-arginine is a non-specific inhibitor of vascular relaxation. *Eur. J. Pharmacol.*, **170**, 123–124.

TODA, N. & OKAMURA, T. (1990). Modification by L-N^G-monomethyl arginine (L-NMMA) of the response to nerve stimulation in isolated dog mesenteric and cerebral arteries. *Jpn. J. Pharmacol.*, **52**, 170–173.

TOPOUZIS, S., SHOTT, C. & STOCLET, J.-C. (1991). Participation of endothelium-derived relaxing factor and role of cyclic GMP in inhibitory effects of endothelium on contractile responses elicited by α-adrenoceptor agonists in rat aorta. *J. Cardiovasc. Pharmacol.*, **18**, 670–678.

URABE, M., KAWASAKI, H. & TAKASAKI, K. (1991). Effect of endothelium removal on the vasoconstrictor response to neuronally released 5-hydroxytryptamine and noradrenaline in the rat isolated mesenteric and femoral arteries. *Br. J. Pharmacol.*, **102**, 85–90.

VALLANCE, P., COLLIER, J. & MONCADA, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet*, **ii**, 997–1000.

VO, P.A., REID, J.J. & RAND, M.J. (1991). Endothelial nitric oxide attenuates vasoconstrictor responses to nerve stimulation and noradrenaline in the rat tail artery. *Eur. J. Pharmacol.*, **199**, 123–125.

WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163–196.

WARNER, T.D., DE NUCCI, G. & VANE, J.R. (1989). Rat endothelin is a vasodilator in the isolated perfused mesentery of the rat. *Eur. J. Pharmacol.*, **159**, 325–326.

WAY, K.J., REID, J.J. & RAND, M.J. (1991). Endothelium-derived nitric oxide attenuates vasoconstriction to noradrenaline and nerve stimulation in rat isolated perfused mesentery. *Proc. Aus. Pharmacol. Physiol. Soc.*, **22**, 185P.

XIAO, X-H. & RAND, M.J. (1989). α_2-Adrenoceptor agonists enhance responses to certain other vasoconstrictor agonists in the rat tail artery. *Br. J. Pharmacol.*, **96**, 539–546.

YANAGISAWA, M. & MASAKI, T. (1989). Endothelin, a novel endothelium-derived peptide. *Biochem. Pharmacol.*, **38**, 1877–1883.

(Received July 31, 1992
Accepted August 11, 1992)

Br. J. Pharmacol. (1992), **107**, 1129–1134

Bradykinin-induced activation of nociceptors: receptor and mechanistic studies on the neonatal rat spinal cord-tail preparation *in vitro*

A. Dray, I.A. Patel, M.N. Perkins & A. Rueff

Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6BN

1 The effects of bradykinin on nociceptors have been characterized on a preparation of the neonatal rat spinal cord with functionally connected tail maintained *in vitro*. Administration of bradykinin to the tail activated capsaicin-sensitive peripheral fibres and evoked a concentration-dependent (EC_{50} = 130 nM) depolarization recorded from a spinal ventral root (L_3–L_5).

2 The response to bradykinin was unaffected by the peptidase inhibitors, bestatin (0.4 mM), thiorphan (1 μM), phosphoramidon (1 μM) and MERGETPA (10 μM) or by the presence of calcium blocking agents, cadmium (200 μM) and nifedipine (10 μM).

3 Inhibition of cyclo-oxygenase with indomethacin (1–5 μM), aspirin (1–10 μM) and paracetamol (10–50 μM) consistently attenuated responses to bradykinin.

4 The effect of bradykinin was mimicked by the phorbol ester PDBu, an activator of protein kinase C. The response to bradykinin was attenuated following desensitization to PDBu but desensitization to bradykinin did not induce a cross-desensitization to PDBu. The protein kinase C inhibitor staurosporine (10–500 nM) consistently attenuated the effects of PDBu and bradykinin.

5 Bradykinin responses were reversibly enhanced by dibutyryl cyclic AMP (100 μM). However dibutyryl cyclic GMP (0.5 mM) and nitroprusside (10 μM) produced prolonged block of responsiveness to bradykinin. Prolonged superfusion with pertussis toxin did not affect responses to bradykinin.

6 The B_1-receptor agonist des Arg^9-bradykinin (10–100 μM) was ineffective alone or after prolonged exposure of the tail to lipopolysaccharide (100 ng ml^{-1}) or epidermal growth factor (100 ng ml^{-1}) to induce B_1 receptors. The B_1-receptor antagonist, des Arg^9 Leu^8-bradykinin (10 μM) did not attenuate the response to bradykinin. A number of bradykinin B_2 antagonists selectively and reversibly attenuated the response to bradykinin. The rank order potency was Hoe 140 > LysLys [Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin > D-Arg[Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin = D-Arg[Hyp^2,$Thi^{5,8}$, D-Phe^7]-bradykinin.

7 These data show that bradykinin produces concentration-dependent activation of peripheral nociceptors in the neonatal rat tail. The responses were unaffected by calcium channel block and were partially dependent on the production of prostanoids. Bradykinin-evoked responses were consistent with the activation of protein kinase C-dependent mechanisms. Cyclic GMP-dependent mechanisms may be involved in bradykinin-receptor desensitization whereas cyclic-AMP dependent mechanisms increase fibre excitability and facilitate bradykinin-induced responses. The effects of bradykinin were mediated by a B_2 receptor.

Keywords: Bradykinin; peripheral nociceptors; bradykinin receptors; antagonists; second messengers

Introduction

Bradykinin, a pain producing and pro-inflammatory nonapeptide hormone is formed at sites of tissue trauma from proteolytic enzymes acting on kininogen precursors (Keele & Armstrong, 1964; Wilhelm, 1973; Erdos, 1979). The mechanisms of pain production involve the activation of a sub-population of polymodal nociceptors and the subsequent transmission of nociceptive signals to the spinal cord.

Studies of nociceptor activation by bradykinin have been made in sensory fibres *in vivo* (Beck & Handwerker, 1974; Mense & Schmidt, 1974; Franze & Mense, 1975) but because of the difficulties of studying fine sensory nerve terminals *in situ* these experiments have provided little quantitative information about the events and factors on which the effects of bradykinin depend. Mechanistic studies of bradykinin have been performed mainly on cultured sensory neurones or other hybrid cells. These have shown that bradykinin changes a number of membrane ion conductance mechanisms through interactions with several second messenger systems (Weinreich, 1986; Miller, 1987; Brown & Higashida, 1988; Dunn *et al.*, 1991) including phospholipase C to produce inositol 1,4,5, trisphosphate and diacyl glycerol (Thayer *et al.*, 1988; Burgess *et al.*, 1989b; Gammon *et al.*, 1989) and phospholipase A_2 to generate prostanoids, especially prostaglandins. These effects of bradykinin are likely to be coupled with specific receptors. At present two bradykinin receptors, B_1 and B_2 (Regoli & Barabe, 1980), have been convincingly demonstrated by the use of several generations of peptide antagonists (Vavrek & Stewart, 1985; Hock *et al.*, 1991).

To circumvent some of the disadvantages of *in vivo* studies of nociceptors we have used an *in vitro* preparation of the neonatal rat spinal cord/tail to characterize the receptors and second messengers involved in the activation of peripheral nociceptors by bradykinin. Some findings of this study have been reported previously (Dray *et al.*, 1988a,b).

Methods

The intact spinal cord and the functionally connected tail were taken from 1–2 day old rats following decapitation. The skin was carefully removed from the tail to expose cutaneous fibres and their endings to allow activation by bradykinin and to facilitate activation by capsaicin (Dray *et al.*, 1990a). Histology was not routinely performed but recent unpublished findings using immunological markers for developing neurones (GAP-43; Reynolds *et al.*, 1991) and for

specific neuronal cytoplasmic protein (PGP 9.5, Dalsgaard et al., 1989) did not give any indication of damage to cutaneous fibres following skin removal. Bradykinin is not known to activate axons of nociceptors; it is more likely to stimulate nociceptors via the signal transducing elements localized on the terminations of intact primary afferent fibres. In addition the effects of other peripheral stimuli were robust and reproducible over many hours. This would be unlikely in the face of significant tissue damage.

The preparation was placed in a chamber and the spinal-cord and tail were separately superfused (2 ml min^{-1} on the cord and 4 ml min^{-1} on the tail) with a physiological salt solution (composition mM: NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.16, $NaHCO_3$ 21.0, $NaHPO_4$ 0.58, glucose 10; at 24°C and gassed with 95% O_2/5% CO_2). Peripheral nociceptive fibres were activated by superfusion of the tail with bradykinin, capsaicin and by superfusate heated to 48–50°C (noxious heat). Each stimulus was applied for 10 s with an intervening period of 15 min between stimuli. Bradykinin applications were separated by at least 30–60 min to avoid tachyphylaxis.

The activation of peripheral fibres was assessed by measuring the depolarization produced in a spinal ventral root (L_3–L_5). The ventral root potential was recorded (d.c. with respect to the spinal cord which was earthed) using a low impedance glass pipette which was placed in an electrolyte-filled well containing the selected ventral root. The signals were amplified using conventional means (Neurolog System) and displayed simultaneously on an oscilloscope and on a rectilinear chart recorder.

In the following studies we examined whether bradykinin-induced activation of nociceptors was coupled to a G-protein or mediated via the activation of a second messenger system. In addition we have characterized the receptor involved in the bradykinin-induced activation of nociceptors and compared the potency of a number of bradykinin antagonists. Antagonist activity was expressed as the IC_{50} concentration, determined by measuring the responses to a submaximal concentration of bradykinin (usually the EC_{50}) in the presence of cumulative increases in the concentration of the antagonist. Three or more antagonist concentrations were used in the IC_{50} determination.

Drugs

The following substances were used; capsaicin (Sigma, 10 mM stock solution in dimethylsulpoxide (DMSO) made up to the desired concentration in physiological salt solution), bradykinin (Bachem, Nova), forskolin, sodium nitroprusside, sodium dibutyryl cyclic AMP, dibutyryl cyclic GMP, trifluoperazine, indomethacin, mepacrine, phenobarbital sodium, ruthenuim red (all from Sigma), nifedipine (Research Biochemicals Incorporated); R_p isomer of adenosine 3′-5′-cyclic monophosphathioate (R_p-cAMPS, BIOLOG); β-phorbol 12,13 dibutyrate, (Avanti Polar Lipids); staurosporine (Fluka), H7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine] (Seikagaku America Inc); sodium chromoglycate (gift from Pfizer); DL-2-mercaptomethyl-3-guanidoethylthiopropionic acid (MERGETPA, Calbiochem-Behring), phosphoramidon (Peninsula), des-Arg^9 bradykinin; Leu^8 (des Arg^9)-bradykinin; D-Arg [Hyp^2 Thi^{5,8} D-Phe^7 bradykinin], D-Arg [Hyp^3 Thi^{5,8}, D-Phe^7 bradykinin], Lys,Lys [Hyp^3 Thi^{5,8} D-Phe^7 bradykinin] (where Thi = β (2-thienyl)-L-alanine; Hyp = L-4-hyroxyproline: Peninsula Labs); D-Arg[Hyp^3-Thi^5-D-Tic^7-Oic^8-bradykinin] (HOE 140, where Tic = 1,2,3,4 tetrahydroisoquinolin-2-yl-carbonyl, Oic = (3aS,7aS)-octahydroindol-2-yl-carbonyl: synthesized at the Sandoz Institute, London); pertussis toxin (Porton Products Ltd).

Results

A 10 s administration of bradykinin to the tail evoked a ventral root response. Application of bradykinin (1–5 μM) to the intact tail without prior removal of the superficial skin did not produce a response ($n = 5$). A ventral root response was also evoked by brief applications of capsaicin (0.2–1.0 μM) and noxious heat. In any given tissue the noxious heat stimulus produced the maximal response. Therefore for quantification of data, chemically induced responses were normalized, in each tissue, relative to the noxious heat response.

The amplitude of the ventral root depolarization produced by bradykinin was concentration-related (Figures 1 and 3). A concentration-response curve (EC_{50} = 130 nM, maximum concentration = 1 μM), shown in Figure 1, was determined from the response produced by the first application of bradykinin at any given concentration. This provided only one data point per experiment but avoided possible sensitivity changes due to tachyphylaxis upon repeated administration.

The onset of the effect of a 10 s application of bradykinin occurred within 20–60 s of the start of the superfusion and the duration of the effect ranged from 40–190 s. The onset of the effect was shorter and the duration longer with higher concentrations (e.g. 20 s latency and 190 s duration at 1 μM) but this relationship was not systematically studied. Under the present conditions, responses evoked by a submaximal concentration of bradykinin (0.3 μM) were not significantly influenced by enzymatic degradation since they were unchanged in the presence of the peptidase inhibitors, bestatin (0.4 mM, $n = 4$; control response = 65 ± 5%; test response = 71 ± 9%, $P > 0.05$), thiorphan (1 μM, $n = 4$; control response = 56 ± 8%; test response = 59 ± 5%, $P > 0.05$), phosphoramidon (1 μM, $n = 5$; control response = 60 ± 5%, test response = 63 ± 7%, $P > 0.05$) or MERGETPA (10 μM, $n = 5$, 61 ± 9%; test response = 64 ± 9%, $P > 0.05$), each superfused 15 min prior to and throughout the test with bradykinin. In addition bradykinin-evoked (0.35 μM) responses were unaffected in the presence of cadmium chloride (200 μM, $n = 4$; control response = 100 ± 10%; test response = 88 ± 17%, $P > 0.05$) or nifedipine (10 μM, $n = 5$; control response = 89 ± 11%; test response = 80 ± 8%, $P > 0.05$).

Second messenger studies

The effects of phorbol esters, non-hydrolysable protein kinase C activators (Castagna et al., 1982), and their interactions with bradykinin were tested on peripheral nociceptors. Both phorbol 12, myristate-13,acetate (PMA, 1 μM, $n = 4$, response = 32 ± 9%) and β-phorbol 12,13dibutyrate (PDBu 1 μM, $n = 10$, response = 48 ± 13%) application to the tail

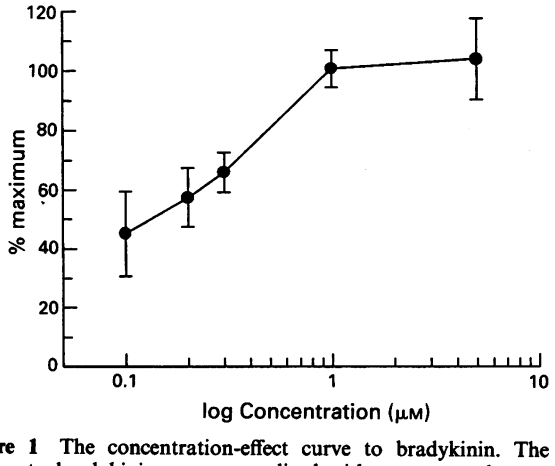

Figure 1 The concentration-effect curve to bradykinin. The responses to bradykinin were normalized with respect to the maximal tissue response evoked by noxious heat response and plotted as % maximal effect vs log concentration of bradykinin (nM). The EC_{50} concentration = 130 nM. Each point is the mean ± s.e.mean (vertical bars) of 4–5 determinations.

evoked ventral root responses due to activation of capsaicin-sensitive afferents (Dray *et al.*, 1988a). In further studies, 1 μM PDBu was used, as responses with this agent were obtained more consistently and were comparable to those produced by bradykinin (0.3 μM).

The onset of responses evoked by PDBu was delayed like those of bradykinin. Frequently repeated (every 10–20 min) or prolonged (2 min) administration of 1 μM PDBu produced a tachyphylaxis at which time the response to bradykinin was also attenuated ($n = 5$, control bradykinin response = 83 ± 10%; post PDBu = 12 ± 9%, $P < 0.01$). On the other hand prolonged administration of bradykinin (10–20 μM, $n = 5$), to induce desensitization to bradykinin did not induce a cross desensitization to PDBu (control PDBu = 60 ± 15%; post bradykinin = 54 ± 12%, $P > 0.05$). The degree of tachyphylaxis to repeated brief administrations of PDBu could be minimized by restricting drug administration to once every 60 min.

Prolonged application of the protein kinase C inhibitor, H7 (Hidaka & Hagiwara, 1987) (100 μM, $n = 3$) did not affect the response to PDBu (control = 40 ± 12%; post H7 = 45 ± 10%, $P > 0.05$) or bradykinin (control = 72 ± 11%; post H7 = 66 ± 14%). However another protein kinase C inhibitor, staurosporine (Tamaoki *et al.*, 1986) consistently (10–500 nM, $n = 8$) attenuated the responses to PDBu (control = 46 ± 10%; post staurosporine = 22 ± 12%, $P < 0.01$) and bradykinin (control = 69 ± 9%; post staurosporine = 14 ± 9%, $P < 0.01$) without affecting responses to other stimulants such as capsaicin (control = 61 ± 8%; post staurosporine = 54 ± 8%, $P > 0.05$) and noxious heat. Prolonged (20–30 min) superfusion of the tail with phenobarbitone (0.1–1.0 mM, $n = 4$), previously suggested to be an inhibitor of protein kinase C (Chaouhan & Brockerhoff, 1987), did not attenuate the responses to bradykinin (control = 72 ± 14%; post phenobarbitone = 67 ± 12%, $P > 0.05$). At higher concentrations (3–10 mM, $n = 3$) phenobarbitone depressed responsiveness to all sensory stimuli.

Previous studies have shown that adenosine 3′:5′-cyclic monophosphate (cyclic AMP) increased membrane excitability by blocking hyperpolarizing potassium conductance (Weinreich & Wonderlin, 1987; Grega & Macdonald, 1987). In our experiments the addition of dibutyryl cyclic AMP (30 s, 100 μM, $n = 13$) or stimulation of adenylate cyclase by the addition of forskolin (30 s, 10 μM, $n = 5$) did not evoke a response. However in 8 of 13 preparations the effects of bradykinin (control = 32 ± 12%; with dibutyryl cyclic AMP (db cyclic AMP) = 122 ± 19%, $P < 0.01$) and capsaicin (control = 74 ± 5%; with db cyclic AMP = 123 ± 12%, $P < 0.01$) were significantly enhanced (Figure 2) by db cyclic AMP but were not significantly affected in the 4 other experiments. The effects of db cyclic AMP were reversed following 10–20 min of washing (Figure 2). The effect of bradykinin was not significantly changed in the presence of forskolin (10 μM, $n = 5$) or by 10 μM, R_p-cAMPS ($n = 4$), a cyclic AMP-dependent kinase inhibitor (Botelho *et al.*, 1988).

Bradykinin has previously been shown to increase guanosine 3′:5′-cyclic monophosphate (cyclic GMP) production in sensory neurones (Burgess *et al.*, 1989a). In our experiments neither db cyclic GMP (0.5 mM, $n = 5$) nor nitroprusside (2–50 μM, $n = 7$) evoked a measurable response. In the presence of these agents the response to bradykinin was selectively attenuated or abolished ($n = 5$, db cyclic GMP, 0.5 mM; control response = 78 ± 11%; test response = 18 ± 9%, $P < 0.01$: sodium nitroprusside, 2–10 μM, $n = 6$, control response = 80 ± 14%; test response = 18 ± 10%, $P < 0.01$). In contrast to the short lived effect of cyclic AMP, that produced by cyclic GMP or nitroprusside was prolonged and was incompletely reversed even by 1–2 h after continuous washing of the tissue. In this respect the loss of bradykinin sensitivity was similar to that seen during bradykinin-induced tachyphylaxis.

Prolonged superfusion of the tail (20–30 min) with the calmodulin-kinase inhibitor trifluoperazine (30 μM, $n = 4$) or the phospholipase A_2 inhibitor, mepacrine (10–30 μM, $n = 4$) did not significantly change the responses to bradykinin, capsaicin or noxious heat. In addition similar applications of sodium chromoglycate (1–100 μM, $n = 4$), reported to depress C-fibre excitability in the lung (Dixon *et al.*, 1980) and bradykinin-induced bronchoconstriction (Fuller *et al.*, 1987), did not significantly affect responses to bradykinin. Finally, ruthenium red (100–500 nM, $n = 6$), which selectively inhibited C-fibre activation by capsaicin (Dray *et al.*, 1990b) did not alter responses to bradykinin.

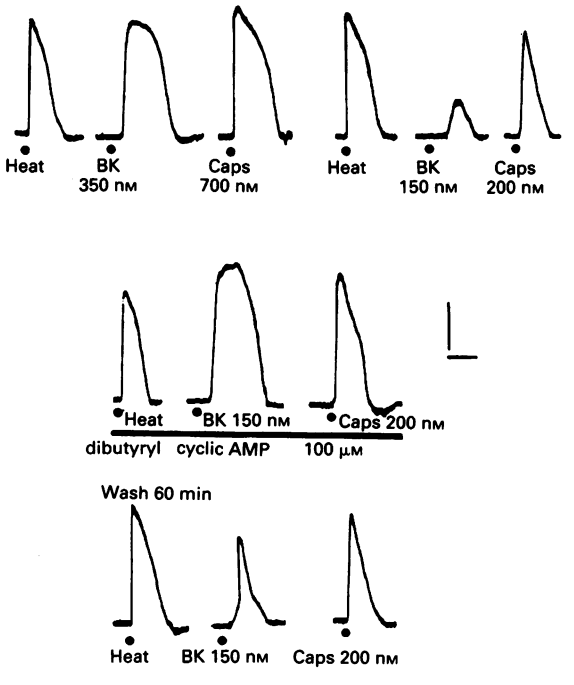

Figure 2 Enhancement of the bradykinin-evoked response during the administration of dibutyryl cyclic AMP. The top row of traces show responses to heat, bradykinin (BK) (350 nM and 150 nM respectively) and capsaicin (Caps, 700 and 200 nM). Middle traces: the responses to bradykinin (150 nM) and capsaicin (200 nM) were enhanced in the presence of dibutyryl cyclic AMP (100 μM). Bottom traces: the effect of cyclic AMP was reversed 60 min after the end of the cyclic AMP superfusion. The calibration bars are 0.2 mV and 60 s.

Indomethacin (1.5 μM) reduced, but did not abolish, the response to bradykinin (300 nM) in 7 of 9 preparations (control responses = 42 ± 15%; with indomethacin = 17 ± 11%, $P < 0.01$, $n = 7$). Higher concentrations of indomethacin (10–50 μM, $n = 5$) produced an additional non-selective depression of responses to capsaicin (43 ± 2% of control) and noxious heat (56 ± 9% of control). Aspirin (1–10 μM, $n = 6$, control response = 65 ± 10%; with aspirin = 21 ± 8%, $P < 0.01$) or paracetamol (10–50 μM, $n = 6$, control response = 57 ± 12%; with paracetamol = 30 ± 7%, $P < 0.01$) also selectively reduced the responses to bradykinin (300 nM).

Superfusion of the tail for 4–6 h with recirculated and reoxygenated pertussis toxin (200 ng–1.0 μg ml^{-1}) (Dolphin, 1987) to inactivate G_i and G_o did not affect the responses to 300 nM bradykinin ($n = 3$, control = 59 ± 15%; test = 66 ± 11%, $P > 0.05$).

Pharmacology of bradykinin on peripheral fibres

Brief (10 s) or prolonged (5 min) application of the B_1 receptor agonist des-Arg^9-bradykinin (10–100 μM, $n = 5$) did not evoke a response. Also prolonged superfusion (1–4 h) with lipopolysaccharide (100 ng ml^{-1}, $n = 4$) and epidermal growth factor (100 ng ml^{-1}, $n = 3$), both shown to induce responses to B_1 receptor agonists *in vitro* (Bouthillier *et al.*, 1987) did not induce any measurable response to a subse

quent administration of des-Arg^9-bradykinin (10 μM). Prolonged superfusion of the tail with the B_1-antagonist, des-Arg^{9}Leu^8-bradykinin (10 μM, $n = 5$) did not change responsiveness to bradykinin (control = 77 ± 10%; test = 84 ± 12%, $P > 0.05$).

The response to bradykinin was reduced, in a concentration-dependent manner, by a number of competitive peptide antagonists. Antagonism was quantified by determining the concentration of the antagonist (IC_{50}), incremented in a cumulative manner, required to reduce the response produced by a submaximal 2x-dose of bradykinin to that produced by a single x-dose (Figure 3). The relative potency of a number of bradykinin antagonists, determined in this manner was Hoe 140 = 1.5 ± 0.4 nM, $n = 5$; LysLys[Hyp^3,Thi^{5,8} D-Phe^7-bradykinin] = 36 ± 14 nM, $n = 5$; D-Arg[Hyp^3,Thi^{5,8}, D-Phe^7-bradykinin] = 44 ± 23 nM, $n = 6$; D-Arg[Hyp^2, Thi^{5,8}, D-Phe^7-bradykinin], = 69 ± 21 nM, ($n = 5$ for each determination). The effect of each antagonist was reversed within a 60 min wash period though more prolonged washing (120–180 min) was required to show partial reversal of the Hoe 140-induced antagonism. This suggested that Hoe 140 may have been more tightly bound to the tissue. The reversibility of the antagonism with each compound readily distinguished this effect from desensitization. None of these substances exhibited any agonistic activity when administered up to 10 μM ($n = 3$ for each substance).

Discussion

By using an *in vitro* preparation we have been able to study in greater detail the receptor-mediated interactions of bradykinin with peripheral nociceptors. In the neonatal rat, cutaneous nociceptors have similar sensitivity to physiological stimuli to those in adults (Fitzgerald, 1987).

The peripheral nerve elements activated by bradykinin were likely to be C and Aδ nociceptive fibres because previous studies have shown that bradykinin affected C-cells but not larger A-cells (Burgess *et al.*, 1989b) and that bradykinin activated a subpopulation of capsaicin-sensitive C and Aδ nociceptors (Lang *et al.*, 1990). In addition the effects

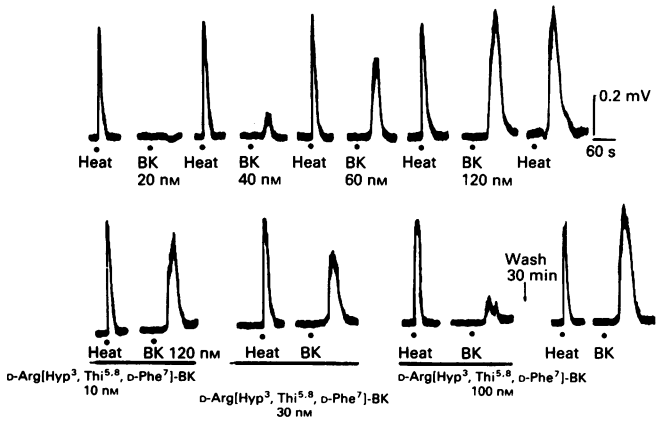

Figure 3 Concentration-related antagonism of bradykinin by D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin. The top traces show response to brief administration of noxious heat and concentration related responses to bradykinin (20, 40, 60 and 120 nM). The response to 120 nM bradykinin was chosen as the test concentration of bradykinin and in the bottom traces the antagonistic effect of D-Arg [Hyp^3,Thi^{5,8}D-Phe^7]-bradykinin was determined. The antagonist was administered 15 min before and throughout the repeated tests with bradykinin. The concentration of the antagonist (10, 30, 100 nM) was cumulatively increased until the response to bradykinin was reduced to or beyond that produced by the 60 nM control concentration. The IC_{50} was subsequently determined. The effect of the antagonist was readily reversed and therefore distinguishable from any possible bradykinin-induced tachyphylaxis. The calibration bars are 0.2 mV and 60 s.

of bradykinin could be abolished by pretreatment with capsaicin (Dray *et al.*, 1989) which has a selective action on these classes of fibres (Buck & Burks, 1986). Finally the effect of bradykinin or other peptide analogues (see later) were unlikely to be limited by the actions of endogenous degrading enzymes since the responses to bradykinin were unchanged in the presence of a variety of kinase inhibitors. The responses evoked by bradykinin were concentration-dependent with an EC_{50} = 130 nM, somewhat higher than that found for activation of sensory neurones in culture (Burgess *et al.*, 1989b; Thayer *et al.*, 1988).

Activation of peripheral fibres by bradykinin was unlikely to be due to an increase in permeability to extracellular calcium ions since bradykinin-evoked responses were not significantly changed by the calcium channel blockers, cadmium and nifedipine. Similar findings were reported with cultured sensory neurones where bradykinin-induced membrane currents were unaffected by calcium-free solution or by the presence of calcium channel blocking drugs. Moreover any intracellular calcium flux was considered to be secondary to membrane depolarization and the activation of voltage-sensitive calcium channels (Burgess *et al.*, 1989b). Indeed the depolarization of sensory neurones has been suggested to depend mainly on an increase membrane permeability to sodium ions (Burgess *et al.*, 1989b).

The effect of bradykinin appeared to be mediated in part via protein kinase C. Thus bradykinin and phorbol esters activated peripheral fibres. Responses to both agents were sensitive to the protein kinase C inhibitor, staurosporine (Tamaoki *et al.*, 1986) and bradykinin responses were attenuated or abolished after PDBu-induced tachyphylaxis. These observations confirm similar interactions in sensory neurones in culture (Burgess *et al.*, 1989b). On the other hand PDBu was still active following bradykinin-induced desensitization, suggesting that the mechanisms of desensitization to bradykinin and PDBu were different.

The response to bradykinin was also consistently and selectively attenuated, but not abolished, by a number of cyclo-oxygenase inhibitors including indomethacin, aspirin and paracetamol. These data indicate that bradykinin stimulated the production of prostanoids, as occurs in many tissues (Griesbacher & Lembeck, 1987; Conklin *et al.*, 1988). However, bradykinin was unlikely to have stimulated phospholipase A_2 (PLA_2) activity to any significant extent as the PLA_2 inhibitor, mepacrine, had litte effect on bradykinin-induced responses. Also it is unlikely that endogenously produced prostanoids directly activated nociceptors since in our preparation, exogenously administered prostaglandins only sensitized nociceptors to other direct activators, including bradykinin.

It is unclear how bradykinin-induced protein kinase C activation contributed to an increased excitability of nociceptors. On the one hand bradykinin or phorbol esters, which stimulate protein kinase C, depolarize C-fibres (Rang & Ritchie, 1988) and increase membrane conductance, to sodium ions, in sensory neurones (Baccaglini & Hogan, 1983; McGhee & Oxford, 1989; Burgess *et al.*, 1989b). Other studies however indicate that bradykinin and phorbol esters inhibit membrane calcium conductance (Rane *et al.*, 1989; Boland *et al.*, 1991). In visceral sensory neurones, a reduced calcium permeability (Gross & Macdonald, 1989) may account for the inhibition of calcium-dependent potassium conductance, the reduction of membrane spike after-hyperpolarization and the consequent increase in cell excitability (Weinreich, 1986). Bradykinin induced prostaglandin production may also increase sensory neurone excitability by inhibiting potassium permeability (Weinreich, 1986; Weinreich & Wonderlin, 1987). Clearly the actions of bradykinin on membrane excitability of sensory neurones are complex and depend on a multitude of factors including the prevailing level of membrane polarization.

Bradykinin-induced activation did not appear to involve cyclic AMP or cyclic GMP-dependent mechanisms as neither

the addition of nucleotide analogues nor stimulators of cyclic nucleotide formation (forskolin and nitroprusside) mimicked the effects of bradykinin. Moreover the effect of bradykinin was unchanged by the cyclic AMP-kinase inhibitor R_p-cAMPS (Botelho *et al.*, 1988). However cyclic AMP produced a short lasting increase in the responsiveness of nociceptors to bradykinin and capsaicin. This may have been due to a generalized increase in excitability of nociceptors, in keeping with findings *in vivo* which have suggested the participation of cyclic AMP mechanisms in peripheral hyperalgesia (Taiwo & Levine, 1991). On the other hand cyclic GMP consistently attenuated the response to bradykinin for a prolonged period. The reason for this is unclear at present but may have involved desensitization of bradykinin receptors due to receptor phosphorylation by a cyclic GMP-dependent kinase or the inhibition of bradykinin-induced second messenger production e.g. IP_3 and DAG, important for mediating the bradykinin response (Burgess & McNeill, 1989). In addition we were unable to show that the effect of bradykinin was coupled with a pertussis toxin-sensitive G-protein. Indeed evidence for the involvement of a G-protein in the effects of bradykinin on sensory neurones has been controversial. Neither Burgess *et al.* (1989b) nor McGehee & Oxford (1989) found altered sensitivity following pertussis toxin treatment while McGuirk *et al.* (1989) measured an increase in sensory neurone activity following treatment with a non-hydrolysable GTP-analogue, though it was unclear that this was related to a bradykinin mechanism.

Studies with the bradykinin analogues showed that the effects of bradykinin were mediated by a B_2 receptor. Thus no activity was observed with either the B_1 receptor agonist or antagonist. Moreover, we were unable to show the presence of B_1 receptors by incubating tissues with lipopolysaccharide or epidermal growth factor. These substances have been shown to enhance B_1-receptor expression in a number of smooth muscle preparations *in vitro* (Bouthillier *et al.*, 1987). On the other hand several peptide antagonists of bradykinin B_2 receptors produced a concentration-related attenuation of bradykinin-responses. The rank order potency of these substances was Hoe 140 > LysLys [Hyp^3,$Thi^{5,8}$D-Phe^7]-bradykinin > D-Arg^0[Hyp^3,$Thi^{5,8}$D-Phe^7]-bradykinin = D-Arg^0 [Hyp^2,$Thi^{5,8}$D-Phe^7]-bradykinin. In keeping with their *in vitro* activity, several of these agents have been shown to be anti-inflammatory and antinociceptive in a number of *in vivo* studies (Hargreaves *et al.*, 1988; Steranka *et al.*, 1988; 1989; Costello & Hargreaves, 1989; Burch & deHaas, 1990; Haley *et al.*, 1989). This also supports the likely involvement of endogenous bradykinin in inflammatory hyperalgesia.

In summary, we have shown that bradykinin produces a prolonged and concentration-related activation of peripheral nociceptors of the neonatal rat. The activation of nociceptors occurred independently of extracellular calcium but involved a number of cellular second messenger systems including cyclo-oxygenase products and protein kinase C. Finally a number of selective peptide antagonists of bradykinin suggested that these mechanisms are coupled to a bradykinin B_2 receptor.

References

BACCAGLINI, P.I. & HOGAN, P.G. (1983). Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 594–598.

BECK, P.W. & HANDWERKER, H.O. (1974). Bradykinin and serotonin effects on various types of cutaneous nerve fibres. *Pflügers Arch.*, **347**, 209–222.

BOLAND, L.M., ALLEN, A.C. & DINGLEDINE, R. (1991). Inhibition by bradykinin of voltage-activated barium current in a rat dorsal root ganglion cell line: role of protein kinase C. *J. Neurosci.*, **11**, 1140–1149.

BOTELHO, L.H.P., ROTHERMAN, J.D., COOMBS, R.V. & JOSTORFF, B. (1988). cAMP analog antagonist of cAMP action. *Methods Enzymol.*, **159**, 159–172.

BOUTHILLIER, J., DE BOIS, D. & MARCEAU, F. (1987). Studies on the induction of pharmacological responses to des-Arg^9-bradykinin *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **92**, 257–264.

BROWN, D.A. & HIGASHIDA, A. (1988). Inositol 1,4,5- trisphosphate and diacylglycerol mimic bradykinin effects on mouse neuroblastoma x rat glioma hybrid cells. *J. Physiol.*, **397**, 185–207.

BUCK, S.H. & BURKS, T.F. (1986). The neuropharmacology of capsaicin: review of some recent observations. *Pharmacol. Rev.*, **38**, 179–226.

BURCH, R.M. & DEHAAS, C. (1990). A bradykinin antagonist inhibits carageenan edema in rat. *Naunyn-Schmiedebergs Arch Pharmacol.*, **342**, 189–193.

BURGESS, G.M. & MCNEILL, M. (1989). Homologous desensitization of bradykinin-induced inositol polyphosphate formation in neonatal rat dorsal root ganglion neurones in culture. *Soc Neurosci.*, **15**, 1007.

BURGESS, G.M., MULLANEY, I., MCNEILL, M., COOTE, P.R., MINHAS, A. & WOOD, J.N. (1989a). Activation of guanylate cyclase by bradykinin in rat sensory neurones is mediated by calcium influx: possible role of the increase in cyclic GMP. *J. Neurochem.*, **53**, 1212–1218.

BURGESS, G.M., MULLANEY, I., MCNEIL, M., DUNN, P.M. & RANG, H.P. (1989b). Second messengers involved in the mechanism of action of bradykinin in sensory neurones in culture. *J. Neurosci.*, **9**, 3314–3325.

CASTAGNA, M., TAKAI, Y., KAIBACHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated phospholipid dependent protein kinase by tumor promoting phorbol esters. *J. Biol. Chem.*, **257**, 335–363.

CHAOUHAN, V.P.S. & BROCKERHOFF, H. (1987). Phenobarbital competes with diacylglycerol for protein kinase C. *Life Sci.*, **40**, 89–93.

CONKLIN, B.R., BURCH, R.M., STERANKA, L.R. & AXELROD, J. (1988). Distinct bradykinin receptors mediate stimulation of prostaglandin synthesis by endothelial cells and fibroblasts. *J. Pharmacol. Exp. Ther.*, **244**, 646–649.

COSTELLO, A.H. & HARGREAVES, K.M. (1989). Suppression of carageenin induced hyperalgesia, hyperthermia and edema by a bradykinin antagonist. *Eur. J. Pharmacol.*, **171**, 259–263.

DALSGAARD, C.-J., RYDH, M. & HAEGERSTRAND, A. (1989). Cutaneous innervation in man visualized with protein gene product 9.5. (PGP 9.5) antibodies. *Histochem.*, **92**, 385–390.

DIXON, M., JACKSON, D.M. & RICHARDS, I.M. (1980). The actions of sodium chromoglycate on 'C' fibre endings in the dog lung. *Br. J Pharmacol.*, **70**, 11–13.

DOLPHIN, A.C. (1987). Nucleotide binding proteins in health and disease. *Trends Neurol. Sci.*, **10**, 53–57.

DRAY, A., BETTANEY, J., FORSTER, P. & PERKINS, M.N. (1988a). Bradykinin-induced stimulation of afferent fibres is mediated through protein kinase C. *Neurosci. Lett.*, **91**, 301–307.

DRAY, A., BETTANEY, J., FORSTER, P. & PERKINS, M.N. (1988b). Activation of bradykinin receptor in peripheral nerve and spinal cord in the neonatal rat *in vitro*. *Br. J. Pharmacol.*, **95**, 1008–1010.

DRAY, A., BETTANEY, J. & FORSTER, P. (1989). Capsaicin desensitization of peripheral nociceptive fibres does not impair sensitivity to other noxious stimuli. *Neurosci. Letts.*, **99**, 50–54.

DRAY, A., BETTANEY, J. & FORSTER, P. (1990a). Action of capsaicin on peripheral nociceptors of the neonatal rat spinal cord-tail preparation in vitro: dependence of extracellular ions and independence of second messengers. *Br. J. Pharmacol.*, **101**, 727–733.

DRAY, A., FORBES, C.A. & BURGESS, G.M. (1990b). Ruthenium red blocks capsaicin-induced increase in intracellular calcium and activation of membrane currents in sensory neurones as well as the activation of peripheral nociceptors in vitro. *Neurosci. Letts.*, **110**, 52–59.

DUNN, P.M., COOTE, P.R., WOOD, J.N., BURGESS, G.M. & RANG, H.P. (1991). Bradykinin evoked depolarization of a novel neuroblastoma x DRG neurone hybrid cell line (ND 7/23). *Brain Res.*, **545**, 80–86.

ERDOS, E. (1979). *Bradykinin, Kallidin and Kallikrein. Handbook of Experimental Pharmacology*. Supplement to vol 25, Berlin: Springer Verlag.

FITZGERALD, M. (1987). Cutaneous primary afferent properties in the hind limb of the neonatal rat. *J. Physiol.*, **383**, 79–92.

FRANZ, M. & MENSE, S. (1975). Muscle receptors with Group IV afferent fibres responding to application of bradykinin. *Brain Res.*, **92**, 369–383.

FULLER, R.W., DIXON, C.M.S., CUSS, F.M.C. & BARNES, P.J. (1987). Bradykinin-induced bronchoconstriction in man. *Am. Rev. Respir. Dis.*, **135**, 176–180.

GAMMON, C.M., ALLEN, A.C. & MORELL, P. (1989). Bradykinin stimulates phosphoinositide hydrolysis and mobilization of arachidonic acid in dorsal root ganglion neurons. *J. Neurochem.*, **53**, 95–101.

GREGA, D.S. & MACDONALD, R.L. (1987). Activators of adenylate cyclase and cyclic AMP prolong calcium-dependent action potentials of mouse sensory neurons in culture by reducing a voltage-dependent potassium conductance. *J. Neurosci.*, **7**, 700–707.

GRIESBACHER, T. & LEMBECK, F. (1987). Effects of bradykinin antagonists on bradykinin-induced plasma extravasation, venoconstriction, prostaglandin E_2 release, nociceptor stimulation and contraction of iris sphincter muscle in the rabbit. *Br. J. Pharmacol.*, **92**, 333–340.

GROSS, R.A. & MACDONALD, R.L. (1989). Activators of protein kinase C selectively enhance inactivation of a calcium current component of cultured sensory neurons in a pertussis toxin-sensitive manner. *J. Neurophysiol.*, **61**, 1259–1269.

HALEY, J.E., DICKENSON, A.H. & SCHACHTER, (1989) Electrophysiological evidence for a role of bradykinin in chemical nociception. *Neurosci. Letts.*, **97**, 198–202.

HARGREAVES, K.M., TROULLOS, E.S., DIONNE, R.A., SCHMIDT, E.A., SCHAFER, S.C. & JORIS, J.L. (1988). Bradykinin is increased during acute and chronic inflammation: therapeutic implications. *Clin. Pharmacol. Ther.*, **44**, 613–621.

HIDAKA, H. & HAGIWARA, M. (1987). Pharmacology of the isoquinoline sulfonamide protein kinase C inhibitors. *Trends Pharmacol. Sci.*, **8**, 162–164.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMAR, G., HENKE, ST., BREIPOL, G., KONIG, W., KNOLLE, J. & SCHOLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.*, **102**, 769–773.

KEELE, C.A. & ARMSTRONG, D. (1964). *Substances Producing Pain and Itch*. London: Edward Arnold.

LANG, E., NOVAK, A., REEH, P.W. & HANDWERKER, H.O. (1990). Chemosensitivity of fine afferents from rat skin in vitro. *J Neurophysiol.*, **63**, 887–901.

MCGEHEE, D.S. & OXFORD, G.S. (1989). Bradykinin-induced currents in rat DRG neurons and F-11 cells are insensitive to pertussis toxin. *Soc. Neurosci.*, **15**, 217.

MCGUIRK, S.M., VALLIS, PASTERNAK, C.A. & DOLPHIN, A.C. (1989). Bradykinin enhances excitability in cultured rat sensory neurons by a GTP-dependent mechanism. *Neurosci. Lett.*, **99**, 85–89.

MENSE, S. & SCHMIDT, R.F. (1974). Activation of Group IV afferent units from muscle by algesic agents. *Brain Res.*, **72**, 305–310.

MILLER, R.J. (1987). Bradykinin highlights the role of phospholipid metabolism in the control of nerve excitability. *Trends Neurol. Soc.*, **10**, 226–228.

RANE, S.G., WALSH, M.P., MCDONALD, J.R. & DUNLAP, K. (1989). Specific inhibitors of protein kinase C block transmitter-induced modulation of sensory neuron calcium current. *Neuron.*, **3**, 239–247.

RANG, H.P. & RITCHIE, M. (1988). Depolarization of non-myelinated fibres of the rat vagus nerve produced by activation of protein kinase C. *J. Neurosci.*, **8**, 2606–2617.

REGOLI, D. & BARABE, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–46.

REYNOLDS, M.L., FIZGERALD, M. & BENOWITZ, L.I. (1991). GAP-43 expression in developing cutaneous muscle nerves in the rat hindlimb. *Neuroscience*, **41**, 201–211.

STERANKA, R.R., MANNING, D., DEHASS, C.J., FERKANY, J.W., BOROSKY, S.A., CONNOR, J.R., VAVREK, R.J., STEWART, J.M. & SNYDER, S.H. (1988). Bradykinin as a pain mediator: receptors are localized to sensory neurons, and antagonists have analgesic actions. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3245–3249.

STERANKA, L.R., FARMER, S.G. & BURCH, R.M. (1989). Antagonists of B_2 bradykinin receptors. *FASEB J.*, **3**, 2019–2025.

TAIWO, Y.O. & LEVINE, J.D. (1991). Further confirmation of the role of adenyl cyclase and of cAMP dependent protein kinase in primary afferent hyperalgesia. *Neuroscience*, **44**, 131–135.

TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. *Biochem. Biophys. Res. Commun.*, **135**, 397–402.

THAYER, S.T., EWALD, D.A., PERNEY, T.M. & MILLER, R.J. (1988). Regulation of calcium homeostasis in sensory neurons by bradykinin. *J. Neurosci.*, **8**, 4089–4097.

VAVAREK, R.J. & STEWART, J.M. (1985). Competitive antagonists of bradykinin. *Peptides*, **6**, 161–164.

WEINREICH, D. (1986). Bradykinin inhibits a slow spike afterhyperpolarization of visceral sensory neurons. *Eur. J. Pharmacol.*, **132**, 61–63.

WEINREICH, D. & WONDERLIN, W.F. (1987). Inhibition of calcium-dependent spike after-hyperpolarization increases excitability of rabbit visceral sensory neurones. *J. Physiol.*, **394**, 415–427.

WILHELM, D.L. (1973). Chemical mediators. In *The Inflammatory Process*. 2nd edition. ed. Zweifach, B.W., Grant, L. & McLusky, R.T. pp. 251–301. New York: Academic Press.

(Received April 7, 1992
Revised July 14, 1992
Accepted August 12, 1992)

Br. J. Pharmacol. (1992), **107**, 1135–1139

Effects of cicletanine on haemodynamics, arrhythmias and extent of necrosis during coronary ligation in rabbits

T. Burton, S. Chakrabarty, D.S. Fluck, N.A. Flores & [1]D.J. Sheridan

Academic Cardiology Unit, St. Mary's Hospital Medical School, QEQM Wing, South Wharf Road, London W2 1NY

1 The effects of cicletanine on arrhythmias, haemodynamics and extent of necrosis during myocardial ischaemia were investigated in rabbits subjected to coronary ligation.

2 Cicletanine increased cardiac output prior to coronary occlusion ($P<0.01$) but had no other significant haemodynamic effects at this time and did not significantly alter heart rate, blood pressure or cardiac output during 30 min of ischaemia or 30 min of reperfusion.

3 Ventricular fibrillation and mortality were greater in control (65% and 60% respectively) than treated animals (15.4% and 15.4%, $P<0.01$).

4 The extent of myocardial necrosis expressed as a percentage of the area at risk was also reduced by cicletanine from $61 \pm 8\%$ in controls to $37 \pm 6\%$ ($P<0.05$).

5 These findings indicate that cicletanine attenuates arrhythmias and preserves myocardium in the early phase of ischaemia and this effect appears to be independent of an established antihypertensive action.

Keywords: Cicletanine; arrhythmias; myocardial infarction; necrosis; ligation; ischaemia; reperfusion; cardiac output; vascular resistance

Introduction

Cicletanine (1,3-dihydro-3(4-chlorophenyl)-7 hydroxy-6-methylfuro-(3,4C)-pyridine) is an antihypertensive agent which has been shown to possess antiarrhythmic properties in ischaemic myocardium (Jouve *et al.*, 1986; Tosaki *et al.*, 1990). The mechanism for this effect is unclear although the drug is known to have several pharmacological actions. It increases blood and urine levels of 6-keto-$PGF_{1\alpha}$, a stable metabolite of prostacyclin (PGI_2) in hypertensive patients (Garay *et al.*, 1983), enhances PGI_2 production in rat cultured smooth muscle cells (Dorian *et al.*, 1984) and stimulates potassium fluxes in human erythrocytes (Garay *et al.*, 1984). It has also been shown to reverse potassium ion loss and sodium ion gain in ischaemic myocardium (Tosaki *et al.*, 1990), although it is not clear whether this is the primary mode of protection, or whether it occurs secondary to some other myocardial protective effect. Neither is it clear whether this protective effect is associated with tissue preservation. The purpose of this study was to investigate the effects of cicletanine on haemodynamics, arrhythmias and tissue necrosis during experimental coronary ligation in order to establish whether its antiarrhythmic action in ischaemic myocardium is associated with early tissue preservation and whether such protective effects may be explained on the basis of its antihypertensive action.

Methods

New Zealand White rabbits (2.5–3.0 kg) were maintained at 21–22°C, $50 \pm 5\%$ humidity and 12/12 hourly light/dark cycles for 3 to 5 days before study. Animals were fed on R14 'high fibre without grass' meal and water *ad libitum*. All experiments were carried out in a single laboratory maintained at 21–22°C throughout the year between 09 h 00 min and 18 h 00 min.

Surgical procedures

Anaesthesia was induced with alphaxalone (9 mg kg^{-1}, i.v.) and maintained with pentobarbitone (25 mg kg^{-1}, i.v.). Tracheostomy and tracheal intubation were performed and animals were ventilated with room air by means of a mechanical pump (CF Palmer (London) Ltd., Model No. 16/24) at a fixed rate of 45 strokes per minute and a tidal volume of 25–30 ml. Body temperature was monitored with an oesophageal thermistor and maintained at a constant level with an overhead lamp. Blood pH and Po_2 were measured with a Corning blood gas analyser (Model No. 158). The right carotid artery was cannulated to monitor the arterial pressure with a Lectromed transducer (No. 3552) and ECG leads were connected to the four limbs and the signals amplified by use of a Lectromed ECG amplifier. Arterial pressure and ECG signals were continuously recorded on a Lectromed chart recorder. Systemic vascular resistance was calculated as the quotient of mean arterial pressure and cardiac output.

A thoracotomy was performed via a left parasternal incision between the 4th and 6th ribs. The heart was exposed by incising the pericardium and was then supported in a pericardial cradle. An electromagnetic flow probe was placed around the ascending aorta and connected to a Spectromed blood flow meter, model SP2202B. The left ventricular branch of the circumflex artery was identified and a loose 2/0 polyester ligature was placed around it adjacent to its origin. Each preparation was allowed to stabilize for 45 min before coronary occlusion. Regional ischaemia was produced by use of a snare made of polyethylene tubing 2.5 cm in length and 3 mm in diameter which was threaded over the suture and clamped firmly in place for 30 min. Reperfusion was induced by releasing the clamp. Following 30 min of reperfusion hearts were removed for measurement of the extent of myocardial damage.

Drug administration

Cicletanine 10 mg kg^{-1} (obtained from Institut Henri Beaufour, Le Plessis Robinson, France) was dissolved in 0.5 ml

[1] Author for correspondence.

dimethylsulphoxide (DMSO), dispersed in 2 ml distilled water and given intravenously 10 min before coronary occlusion in 13 animals. Twenty animals received vehicle only and served as controls. Unequal numbers were studied so as to offset the greater survival in the cicletanine group and provide similar numbers of hearts for infarct size measurement.

Arrhythmia analysis

The occurrence of premature ventricular complexes, ventricular tachycardia (VT) and ventricular fibrillation (VF) was noted from ECG recordings. Following 30 min of ischaemia, only those animals which remained in sinus rhythm were reperfused and the incidence of VT and VF during reperfusion was noted. Animals which reverted to sinus rhythm spontaneously and were in sinus rhythm at the end of the reperfusion period were regarded as survivors.

Infarct size measurements

At the end of the experimental period the heart was removed and perfused with warm 6% dextran 70 in normal saline. The ligature was retightened and the coronary circulation perfused with 0.5% monastral blue and 6% dextran 70 in normal saline to determine the area at risk. The heart was then cut into four sections from base to apex parallel to the atrioventricular groove, each approximately 2 mm thick. The sections were photographed onto a reference grid and then washed in cold saline and placed in freshly prepared 0.5% nitrobluetetrazolium at 37°C for 10 min. They were then washed in water and placed in 40% formalin solution for fixation. The sections were photographed with a reference grid on 35 mm colour slides and their projected images were used to measure the area at risk and areas of infarction and non-infarction using computerized planimetry. The volumes of each section were calculated as the product of the planimetered areas and the section thickness. The area at risk was expressed as a percentage of the total left ventricular volume. The volume of infarction was calculated as a percentage of each section, of the total left ventricular volume and of the area at risk.

Statistical analysis

Haemodynamic parameters are presented as means ± standard error. Results were compared by Student's paired t test within groups and the Wilcoxon rank sum test between groups. Arrhythmia analysis was performed with the Chi-squared test and infarct size analysis was performed by use of Student's unpaired t test. Statistical significance was accepted if $P<0.05$.

Results

Haemodynamics

Changes in heart rate, arterial pressure, cardiac output and systemic vascular resistance are illustrated in Figure 1. Coronary ligation produced an immediate fall in blood pressure and cardiac output and both remained significantly depressed at the end of the reperfusion phase. Heart rate remained unchanged throughout the periods of ischaemia and reperfusion in control animals but fell slightly following coronary ligation in the cicletanine-treated group. Cicletanine increased cardiac output from 308 ± 18 ml min^{-1} to 346 ± 21 ml min^{-1} prior to coronary occlusion ($P<0.01$). Heart rate and blood pressure were not significantly altered by cicletanine. Blood pressure and cardiac output were slightly higher in the cicletanine group during ischaemia and reperfusion but these were not statistically significant. Systemic vascular resistance was increased following coronary ligation from 24000 ± 1900 dyn s cm^{-5} to 29350 ± 4600 dyn s cm^{-5} at 30 min, $P<0.01$ and fell sharply on reperfusion. This was not altered by treatment with cicletanine (Figure 1d).

Ventricular arrhythmias

The incidence of ventricular arrhythmias observed during 30 min of ischaemia is illustrated in Figure 2. Premature ventricular complexes (PVCs) were counted as isolated complexes only, excluding beats during ventricular tachycardia. A mean of 53.1 ± 8.7 PVCs occurred in control animals during ischaemia and this was significantly greater than in the cicletanine-treated group (31.4 ± 8.6), $P<0.01$. Ventricular tachycardia occurred in 13 of 20 controls (65%) compared with 5 of 12 (38.5%) cicletanine-treated animals. Ventricular fibrillation occurred in 13 of 20 controls (65%) compared with 2 of 12 (15.4%) cicletanine-treated animals, $P<0.01$. Ventricular tachycardia occurred in 3 of 8 control (37.5%) animals during reperfusion and 3 of 11 (27.3%) treated ones. Ventricular fibrillation occurred during reperfusion in 1 control animal and in none of the treated group.

Area at risk and infarct size

The area of myocardium at risk was identical in both the control (46 ± 6% of total left ventricular volume) and cicletanine-treated groups (47 ± 6%), (Figure 3). The extent of left ventricular necrosis was 31 ± 7% in control animals and 18 ± 4% in rabbits treated with cicletanine. Cicletanine reduced the area of infarction (expressed in terms of area at risk) from 61 ± 8% to 37 ± 6%, ($P<0.05$).

Discussion

This study illustrates that coronary occlusion for 30 min in the rabbit results in substantial myocardial necrosis, haemodynamic impairment characterized by a fall in cardiac output and blood pressure and lethal ventricular arrhythmias. Pretreatment with cicletanine reduced the incidence of ventricular fibrillation and PVCs and attenuated the extent of myocardial necrosis. However, these protective effects occurred in the absence of any significant effect on cardiac output, blood pressure or systemic vascular resistance. These findings demonstrate that cicletanine exerts a myocardial protective effect during ischaemia resulting in early tissue preservation and antiarrhythmic efficacy by a mechanism that is likely to be directly acting on the myocardium or its perfusion rather than on the peripheral circulation.

In the present studies cicletanine did not exert a hypotensive effect as might have been expected. Indeed a small increase in blood pressure and cardiac output may have occurred following treatment prior to ligation, Figure 1. The absence of an acute fall in blood pressure following treatment with cicletanine is nevertheless consistent with its clinical efficacy. Studies in hypertensive patients have demonstrated that development of the full antihypertensive effect of cicletanine requires several weeks of treatment (Tarrade & Guinot, 1988; Fodor & Guinot, 1988). These findings suggest that the myocardial preservation observed here is independent of the antihypertensive effect of cicletanine and that it cannot be explained on the basis of reduced cardiac work resulting from a lowering of systemic vascular resistance. Although systemic vascular resistance was unaffected by cicletanine in the present study this does not exclude an effect of the drug on aortic compliance. A previous study suggests that cicletanine may increase aortic compliance in the absence of any change in systemic vascular resistance (Levy et al., 1989). Such an effect could contribute to myocardial preservation during ischaemia by reducing left ventricular energy requirements.

The antiarrhythmic effect of cicletanine observed here confirms this finding in other studies (Jouve et al., 1986;

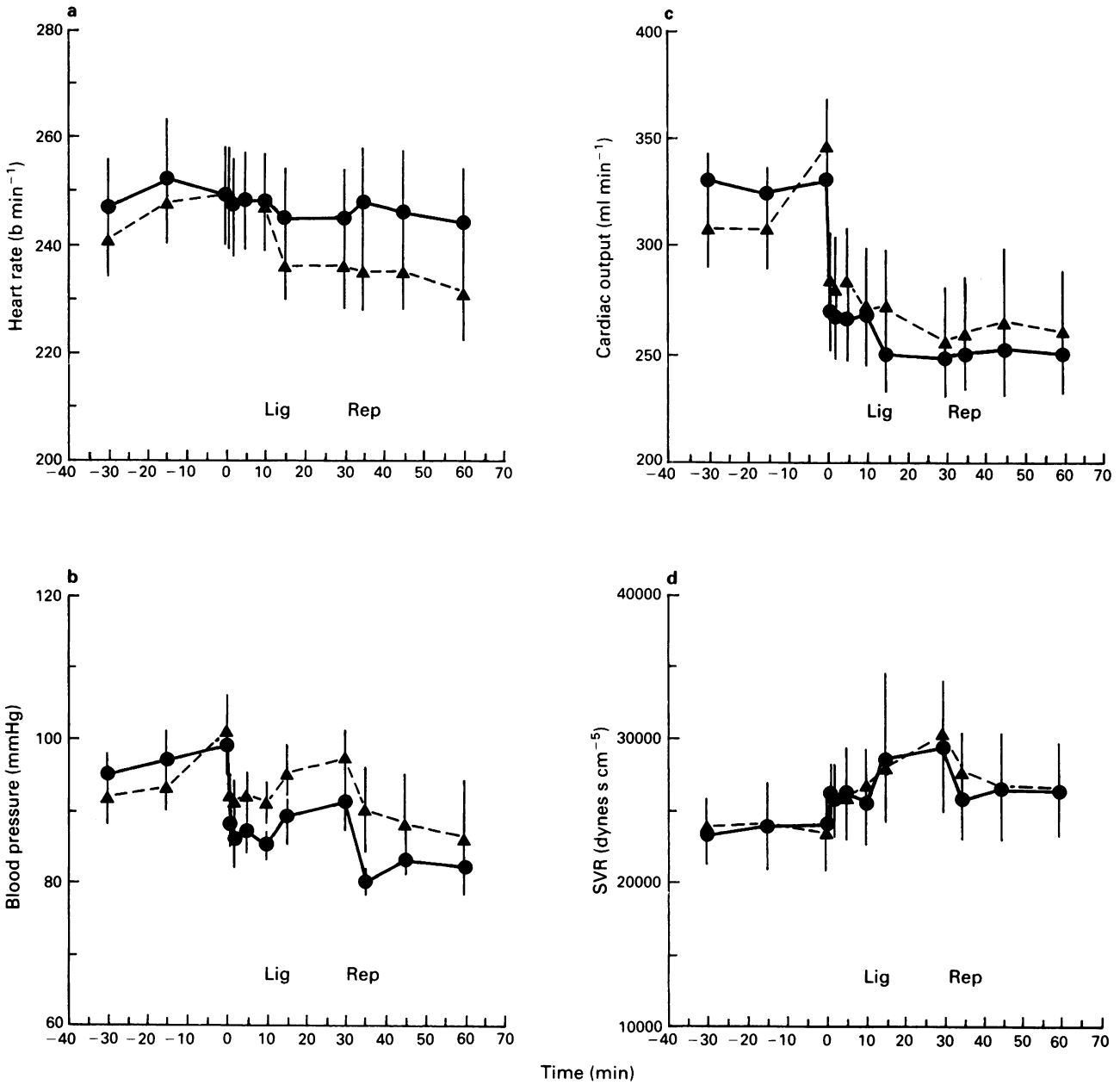

Figure 1 Changes in heart rate (a), blood pressure (b), cardiac output (c) and systemic vascular resistance [SVR] (d) in control (●) and cicletanine-treated (▲) rabbits during coronary ligation (Lig) and reperfusion (Rep). Blood pressure and cardiac output fell significantly following ligation while systemic vascular resistance increased and heart rate remained unchanged. Although blood pressure and cardiac output tended to be higher and heart rate lower during ischaemia and reperfusion in the treated group none was statistically significant.

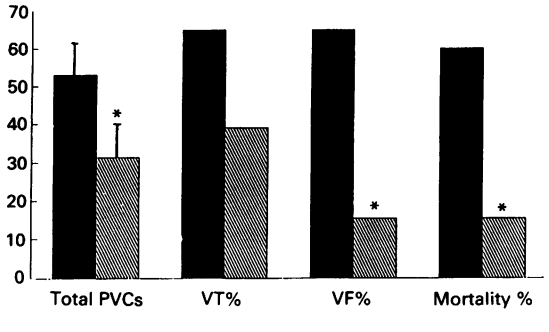

Figure 2 Effect of cicletanine on arrhythmias during ischaemia. Cicletanine (hatched columns) significantly reduced the incidence of premature ventricular complexes (PVCs), ventricular fibrillation (VF) and mortality during ischaemia; controls:solid columns. $*P<0.01$.

Tosaki *et al.*, 1990; Koltai *et al.*, 1992). One possible mechanism for this antiarrhythmic effect is the ability of cicletanine to reduce tissue electrolyte changes associated with ischaemia and reperfusion. Thus cicletanine reduced Na^+ and Ca^{2+} gain and K^+ loss during global ischaemia in rat hearts (Tosaki *et al.*, 1990; Koltai *et al.*, 1992). Since this action was observed in both acutely- and chronically-treated isolated perfused hearts it must reflect a direct action on the heart.

An increase in myocardial perfusion during ischaemia could explain the antiarrhythmic and tissue preservation actions observed here. There is no direct evidence to support such an effect; however, cicletanine is known to relax vascular smooth muscle and to inhibit several vasoconstrictive stimuli (Auguet *et al.*, 1988). A similar effect on coronary vessels could result in increased tissue perfusion during

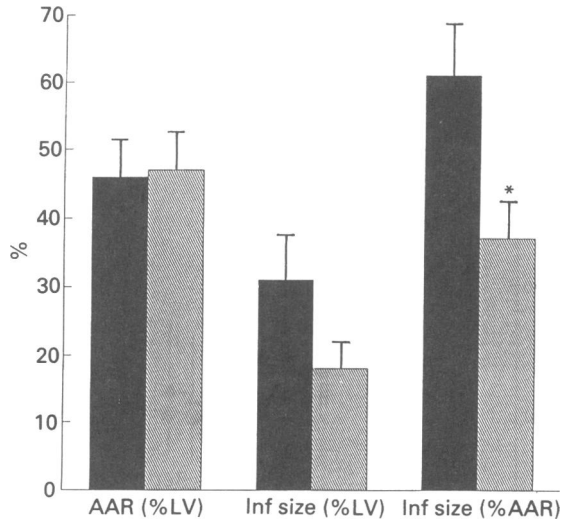

Figure 3 Effect of cicletanine (hatched columns) on the area at risk (AAR) and extent of myocardial necrosis expressed as a percentage of total left ventricular volume (LV); controls:solid columns. Cicletanine significantly reduced the extent of necrosis (Inf size) when expressed as a percentage of the area at risk, $*P<0.05$.

ischaemia, perhaps via collateral vessels. It is more difficult to invoke such a mechanism for beneficial effects observed during global ischaemia (Jouve *et al.*, 1986; Tosaki *et al.*, 1990), where increased collateral flow is not possible, nevertheless reduced coronary resistance could improve tissue perfusion and washout. Further work is needed to clarify the effects of cicletanine on the coronary vasculature.

A number of studies indicate that prostanoids may contribute to the arrhythmogenic effects of ischaemia and reperfusion (Araki & Lefer, 1980; Coker & Parratt, 1985; Parratt & Coker, 1985; Karmazyn, 1986). In particular interest has centred on the deleterious effects of thromboxane A_2 (Coker & Parratt, 1985) and possible beneficial effects of prostacyclin (Araki & Lefer, 1980). For example, inhibition of thromboxane has been shown to reduce ventricular arrhythmias in dogs during ischaemia and reperfusion (Coker & Parratt, 1985), while prostacyclin has been shown by some workers to improve tissue preservation (Araki & Lefer, 1980) but not by others (Moffat, 1987). Cicletanine increased blood and urine levels of 6-keto-$PGF_{1\alpha}$, a stable metabolite of prostacyclin (PGI_2) in hypertensive patients (Garay *et al.*, 1983), increased PGI_2 production in rat aortic smooth muscle cells (Dorian *et al.*, 1984) and increased plasma prostacyclin levels in anaesthetized rabbits (Bourgain *et al.*, 1989). Similar effects on ischaemic myocardium could explain the reduction in arrhythmias and necrosis observed here. Jouve *et al.* (1986) reported that the antiarrhythmic effects of cicletanine which they observed in dogs were associated with increased prostacyclin production, although Tosaki *et al.* (1991) reported that the antiarrhythmic effect of cicletanine seen in rats was not associated with an alteration in release of 6-keto $PGF_{1\alpha}$ or thromboxane during reperfusion but correlated better with alterations in myocardial ion content (Koltai *et al.*, 1990). This may indicate species differences, although such studies are complicated by the fact that reperfusion following an ischaemic period is associated with washout of metabolites accumulated during the preceding period of ischaemia as well as production during reperfusion and therefore may not accurately reflect biological activity. The precise role of prostaglandins in mediating the effects of cicletanine must remain putative.

The antinecrotic effect of cicletanine observed here represents an important degree of myocardial salvage. In these studies necrosis tended to be greater at the apex than base of the heart. This is likely to reflect the artery chosen for ligation; the left ventricular branch of the circumflex is the largest coronary artery in the rabbit and supplies most of the apex (Flores *et al.*, 1984). It should be remembered however that this effect was measured following 30 min of ischaemia plus 30 min of reperfusion. The findings therefore indicate early myocardial salvage. The effects observed could have resulted from either a delay or a reversal of the necrotic process. It is not clear from these experiments whether similar beneficial effects would result in the presence of a maintained occlusion. Neverthless any inhibition of the development of necrosis following coronary occlusion could provide important clinical benefit particularly in the context of thrombolysis.

In conclusion this study confirms the previously observed antiarrhythmic effect of cicletanine in ischaemic myocardium and extends this by demonstrating significant myocardial preservation. These effects appear to be independent of an established antihypertensive effect.

References

ARAKI, H. & LEFER, A.M. (1980). Role of prostacyclin in the preservation of ischemic cardiac tissue in the perfused cat heart. *Circ. Res.*, **47**, 757–763.

AUGUET, M., GUILLON, J.M., DELAFLOTTE, S., LE HEGARAT, M., PIROTZKY, E., CLOSTRE, F. & BRAQUET, P. (1988). In vitro cardiovascular antihistamine properties of cicletanine in comparison with diphenhydramine. *Drugs Exp. Clin. Res.*, **14**, 149–153.

BOURGAIN, R.H., DEBY, C. & ANDRIES, R. (1989). Effet du ciclétanine sur la génération de prostacycline in vivo. *Arch. Mal. Coeur*, **82**, 33–36.

COKER, S.J. & PARRATT, J.R. (1985). AH23848, a thromboxane antagonist, suppresses ischaemic and reperfusion induced arrhythmias in anaesthetised greyhounds. *Br. J. Pharmacol.*, **86**, 259–266.

DORIAN, B., LARRUE, J., DEFEUDIS, F.V., SALARI, H., BORGEAT, P. & BRAQUET, P. (1984). Activation of prostacyclin synthesis in cultured smooth muscle cells by diuretic antihypertensive drugs. *Biochem. Pharmacol.*, **33**, 2265–2269.

FLORES, N.A., DAVIES, R. LI., PENNY, W.J. & SHERIDAN, D.J. (1984). Coronary microangiography in the guinea pig, rabbit and ferret. *Int. J. Cardiol.*, **6**, 459–471.

FODOR, G.P. & GUINOT, P. (1988). Review of three studies to determine the efficacy and tolerance of cicletanine in the short and long term treatment of hypertension. *Drugs Exp. Clin. Res.*, **14**, 195–204.

GARAY, R.P., HORNICH, A., JUIN, G., NAZARET, C., HANAERT, P., PAILLETTE, E. & BRAQUET, P. (1983). K transport, membrane potential and the AA cascade in the vasodilator antihypertensive effects of cicletanine. II Clinical aspects. *Arch. Pharmacol.*, **324**, 240.

GARAY, R.P., NAZARET, C., DIEZ, J., ETIENNE, A., BOURAGIN, R. & BRAQUET, P. (1984). Stimulation of K fluxes by diuretic drugs in red cells. *Biochem. Pharmacol.*, **33**, 2013–2020.

JOUVE, R., LANGLET, F., PUDDU, P.E., ROLLAND, P.H., GUILLEN, J.C., CANO, J.P. & SERRADIMIGNI, A. (1986). Cicletanine improves outcome after left circumflex coronary artery occlusion-reperfusion in the dog. *J. Cardiovasc. Pharmacol.*, **8**, 208–215.

KARMAZYN, M. (1986). Contribution of prostaglandins to reperfusion induced ventricular failure in isolated rat hearts. *Am. J. Physiol.*, **251**, H133–H140.

KOLTAI, M., TOSAKI, A., BERTHET, P., TARRADE, T., ESANU, A. & BRAQUET, P. (1992). Effect of cicletanine on reperfusion-induced arrhythmias and myocardial ion contents: a comparison with furosemide. *Eur. Heart J.*, **13**, 395–403.

LEVY, B.I., CURMI, P., POITEVIN, P. & SAFAR, M.E. (1989). Modifications of the arterial mechanical properties of normotensive and hypertensive rats without arterial pressure changes. *J. Cardiovasc. Pharmacol.*, **14**, 253–259.

MOFFAT, M.P. (1987). Concentration dependent effects of prostacyclin on the response of the isolated guinea pig heart to ischaemia and reperfusion: possible involvement of the slow inward current. *J. Pharmacol. Exp. Ther.*, **242**, 292–299.

PARRATT, J.R. & COKER, S.J. (1985). Arachidonic acid cascade and the generation of ischaemia and reperfusion induced ventricular arrhythmias. *J. Cardiovasc. Pharmacol.*, **7**, (Suppl 5), S65–S70.

TARRADE, T. & GUINOT, P. (1988). Efficacy and tolerance of cicletanine, a new antihypertensive agent: overview of 1226 treated patients. *Drugs Exp. Clin. Res.*, **14**, 205–214.

TOSAKI, A., KOLTAI, M., WILLOUGHBY, D.A. & BRAQUET, P. (1990). Effect of cicletanine on reperfusion induced arrhythmias and ion shifts in isolated rat hearts. *J. Cardiovasc. Pharmacol.*, **15**, 218–226.

TOSAKI, A., HELLEGOUARCH, A. & BRAQUET, P. (1991). Cicletanine and reperfusion injury: Is there any correlation between arrhythmias, 6-keto-$PGF_{1\alpha}$, thromboxane B_2, and myocardial ion shifts (Na^+, K^+, Ca^{++}, and Mg^{++}) induced by ischemia/reperfusion in isolated rat heart. *J. Cardiovasc. Pharmacol.*, **17**, 551–559.

(Received July 31, 1992
Accepted August 12, 1992)

Br. J. Pharmacol. (1992), **107**, 1140–1145

Role of protein kinase C in the regulation of histamine and bradykinin stimulated inositol polyphosphate turnover in adrenal chromaffin cells

[1]Michael R. Boarder & R.A. John Challiss

Department of Pharmacology and Therapeutics, University of Leicester, University Road, Leicester, LE1 9HN

1 The possibility that bradykinin- or histamine-stimulated inositol polyphosphate accumulation may be regulated by protein kinase C (PKC) in bovine adrenal chromaffin cells has been addressed.

2 Initial experiments confirmed that the phorbol ester 12-O-tetradecanoyl-phorbol 13-acetate (TPA) dramatically inhibited agonist-stimulated [^{3}H]-inositol phosphate accumulations in [^{3}H]-inositol prelabelled cells. In contrast, the PKC inhibitor, Ro 31-8220, did not affect this response.

3 Histamine (100 μM) or bradykinin (100 nM) evoked rapid increases in inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) mass accumulations (maximal accumulations within 10 s and 30 s, respectively) which declined towards basal values over a 10 min incubation period. TPA (1 μM) significantly attenuated the peak Ins(1,4,5)P_3 response to bradykinin and histamine by 30% and 70% respectively. In contrast, TPA did not significantly affect agonist-stimulated Ins(1,3,4,5)P_4 responses.

4 Ro 31-8220 (10 μM) significantly enhanced the maximal Ins(1,4,5)P_3 accumulations elicited by both bradykinin and histamine.

5 The results indicate that the initial Ins(1,4,5)P_3 response to either bradykinin or histamine in bovine adrenal chromaffin cells can be attenuated by PKC activation by phorbol ester and enhanced by PKC inhibition by Ro 31-8220. In contrast, agonist-stimulated Ins(1,3,4,5)P_4 accumulation does not appear to be affected by these manipulations of PKC activity. Possible bases for differential modulation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are discussed.

Keywords: Inositol polyphosphates; protein kinase C; histamine; bradykinin; phorbol ester; Ro 31-8220; adrenal chromaffin cell

Introduction

The role and regulation of agonist-stimulated hydrolysis of inositol phospholipids in neuronal function is poorly understood. This is true for one of the most widely studied neurone-like cells in culture, the bovine adrenal chromaffin cell, in which a variety of agonists stimulate both polyphosphoinositide hydrolysis and catecholamine release (Livett & Marley, 1986; Eberhard & Holz, 1987; Plevin & Boarder, 1988; Plevin *et al.*, 1990; Burgoyne, 1991; Challiss *et al.*, 1991). One such agonist, bradykinin, has been shown to stimulate inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) accumulation and to cause an elevation of free cytosolic Ca^{2+}, in part, by mobilization of intracellular pools (O'Sullivan & Burgoyne, 1989; Challiss *et al.*, 1991). However, the role this plays in stimulus-secretion coupling is unclear, since release of noradrenaline in response to bradykinin appears to be dependent upon agonist-stimulated Ca^{2+} influx via a cadmium-sensitive, dihydropyridine-insensitive route (Owen *et al.*, 1989).

The role of agonist-stimulated inositol phospholipid-specific phospholipase C (PLC) may be related to protein kinase C (PKC) stimulation via the formation of *sn* 1,2-diacylglycerol (DAG). One of several possible consequences for the activation of this arm of the second messenger pathway is the presence of a short inhibitory feedback loop in which stimulated DAG accumulation and PKC activity cause an attenuation of agonist-stimulated PLC. This mechanism has been proposed for the regulation of agonist-stimulated PLC in a number of non-neuronal cells (Helper *et al.*, 1988; King & Rittenhouse, 1989; Pfeilschifter *et al.*, 1989). We, and others, have previously shown that activation of PKC does attenuate histamine-stimulated formation of total [^{3}H]-inositol phosphates in [^{3}H]-inositol prelabelled adrenal chromaffin cells (Wan *et al.*, 1989; Jones *et al.*, 1990).

In this study we have investigated the effects of altering PKC activity upon agonist stimulation of inositol polyphosphate accumulation using both the PKC activator 12-O-tetradecanoylphorbol 13-acetate (TPA) and the selective PKC-inhibitor Ro 31-8220 (referred to as compound 3 in Davis *et al.*, 1989). We have measured the response to stimulation by bradykinin and histamine of Ins(1,4,5)P_3 and the putative second messenger inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) concentrations by the mass assays previously described for these inositol polyphosphate isomers (Challiss *et al.*, 1988; Challiss & Nahorski, 1990).

Methods

Cell culture

Bovine adrenal glands were obtained from a local abattoir within 30 min of slaughter. Cells from the medulla were dissociated by digestion with collagenase/protease, and the chromaffin cells purified (routinely to 80% or greater of total cell number) by differential centrifugation and differential plating as described earlier (Owen *et al.*, 1989). Cells were cultured at 0.5×10^6 cells per well on 24-well primaria plates at a cell protein concentration of about 50 μg/well. Cells were used after 4–7 days in culture.

[^{3}H]-inositol labelling and [^{3}H]-inositol phosphate determination

For 48 h preceding the experiments, cells were cultured in M199 medium (0.5 ml) containing 37 kBq [^{3}H]-inositol per

[1] Author for correspondence.

well and supplemented with streptomycin (50 $\mu g\ ml^{-1}$), penicillin (50 i u ml^{-1}) and cytosine arabinoside (5 μM). Cells were then incubated for 10 min in a balanced salt solution (BSS (in mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid 15, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8 and glucose 5.5) pH 7.4 following oxygenation with O_2/CO_2 (19:1). This medium was removed and the same BSS supplemented with 10 mM LiCl used for the incubations (30 min). Reactions were terminated with cold methanol, followed by chloroform extraction and the total [^{3}H]-inositol phosphate ([^{3}H]-$InsP_x$) fraction recovered by use of Dowex-1 (Cl^- form) as described by Rooney & Nahorski (1986).

Measurement of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulations

Cells cultured as described above were washed twice with the BSS solution prewarmed to 37°C. Preincubations were for 15 min, followed by incubation for the time periods stated in the Results section. Drugs present during the preincubation (TPA and/or Ro 31-8220) were also present for the period of stimulation with histamine or bradykinin (or control). Reactions were terminated by rapid removal of the incubation medium followed by immediate replacement with 0.5 ml of ice-cold trichloroacetic acid (0.5 M). Wells were thoroughly scraped and the recovered extract centifuged (10,000 *g*, 4 min). The acid extract was washed with 3 × 3 vol of water-saturated diethylether, and the extract buffered to pH 7 with $NaHCO_3$. Radioreceptor-based mass assays for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were performed as described previously (Challiss *et al.*, 1988; Challiss & Nahorski, 1990).

Statistical analysis

Data are expressed as means ± s.e.mean for the indicated number of experiments performed on different preparations of chromaffin cells. Time-course data were analyzed by unpaired Student's *t* tests. Concentration-response data were analyzed by one-way analysis of variance; where the *F* ratio gave $P<0.05$, comparisons between individual groups were made by Duncan's multiple range test at significance levels of 0.05 and 0.01.

Materials

Myo-[2-^{3}H]-inositol (17 Ci $mmol^{-1}$) was purchased, whilst [^{3}H]-Ins(1,4,5)P_3 (17 Ci $mmol^{-1}$) and [^{32}P]-Ins(1,3,4,5)P_4 (100–200 Ci $mmol^{-1}$) were obtained as gifts, from DuPont (U.K.) Ltd. (Stevenage, Herts.). Bradykinin was from Cambridge Research Biochemicals Ltd. (Northwich, Cheshire). Ro 31-8220, a bis indolylmaleimide derivative whose structure is given by Davis *et al.* (1989), was a kind gift from Dr. G. Lawton, Roche Products Ltd. (Welwyn Garden City, Herts). Cell culture supplies were from GIBCO (Paisley, Scotland) and other chemicals and drugs were from Sigma Chemical Co. Ltd. (Poole, Dorset) or Fisons plc (Loughborough, Leics.).

Results

Effect of TPA and Ro 31-8220 on agonist-stimulated [^{3}H]-$InsP_x$ accumulation

In agreement with our previous studies (Plevin & Boarder, 1988; Jones *et al.*, 1990), preliminary experiments demonstrated that exposure of chromaffin cells to 100 nM bradykinin or 100 μM histamine for 30 min resulted in 2–3 fold and 5–7 fold increases in [^{3}H]-$InsP_x$ accumulations, respectively (Figure 1a).

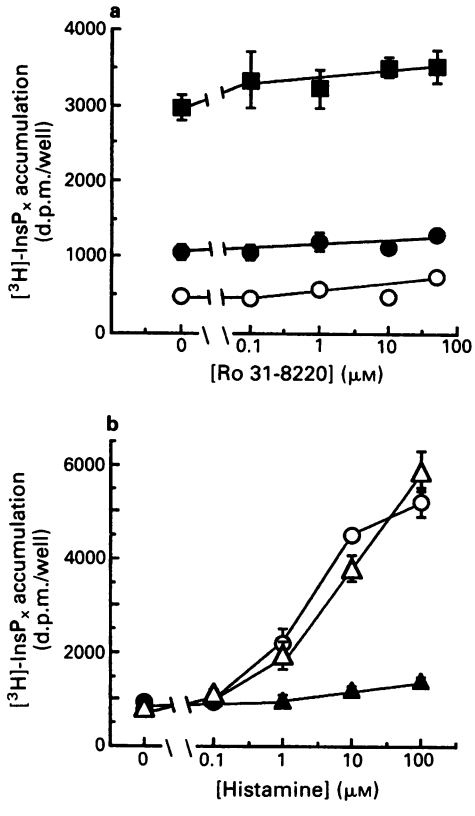

Figure 1 Effects of 12-O-tetradecanoyl-phorbol 13-acetate (TPA) and Ro 31-8220 on total [^{3}H]-inositol phosphate ([^{3}H]-$InsP_x$) responses to bradykinin and histamine in chromaffin cells. (a) Concentration-response relationship for the effect of Ro 31-8220 on [^{3}H]-$InsP_x$ accumulation at 30 min in the absence (O) or presence of bradykinin (100 nM: ●) or histamine (100 nM: ■). (b) Concentration-response relationship for the effect of 30 min exposure to histamine in the absence (O) or presence of TPA (1 μM: ▲) or Ro 31-8220 (10 μM: △). Values are shown as means ± s.e.mean (vertical bars) for a representative experiment of three separate experiments performed in triplicate.

The [^{3}H]-$InsP_x$ accumulation elicited in response to either agonist was dramatically attenuated in the presence of 1 μM TPA; in contrast, the protein kinase C inhibitor Ro 31-8220 (at concentrations up to 50 μM) had little effect on basal, histamine- or bradykinin-stimulated [^{3}H]-$InsP_x$ accumulation (Figure 1a). Time-course studies confirmed these effects of TPA and Ro 31-8220; for example, 1 μM TPA significantly attentuated the histamine-stimulated [^{3}H]-$InsP_x$ response at the earliest time-point investigated (5 min) and caused an 85–95% inhibition of this response at 30 min after histamine challenge (data not shown). Similarly, concentration-response relationships for histamine-stimulated [^{3}H]-$InsP_x$ accumulation demonstrated the inhibitory action of TPA at all stimulatory concentrations of histamine (1–100 μM), whilst Ro 31-8220 (10 μM) had no significant effect (Figure 1b).

It should be noted that despite the lack of effect of Ro 31-8220, this agent was able to antagonize completely the inhibitory action of TPA on histamine-stimulated [^{3}H]-$InsP_x$ accumulation (data expressed as a % of the control response (100 ± 8): 100 μM histamine 724 ± 70; histamine + TPA (1 μM) 189 ± 18; histamine + TPA + Ro 31-8220 (10 μM) 744 ± 67, for 2 experiments performed in quadruplicate).

Modification of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 responses

We have previously characterized the rapid, transient changes in both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 mass accumulations

elicited by maximally effective concentrations of bradykinin and histamine in cultured bovine adrenal chromaffin cells (Challiss *et al.*, 1991): since publication of our original data, a number of studies have reported a biphasic accumulation of Ins(1,4,5)P_3 mass, where the initial, rapid rise and fall in Ins(1,4,5)P_3 level is followed by a secondary increase in Ins(1,4,5)P_3 over the subsequent time-course of continued agonist exposure (Roche *et al.*, 1991; Tobin *et al.*, 1992). For example, Roche *et al.* (1991) have reported that gastrin stimulates an initial Ins(1,4,5)P_3 accumulation in parietal cells which is maximal at 15 s and decreases towards pre-stimulation levels by 30 s; however, over the subsequent 4–5 min period of continued agonist exposure the Ins(1,4,5)P_3 level increases to, or beyond, the 15 s peak value. Some evidence for this unusual response has also been presented recently by Stauderman & Pruss (1990) for histamine-stimulated [^{3}H]-Ins(1,4,5)P_3 accumulation in [^{3}H]-inositol pre-labelled bovine adrenal chromaffin cells. We have therefore carried out preliminary extended time-course studies to ascertain whether a similar biphasic inositol polyphosphate mass accumulation occurs in response to bradykinin or histamine.

The results summarized in Table 1 substantiate our previous data with regard to the relative stimulatory effects of bradykinin and histamine on Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulation, and indicate that no secondary increases, subsequent to the detailed 0–120 s time courses reported previously (and see Figures 2 and 3) are observed. Thus, bradykinin (100 nM) elicited an 156% in-crease in Ins(1,4,5)P_3 accumulation at 10 s and the accumulation of this metabolite then declined, such that by 10 min after agonist addition the Ins(1,4,5)P_3 accumulation was not significantly elevated above basal levels. Although histamine (100 μM) evoked a smaller 51% increase in Ins(1,4,5)P_3, this metabolite was maintained at a significantly elevated level over the 10 min investigation period. The transient nature of the agonist-stimulated Ins(1,3,4,5)P_4 response was also well-illustrated by these data. Thus, bradykinin and histamine evoked respectively 675% and 250% increases in Ins(1,3,4,5)P_4 accumulation at 30 s which returned to levels not significantly different from basal values by 5 min post-agonist addition.

For further investigations to evaluate the effects of agents which alter PKC activity, we have concentrated on the initial 2 min period after addition of the agonist. The effect of 1 μM TPA upon the time-course of bradykinin-stimulated Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulation is shown in Figure 2. TPA significantly inhibited the Ins(1,4,5)P_3 response, producing a 30% attenuation of the maximal increase in mass accumulation. In contrast, TPA was without effect on the Ins(1,3,4,5)P_4 response elicited by bradykinin (Figure 2b). TPA exerted a more profound effect upon histamine-stimulated Ins(1,4,5)P_3 accumulation producing a 70% attenuation of the maximal response observed 10 s after

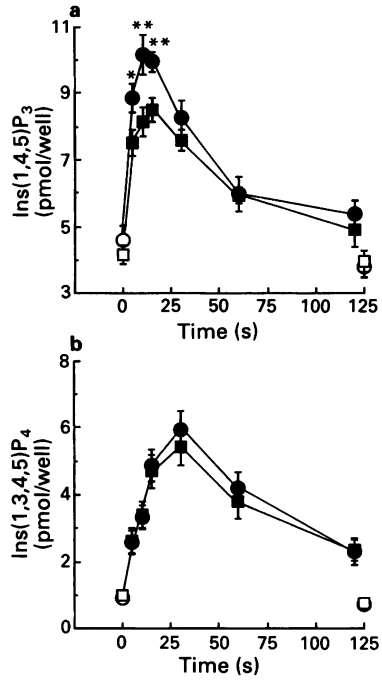

Figure 2 Effect of 12-O-tetradecanoyl-phorbol 13-acetate (TPA) on bradykinin-stimulated inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) (a) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) (b) mass accumulations in chromaffin cells. Cultured bovine adrenal chromaffin cells were pre-incubated for 10 min in the absence (○, ●) or presence of 1 μM TPA (□, ■). Additions of 100 nM bradykinin (●, ■) or vehicle (○, □) were made at zero-time. Values are means ± s.e.mean (vertical bars) from either 3 (Ins(1,3,4,5)P_4) or 4 (Ins(1,4,5)P_3) separate experiments performed in triplicate.

Statistically significant differences between cell responses in the presence and absence of TPA are indicated as $*P<0.05$, $**P<0.01$.

agonist addition (Figure 3a); despite this dramatic attenuation of the Ins(1,4,5)P_3 response, TPA again failed to exert any significant effect on agonist-stimulated Ins(1,3,4,5)P_4 accumulation (Figure 3b).

The effects of increasing concentrations of TPA on Ins(1,4,5)P_3 accumulation stimulated by bradykinin, histamine or control additions to chromaffin cells for 10 s are shown in Figure 4. Statistically significant attenuations of the bradykinin-stimulated response were observed in the presence of 100 nM TPA (31%) and 1 μM TPA (38%), whereas 1 μM TPA caused a 64% attenuation of the histamine-stimulated Ins(1,4,5)P_3 response. In contrast, the relatively inactive phorbol ester 4-methoxy TPA failed to affect bradykinin-stimulated Ins(1,4,5)P_3 accumulation at concentrations of 100 nM or 1 μM.

Table 1 Initial time-courses of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulations elicited by bradykinin and histamine

	Time (s)				
	10	30	60	300	600
Ins(1,4,5)P_3 (pmol/well)					
Control	4.60 ± 0.43	4.97 ± 0.46	4.55 ± 0.36	4.71 ± 0.16	4.92 ± 0.26
Bradykinin	11.78 ± 1.25***	8.40 ± 0.27***	7.66 ± 0.55**	7.27 ± 0.43***	5.31 ± 0.46
Histamine	6.93 ± 0.59**	6.83 ± 0.33**	6.05 ± 0.45*	6.03 ± 0.36*	5.87 ± 0.27*
Ins(1,3,4,5)P_4 (pmol/well)					
Control	0.93 ± 0.31	0.79 ± 0.13	0.90 ± 0.15	0.80 ± 0.16	0.85 ± 0.18
Bradykinin	4.50 ± 0.59**	6.12 ± 0.62***	5.43 ± 0.41***	1.24 ± 0.21	0.90 ± 0.04
Histamine	1.98 ± 0.24*	2.79 ± 0.42**	2.74 ± 0.24***	0.73 ± 0.16	0.75 ± 0.11

Cultured bovine adrenal chromaffin cells were exposed to bradykinin (100 nM), histamine (100 μM) or vehicle for 10 s. Values are shown as means ± s.e.mean for 3 separate experiments performed in triplicate.

Statistically significant differences between agonist-stimulated inositol polyphosphate accumulations compared to respective control values are indicated as $*P<0.05$ $**P<0.01$ or $***P<0.001$.

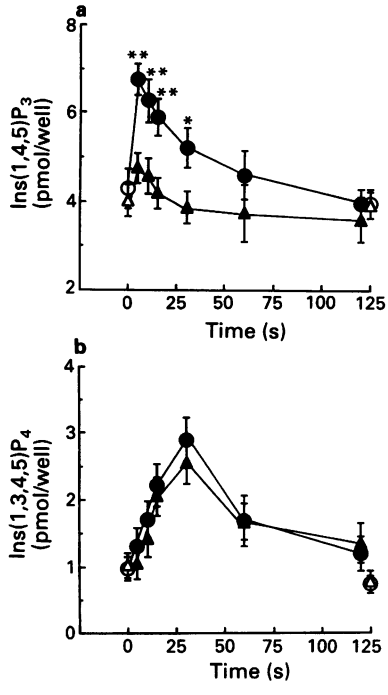

Figure 3 Effect of 12-O-tetradecanoyl-phorbol 13-acetate (TPA) on histamine-stimulated inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) (a) and inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) (b) mass accumulations in chromaffin cells. Cultured bovine adrenal chromaffin cells were pre-incubated for 10 min in the absence (○, ●) or presence of 1 μM TPA (△, ▲). Additions of 100 μM histamine (●, ▲) or vehicle (○,△) were made at zero-time. Values are means ± s.e.mean (vertical bars) from either 3 ($Ins(1,3,4,5)P_4$) or 4 ($Ins(1,4,5)P_3$) separate experiments performed in triplicate.

Statistically significant differences between cell responses in the presence and absence of TPA are indicated as $*P<0.05$, $**P<0.01$.

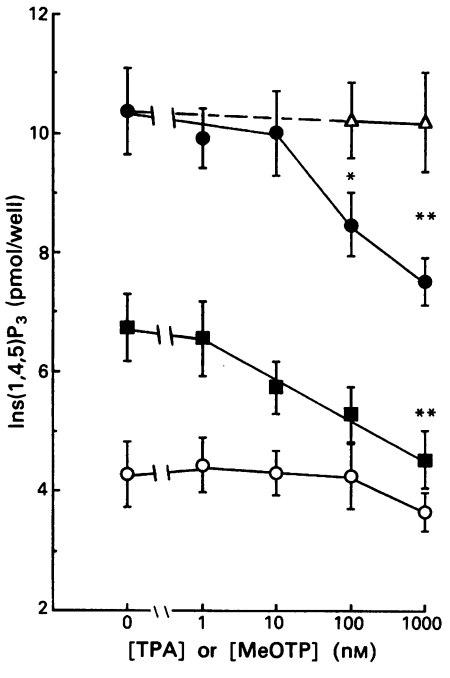

Figure 4 Concentration-response relationships for 12-O-tetradecanoyl-phorbol 13-acetate (TPA) inhibition of agonist-stimulated inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) mass accumulations in chromaffin cells. Cultured bovine adrenal chromaffin cells were pre-incubated for 10 min in the various concentrations of TPA (○,●,■) or 4-methoxy TPA (△), followed by exposure to bradykinin (100 nM: ●, △), histamine (100 μM: ■) or vehicle (○) for 10 s. Values are means ± s.e.mean (vertical bars) for 3 separate experiments performed in triplicate.

Statistically significant differences for the effect of a given concentration of TPA compared to the absence of TPA are shown as $*P<0.05$, $**P<0.01$.

Although Ro 31-8220 was without apparent effect on [^{3}H]-$InsP_x$ accumulation in response to 30 min stimulation with either bradykinin or histamine, we wished to investigate whether inhibition of PKC would affect the initial response of the cell, the generation of $Ins(1,4,5)P_3$ during the first few seconds of agonist exposure. The results from experiments where chromaffin cells were pre-incubated in the presence of 0.1 to 10 μM Ro 31-8220 and then stimulated with histamine or bradykinin for 10 s are shown in Table 2. Ro 31-8220 (10 μM) significantly increased the agonist-stimulated $Ins(1,4,5)P_3$ responses. Furthermore, if the relative increases in $Ins(1,4,5)P_3$ accumulation are expressed as a % increase over basal values, then PKC inhibition resulted in 55% and 61% greater accumulations respectively for bradykinin- and histamine-stimulated responses (Table 2).

Table 2 Effect of Ro 31-8220 on bradykinin- or histamine-stimulated $Ins(1,4,5)P_3$ accumulation in chromaffin cells

Ro 31-8220 (μM)	*Ins(1,4,5)P$_3$* (pmol/well)		
	Control	*Bradykinin*	*Histamine*
0	4.1 ± 0.3	9.6 ± 0.4	7.4 ± 0.4
0.1	4.3 ± 0.3	10.1 ± 0.8	7.4 ± 0.4
1	4.5 ± 0.4	10.9 ± 0.6	8.3 ± 0.5
10	4.4 ± 0.2	13.1 ± 1.1**	9.9 ± 1.1*

Cultured bovine adrenal chromaffin cells were pre-incubated for 10 min in the presence of the indicated concentrations of Ro 31-8220. Additions of bradykinin (100 nM), histamine (100 μM) or vehicle were made for 10 s. Values are shown as means ± s.e.mean for 3 separate experiments performed in triplicate.

Statistically significant differences between agonist-stimulated $Ins(1,4,5)P_3$ accumulation in the presence compared with the absence of Ro 31-8220 are indicated as $*P<0.05$; $**P<0.01$.

Discussion

In the present study we have assessed the effects of acute modulations of PKC activity on phosphoinositide metabolism stimulated by bradykinin and histamine in chromaffin cells. When considering evidence relating to the possible role of a short inhibitory feedback loop which may regulate the activation of PLC by PKC, it is perhaps helpful to distinguish between two types of experimental design. Firstly, the exogenous activation of PKC, usually by a phorbol ester such as TPA, can show whether the components of the loop, i.e. inhibition of agonist stimulation by PKC, are present. Secondly, however, it is necessary to ask whether this feedback circuit is activated by particular conditions of agonist stimulation, and here it is necessary to look at the consequences of interruption of the loop: this has been achieved by the down-regulation of PKC following long term (24 h or 48 h) treatment with TPA, or by use of a limited range of PKC inhibitors. With respect to the first experimental design, there are numerous examples of agonist stimulation being attenuated by TPA (Drummond, 1985; Rittenhouse & Sasson, 1985; Aiyar *et al.*, 1986) including studies on cultured chromaffin cells previously referred to (Wan *et al.*, 1989; Jones *et al.*, 1990). The conclusion of this earlier work, that the components of the feedback circuit are present in

chromaffin cells stimulated by histamine and bradykinin, are confirmed by the experiments with TPA on [^{3}H]-InsP_x accumulation described in this paper. With respect to the second experimental design, there are indications in other cell systems that agonist-initiated PKC stimulation may activate this inhibitory feedback circuit (Helper *et al.*, 1988; King & Rittenhouse, 1989; Pfeilschifter *et al.*, 1989); however, interpretation of such studies are subject to the complexities of long-term treatment with phorbol esters and the recognised limited selectivity of many of the PKC inhibitors used (Ruegg & Burgess, 1989). Our earlier study in this area used down regulation of PKC by 24 h TPA pretreatment (Jones *et al.*, 1990) and showed that agonist-stimulated [^{3}H]-InsP_x production was reduced, not enhanced as the inhibitory feedback circuit hypothesis would predict. While difficult to interpret, these results offered no support for the agonist activation of such a feedback mechanism. In the present study we have attempted a more direct approach using the selective PKC inhibitor Ro 31-8220 (Davis *et al.*, 1989; Nixon *et al.*, 1992). Preliminary studies measuring histamine- and bradykinin-stimulated [^{3}H]-InsP_x, demonstrated no effect of PKC inhibition and therefore suggested that there is no functional negative-feedback circuit.

We have previously reported that stimulation of chromaffin cells with bradykinin or histamine rapidly increases the levels of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Challiss *et al.*, 1991). Considering the observations, discussed above, that short term TPA treatment substantially attenuated agonist-stimulated [^{3}H]-InsP_x in the presence of lithium, it was to be expected that a similar attenuation of the inositol polyphosphate responses would also be seen. In fact, attenuations of 30% and 70% were observed for the bradykinin and histamine Ins(1,4,5)P_3 responses respectively. In contrast, the agonist-stimulated Ins(1,3,4,5)P_4 responses were unaffected by the presence of TPA, even in the case of histamine where the Ins(1,4,5)P_3 response was almost eliminated by phorbol ester treatment. This action of TPA to affect differentially agonist-stimulated inositol polyphosphate accumulations cannot be categorically interpreted from these experimental data, but a number of possible mechanisms can be suggested. Consideration of the TPA inhibition of agonist-stimulated Ins(1,4,5)P_3 accumulation and the dramatic attenuation of [^{3}H]-InsP_x accumulation (in the presence of Li^+) lead to the conclusion that a primary site of action is at the level of PLC activation. Furthermore, the similar increase in agonist-stimulated Ins(1,3,4,5)P_4 accumulation observed in the presence and absence of TPA suggests that this response is not dependent upon the magnitude of the initial increase in Ins(1,4,5)P_3 concentration, but is likely to be regulated independently of changes in substrate concentration, at least within the variations in Ins(1,4,5)P_3 levels observed under basal or agonist-stimulated conditions in chromaffin cells. Certainly, the low K_m values generally reported for Ins(1,4,5)P_3 3-kinase with respect to Ins(1,4,5)P_3 (Shears, 1991) would be consistent with this enzyme approaching saturation with its substrate, even under basal conditions, and external regulatory factors (e.g. increases in $[Ca^{2+}]_i$) being necessary and sufficient to increase Ins(1,3,4,5)P_4 accumulation.

Although PKC-mediated modifications of the activities of 5-phosphatase (Molina y Vedia & Lapetina, 1986; King & Rittenhouse, 1989) and 3-kinase (Sim *et al.*, 1990) have been reported, the findings in the present study are not easily accounted for by the 5-phosphatase activation or 3-kinase inhibition found by these workers.

The experiments on the effects of PKC inhibition on Ins(1,4,5)P_3 accumulation in response to stimulation with either histamine and bradykinin show that Ro 31-8220 does enhance this response (Table 2). These data provide support for the existence of a short inhibitory-feedback circuit which can be elicited by agonist stimulation. This is apparently in conflict with our earlier conclusion, based on [^{3}H]-InsP_x measurements, where no evidence for such feedback was obtained. However, it should be noted that there were significant differences between the two experimental designs (i.e. the presence *vs* the absence of lithium; the stimulation for 30 min *vs* 10 s; the measurement of [^{3}H]-InsP_x as an index of PLC acting on inositol phospholipids *vs* the direct measurement of Ins(1,4,5)P_3 mass changes). Therefore it is possible that activation of PKC does inhibit agonist-stimulated PLC activity, but the extent to which this occurs in response to histamine and bradykinin is time-limited, and is only effective during the first few seconds following agonist addition. Thus, the consequence for [^{3}H]-InsP_x accumulation at longer incubation times is not discernible. This proposal is consistent with our previous observations that bradykinin stimulation produces no measurable elevation in DAG in chromaffin cells, but does produce an elevation of phosphatidic acid (Owen & Boarder, 1991). Thus, diacylglycerol accumulation and consequent activation of a PKC inhibitory feedback loop may be curtailed by diacylglycerol kinase activity, producing phosphatidic acid. It is curious in this regard that chromaffin cells are unusual in that they express no phorbol ester- or agonist-stimulated phospholipase D (PLD) activity (Purkiss *et al.*, 1991). Thus, they also lack another route for agonist-stimulated DAG formation (synthesis of phosphatidic acid by PLD and subsequent DAG formation by phosphatidate phosphohydrolase) which may play a major role in activating a feedback circuit in other systems.

We thank the Wellcome Trust for financial support. The generous gifts of [^{3}H]-Ins(1,4,5)P_3 and [^{32}P]-Ins(1,3,4,5)P_4 from DuPont (U.K.) Ltd. are gratefully acknowledged. We thank Alison Jones and Neela Patel for expert technical assistance at various stages during this project.

References

AIYAR, N., NAMBI, P., WHITMAN, M., STASSEN, F.L. & CROOKE, S.T. (1986). Phorbol ester mediated inhibition of vasopressin and β-adrenergic responses in a vascular smooth muscle cell line. *Mol. Pharmacol.*, **13**, 180–184.

BURGOYNE, R.D. (1991). Control of exocytosis in adrenal chromaffin cells. *Biochim. Biophys. Acta*, **1071**, 174–202.

CHALLISS, R.A.J., BATTY, I.H. & NAHORSKI, S.R. (1988). Mass measurements of inositol 1,4,5-trisphosphate in rat cerebral cortical slices using a radioreceptor assay: effects of neurotransmitters and depolarisation. *Biochem. Biophys. Res. Commun.*, **157**, 684–691.

CHALLISS, R.A.J., JONES, J.A., OWEN, P.J. & BOARDER, M.R. (1991). Changes in inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate mass accumulations in cultured adrenal chromaffin cells in response to bradykinin and histamine. *J. Neurochem.*, **56**, 1083–1086.

CHALLISS, R.A.J. & NAHORSKI, S.R. (1990). Neurotransmitter and depolarization-stimulated accumulation of inositol 1,3,4,5-tetrakisphosphate mass in rat cerebral cortical slices. *J. Neurochem.*, **54**, 2138–2141.

DAVIS, P.D., HILL, C.H., KEECH, E., LAWTON, G., NIXON, J.S., SEDGWICK, A.D., WADSWORTH, J., WESTMACOTT, D. & WILKINSON, S.E. (1989). Potent selective inhibitors of protein kinase C. *FEBS Lett.*, **259**, 61–63.

DRUMMOND, A.H. (1985). Bidirectional control of cytosolic free calcium by thryrotropin-releasing hormone in pituitary cells. *Nature*, **315**, 32–35.

EBERHARD, D.A. & HOLZ, R.W. (1987). Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms. *J. Neurochem.*, **49**, 1634–1643.

HELPER, J.R., BARP, H.S. & HARDEN, T.K. (1988). Longterm phorbol ester treatment down-regulates protein kinase C and sensitizes the phosphoinositide signalling pathway to hormone growth factor stimulation. *J. Biol. Chem.*, **263**, 7610–7619.

JONES, J.A., OWEN, P.J. & BOARDER, M.R. (1990). Influence of phorbol esters, and diacylglycerol kinase and lipase inhibitors on noradrenaline release and phosphoinositide hydrolysis in chromaffin cells. *Br. J. Pharmacol.*, **101**, 521–526.

KING, W.G. & RITTENHOUSE, S.E. (1989). Inhibition of protein kinase C by staurosporine promotes elevated accumulations of inositol trisphosphates and tetrakisphosphate in human platelets exposed to thrombin. *J. Biol. Chem.*, **264**, 6070–6074.

LIVETT, B.G. & MARLEY, P.D. (1986). Effects of opioid peptides and morphine on histamine induced catecholamine secretion from cultured bovine adrenal chromaffin cells. *Br. J. Pharmacol.*, **89**, 327–334.

MOLINA Y VEDIA, L.M. & LAPETINA, E.G. (1986). Phorbol 12,13-dibutyrate and 1-oleyl-2-acetyldiacylglycerol stimulate inositol trisphosphate dephosphorylation in human platelets. *J. Biol. Chem.*, **261**, 10493–10495.

NIXON, J.S., BISHOP, J., BRADSHAW, D., DAVIS, P.D., HILL, C.H., ELLIOTT, L.H., KUMAR, H., LAWTON, G., LEWIS, E.J., MULQUEEN, M., WESTMACOTT, D., WADSWORTH, J. & WILKINSON, S.E. (1992). The design and biological properties of potent and selective inhibitors of protein kinase C. *Biochem. Soc. Trans.*, **20**, 419–425.

O'SULLIVAN, A.J. & BURGOYNE, R.D. (1989). A comparison of bradykinin, angiotensin II and muscarinic stimulation of cultured bovine adrenal chromaffin cells. *Biosci. Rep.*, **9**, 243–252.

OWEN, P.J. & BOARDER, M.R. (1991). Influence of bradykinin on diacylglycerol and phosphatidic acid accumulation in cultured bovine adrenal chromaffin cells. *J. Neurochem.*, **57**, 760–768.

OWEN, P.J., PLEVIN, R. & BOARDER, M.R. (1989). Characterisation of bradykinin stimulated release of noradrenaline from cultured bovine adrenal chromaffin cells. *J. Pharmacol. Exp. Ther.*, **248**, 1231–1236.

PFEILSCHIFTER, J., OCHSNER, M., WHITEBREAD, S. & DEGASPARO, M. (1989). Down-regulation of protein kinase C potentiates angiotensin II-stimulated phosphoinositide hydrolysis in vascular smooth muscle cells. *Biochem. J.*, **262**, 285–291.

PLEVIN, R. & BOARDER, M.R. (1988). Stimulation of formation of inositol phosphates in primary cultures of bovine chromaffin cells by angiotensin II, histamine, bradykinin, and carbachol. *J. Neurochem.*, **51**, 634–641.

PLEVIN, R., OWEN, P.J., MARRIOTT, D.B., JONES, J.A. & BOARDER, M.R. (1990). The role of phosphoinositide turnover and cyclic AMP accumulation in prostaglandin stimulated noradrenaline release from cultured adrenal chromaffin cells. *J. Pharmacol. Exp. Ther.*, **252**, 1296–1303.

PURKISS, J., MURRIN, R.A., OWEN, P.J. & BOARDER, M.R. (1991). Lack of phospholipase D activity in chromaffin cells: bradykinin-stimulated phosphatidic acid formation involves phospholipase C in chromaffin cells but phospholipase D in PC12 cells. *J. Neurochem.*, **57**, 1084–1087.

RITTENHOUSE, S.E. & SASSON, J.P. (1985). Mass changes in *myo*-inositol triphosphate in human platelets stimulated by thrombin: inhibitory effects of phorbol ester. *J. Biol. Chem.*, **260**, 8657–8660.

ROCHE, S., GUSDINAR, T., BALI, J.-P. & MAGOUS, R. (1991). Biphasic kinetics of inositol 1,4,5-trisphosphate accumulation in gastrin-stimulated parietal cells. *FEBS Lett.*, **282**, 147–151.

ROONEY, T.A. & NAHORSKI, S.R. (1986). Regional characterisation of agonist and depolarisation induced phosphoinositide hydrolysis in rat brain. *J. Pharmacol. Exp. Ther.*, **239**, 873–879.

RUEGG, U.T. & BURGESS, G.M. (1989). Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends Pharmacol. Sci.*, **10**, 218–220.

SHEARS, S.B. (1991). Regulation of the metabolism of 1,2-diacylglycerols and inositol phosphates that respond to receptor activation. *Pharmacol. Ther.*, **49**, 79–104.

SIM, S.S., KIM, J.W. & RHEE, S.G. (1990). Regulation of D-*myo*-inositol 1,4,5-trisphosphate 3-kinase by cAMP-dependent protein kinase and protein kinase C. *J. Biol. Chem.*, **265**, 10367–10372.

STAUDERMAN, K.A. & PRUSS, R.M. (1990). Different patterns of agonist-stimulated increases of ^{3}H-inositol phosphate isomers and cytosolic Ca^{2+} in bovine adrenal chromaffin cells: comparison of the effects of histamine and angiotensin II. *J. Neurochem.*, **54**, 946–953.

TOBIN, A.B., LAMBERT, D.G. & NAHORSKI, S.R. (1992). Desensitization of recombinant muscarinic M_3 receptor responses following short-term exposure to agonist. *Br. J. Pharmacol.*, **105**, 65P.

WAN, D.C.C., BUNN, S.J. & LIVETT, B.G. (1989). Effects of phorbol esters and forskolin on basal and histamine-stimulated accumulation of inositol phosphates in cultured bovine chromaffin cells. *J. Neurochem.*, **53**, 1219–1277.

(Received May 29, 1992
Revised July 30, 1992
Accepted August 12, 1992)

Br. J. Pharmacol. (1992), **107**, 1146–1151

Subclassification of release-regulating α_2-autoreceptors in human brain cortex

[1]Maurizio Raiteri, Giambattista Bonanno, Guido Maura, Mario Pende, *Gian Carlo Andrioli & *Antonio Ruelle

Institute of Pharmacology and Pharmacognosy, University of Genoa, Viale Cembrano 4, 16148 Genoa, Italy and *Division of Neurosurgery, Galliera Hospital, Via A. Volta 8, 16128 Genoa, Italy

1 Release-regulating α_2-autoreceptors in human brain were characterized pharmacologically in cortical slices from patients undergoing neurosurgery to remove subcortical tumours; the slices were prelabelled with [^{3}H]-noradrenaline ([^{3}H]-NA) and stimulated electrically (3 Hz, 2 ms, 24 mA) under superfusion conditions.

2 The stimulus-evoked tritium overflow was almost totally Ca^{2+}-dependent and tetrodotoxin-sensitive.

3 Clonidine and oxymetazoline 0.01 to 1 μM inhibited in a concentration-dependent manner the evoked overflow of tritium. The two drugs were equipotent ($EC_{50} = 0.03\ \mu M$) and their maximal effect was approx. 45%. Phenylephrine and methoxamine, up to 1 μM, did not affect tritium overflow.

4 Yohimbine (0.01–0.1 μM) shifted the concentration-response curve of clonidine to the right. The calculated pA_2 value was 8.29.

5 Prazosin and 2-[2-[4-(*o*-methoxyphenyl)piperazine-1-yl]ethyl]-4,4-dimethyl-1,3(2H,4H)-isoquinolinedione (AR-C 239), tested at 0.3 μM, did not modify the concentration-response curve of clonidine.

6 The effect of clonidine was antagonized by (+)-mianserin ($pA_2 = 7.74$), but not by up to 0.3 μM of the (−)-enantiomer. The concentration-response curve of clonidine was shifted to the right by the novel α_2-adrenoceptor antagonist, 5-chloro-4-(1-butyl-1,2,5,6-tetrahydropyridin-3-yl)-thiazole-2-amine (Z)-2-butenedioate (1:1) salt (ORG 20350) ($pA_2 = 7.55$).

7 Yohimbine, (+)-mianserin and ORG 20350, but not prazosin and (−)-mianserin, increased the electrically-evoked tritium overflow, suggesting that autoreceptors may be tonically activated by endogenous NA.

8 Desipramine (1 μM) increased evoked tritium overflow from human cortex slices. The effect of clonidine (0.01– 1 μM) on the evoked overflow of tritium was reduced in presence of 1 μM desipramine.

9 It is proposed that autoregulation of NA release can occur in human cerebral cortex. The process involves activation of α_2-adrenoceptors which may be either the α_{2A} or the α_{2D} subtype.

Keywords: Human cerebral cortex; noradrenaline release; noradrenaline autoreceptors; α_2-adrenoceptor subtypes; clonidine; desipramine

Introduction

Our knowledge of noradrenaline (NA) release and of its autoregulation in the central nervous system originates essentially from experiments carried out with laboratory animals (see, for a review, Starke, 1987). The existence of NA autoreceptors in human brain was reported only in a recent study in which the subtype to which they belong was not determined (Feuerstein *et al.*, 1990).

Fresh tissues are required for release experiments and this poses obvious difficulties in 'normal' human brain specimens. On the other hand, the possibility of species differences between humans and laboratory animals appears more and more likely due to the surprising heterogeneity of receptors which has emerged in the recent years. Experiments with human brain tissue, hence, seem necessary.

Substantial pharmacological evidence supports the existence of α_2-adrenoceptor heterogeneity in animals. Such evidence comes from functional (Doxey & Everitt, 1977; Dubocovich, 1979; Raiteri *et al.*, 1983; Alabaster *et al.*, 1986; Kapocsi *et al.*, 1987; Ruffolo *et al.*, 1987; Connaughton & Docherty, 1990; Akers *et al.*, 1991; Maura *et al.*, 1992) as well as from binding (Bylund, 1981; 1985; Cheung *et al.*, 1982; Michel *et al.*, 1989; Brown *et al.*, 1990; Uhlén & Wikberg, 1991) studies. Multiple α_2-adrenoceptors in human tissues were predicted on the basis of molecular biology studies (Kobilka *et al.*, 1987; Regan *et al.*, 1988).

In this functional study we have characterized pharmacologically the NA autoreceptor involved in the negative feedback regulation of the release of the catecholamine in the human cerebral cortex.

Methods

Characteristics of human brain specimens

Samples of human cerebral cortex were obtained from patients undergoing neurosurgery. The tissues used had to be removed by the surgeon to reach deeply located tumours. After premedication with atropine and meperidine, anaesthesia was induced with thiopentone and maintained with 70% nitrous oxide, 30% oxygen and 0.5–1% isoflurane. Pancuronium was employed to obtain muscular relaxation. The samples removed represented parts of frontal (4), temporal (11), and parietal (3) lobes and were obtained from 7 male and 11 female patients (aged 42–72 years). The tissues were obtained and processed separately on different days.

[1] Author for correspondence.

Preparation of slices

Immediately after removal, the tissue was placed in a physiological salt solution (see below) kept at 0–4°C and cortical slices were prepared within 60 min. Slices (0.4 mm thick) perpendicular to the surface were prepared by use of a McIlwain tissue chopper after cubic pieces had been obtained from human specimens. In some experiments (effect of clonidine in the presence of desipramine and Ca^{2+}-and tetrodotoxin-dependence) surface-parallel slabs, 0.4 mm thick, were cut from the human brain cortex samples after the superficial 0.3 mm had been removed. Slices of 5 mm diameter were punched out from the slabs. The results obtained did not depend on the method of slice preparation.

Release experiments

The slices were incubated with 0.1 μM [^{3}H]-NA, in the presence of 0.3 μM citalopram, for 15 min at 37°C in 5 ml of a medium having the following composition (mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, Na_2HPO_4 1, $NaHCO_3$ 22, glucose 10 (aerated with 95% O_2 and 5% CO_2), pH 7.2–7.4. After washing with tracer-free medium, one slice was transferred to each of 12 parallel superfusion chambers equipped with platinum electrodes 20 mm apart (Reichenbacher *et al.*, 1982) and superfused for 155 min at a rate of 1 ml min^{-1}. After 55 min of superfusion to achieve a constant baseline of tritium efflux, 5-min fractions were collected until the end of the experiment. The slices were then solubilized in 0.5 ml of Soluene 350 (Canberra Packard, Milan, Italy) and tritium in the superfusate samples and solubilized tissue was measured by liquid scinitillation counting.

Continuous electrical stimulation with biphasic square pulses (24 mA, 3 Hz, 2 ms) started at $t = 65$ min ($t = 0$ being the start of superfusion), after the second fraction had been collected (see Figure 1). Some unstimulated slices were superfused in parallel in order to estimate the basal efflux of tritium and the effects of drugs on the spontaneous outflow.

The NA receptor agonists, clonidine, oxymetazoline, phenylephrine and methoxamine were added to the superfusion medium after the peak of the electrically-evoked tritium efflux was reached (at $t = 75$ min, see Figure 1). The agonist concentrations were then increased every 20 min (at $t = 95$, 115, 135 min) in order to construct a cumulative concentration-response curve (Frankhuyzen & Mulder, 1982; Richards, 1985; Bonanno & Raiteri, 1987). The NA receptor antagonists, yohimbine, prazosin, 2-[2-[4-(*o*-methoxyphenyl) piperazine-1-yl]ethyl]-4,4-dimethyl-1,3 (2H,4H)-isoquinolinedione (AR-C 239), (+)-mianserin, (−)-mianserin, 5-chloro-4-(1-butyl-1,2,5,6-tetrahydropyridin-3-yl)-thiazole-2-amine (Z)-2-butenedioate (1:1) salt (ORG 20350) and the NA uptake inhibitor, desipramine, when used, were present throughout the experiment. Controls in standard medium or in the presence of desipramine or antagonists but without agonists were always run in parallel in the same experiment so that each experimental condition had its own control group.

In some experiments, slices were superfused from $t = 75$ to $t = 115$ min with a Ca^{2+}-free medium or in the presence of 1 μM tetrodotoxin.

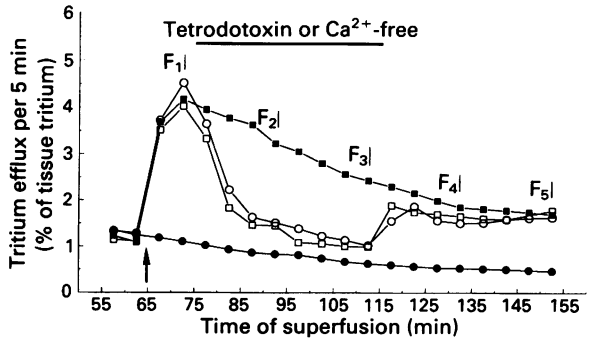

Figure 1 Tetrodotoxin- and Ca^{2+}-dependence of tritium overflow evoked by continuous electrical stimulation of human brain cortex slices. Slices prelabelled with [^{3}H]-noradrenaline ([^{3}H]-NA) were superfused. Electrical stimulation (24 mA, 3 Hz, 2 ms) was applied from $t = 65$ min to $t = 155$ min. Five-min fractions were collected and the radioactivity present in each fraction was expressed as a percentage of the total tritium content of the tissue at the onset of the fraction considered. As indicated in the figure, tetrodotoxin-containing or Ca^{2+}-free medium was introduced from $t = 75$ min to $t = 115$ min. Points represent the mean of three experiments in duplicate. The s.e.mean (not shown) was always within 10% of the respective mean value. (■) Experiments with electrical stimulation; (□) experiments with electrical stimulation and 1 μM tetrodotoxin; (○) experiments with electrical stimulation and Ca^{2+}-free medium; (●) experiments without electrical stimulation.

Calculations

The efflux of radioactivity collected in each fraction was calculated as a percentage of the total radioactivity present in the tissue at the onset of the fraction considered. The evoked overflow in each fraction was calculated by subtraction, from the efflux in the particular fraction, of the respective basal efflux. The basal efflux was calculated from the efflux of each slice in the fraction preceding the stimulation ($t = 60$–65 min), assuming a decline similar to that of unstimulated slices processed in the same experiment. The evoked overflow in the fraction F_1 ($t = 70$–75 min) and that in every fourth fraction collected after the addition of the various agonist concentrations (F_2, F_3, F_4, F_5) was calculated (see Figure 1). Effects of the various agonists on the electrically-evoked overflow of tritium were evaluated by forming the ratios F_2/F_1, F_3/F_1, F_4/F_1, F_5/F_1. These ratios were compared to the corresponding ratios obtained under control conditions (i.e. in standard medium or in the presence of the antagonist or desipramine, as appropriate). In each experiment the average of appropriate F_n/F_1 values from the two superfusion chambers containing the same drug were compared to the average value of the two control chambers run in the same experiment.

The EC_{50} values for the agonists (alone or in the presence of an antagonist) were determined from a curve obtained using a function fitting routine (software Sigma Plot, version 4.0). The following four parameter logistic equation was used:

$$Y = a + ((b-a)/(1 + (10^c/10^x)^d))$$

where (b) is the maximum effect; (c) the EC_{50}; (d) the slope of the curve. The minimum effect of an agonist (a) was always considered constant (zero % inhibition). Each EC_{50} value was the average of n EC_{50}s calculated in the different experiments.

The pA_2 values of (+)-mianserin and ORG 20350 were calculated according to Furchgott (1972, page 290); the values obtained with the two concentrations of antagonist used were averaged. The pA_2 value of yohimbine was calculated according to Arunlakshana & Schild (1959), using three concentrations of the antagonist. The antagonism was considered competitive if the slope was not significantly different from unity.

The effects of the adrenoceptor antagonists and of desipramine, shown in Figure 6, were calculated as follows: $[(F_1\text{drug} - F_1\text{control})/F_1\text{control}] \times 100$ where F_1drug and F_1control was the depolarization-evoked overflow of tritium in the fraction collected at $t = 70$–75 min in the presence of the antagonist and in standard medium, respectively.

Student's t test was used to analyze the significance of the difference between two means.

Drugs

(−)-(7,8-^{3}H)-noradrenaline (specific activity 32 Ci mmol^{-1}) was obtained from Amersham Radiochemical Centre (Buckinghamshire); (−)-phenylephrine hydrochloride, yohimbine hydrochloride and tetrodotoxin from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The following drugs were gifts from the companies indicated: clonidine hydrochloride (Boehringer Ingelheim, Florence, Italy); (±)-methoxamine hydrochloride (Wellcome Research Laboratories, Beckenham); citalopram hydrobromide (Lundbeck, Copenhagen, DK); desipramine hydrochloride (Ciba Geigy, Basel, Switzerland); (+)-mianserin maleate, (−)-mianserin maleate and 5-chloro-4-(1-butyl-1,2,5,6-tetrahydropyridin-3-yl)-thiazole-2-amine (Z)-2-butenedioate (1:1) salt (ORG 20350) (Organon, Oss, The Netherlands); 2-[2-[4-(o-methyoxyphenyl) piperazine-1-yl] ethyl]-4,4-dimethyl-1,3(2H,4H)-isoquinolinedione (AR-C 239) (Karl Thomae, Biberach, Germany); oxymetazoline hydrochloride (Avondale Chemical, Wicklow, Ireland); prazosin hydrochloride (Pfizer, New York, U.S.A.).

Prazosin was dissolved in ethanol and (+)- and (−)-mianserin in 0.1 M HCl. Dilution to 1 mM was made with distilled water. Other compounds were first dissolved (1 mM) in distilled water. Final dilutions were made with standard medium.

Results

Ca^{2+}-dependence and tetrodotoxin-sensitivity of electrically-evoked tritrium overflow

Under continuous field stimulation, the evoked ^{3}H-overflow from human cerebral cortex slices prelabelled with [^{3}H]-NA was largely sensitive to tetrodotoxin (1 μM) and Ca^{2+}-dependent (Figure 1).

Effects of α_1- and α_2-adrenoceptor agonists

As shown in Figure 2, the α_2-adrenoceptor agonists, clonidine and oxymetazoline, tested at 0.01–1 μM, inhibited the evoked overflow of tritium in a concentration-dependent manner. The concentration-response curves for the two drugs were superimposable. The maximal effect was about 45% and the EC_{50} was 0.03 μM. In contrast, the α_1-adrenoceptor agonists, phenylephrine or methoxamine, did not affect the overflow of tritium at concentrations up to 1 μM. At the concentrations used the drugs did not affect the spontaneous outflow of radioactivity.

Antagonism against clonidine

The α_2-adrenoceptor antagonist, yohimbine (0.01–0.1 μM), shifted the clonidine concentration-response curve increasingly to the right (Figure 3). The calculated pA_2 value was 8.29 (Schild slope = 0.98). In contrast, prazosin or AR-C 239, antagonists with clear preference for the α_{2B}- and the α_{2C}-adrenoceptor subtypes as opposed to the α_{2A} and α_{2D} subtype (Paris *et al.*, 1990; Blaxall *et al.*, 1991; Simonneaux *et al.*, 1991), did not change the effect of clonidine at 0.3 μM (Figure 4).

Mianserin antagonized the effect of clonidine in a stereoselective manner (Figure 5). The concentration-response curve of the agonist was shifted to the right in presence of 0.1 and 0.3 μM of (+)-mianserin ($pA_2 = 7.74$), whereas the (−)-enantiomer was ineffective at 0.3 μM. The novel selective α_2-adrenoceptor blocker ORG 20350 (Maura *et al.*, 1992) also antagonized clonidine ($pA_2 = 7.55$). The antagonists used did not affect the basal outflow of tritium.

Effects of α_2-adrenoceptor antagonists and of desipramine

Figure 6 illustrates changes of the electrically-evoked tritium overflow that were observed when slices of human brain were exposed to various α_2-adrenoceptor antagonists or to the NA reuptake inhibitor, desipramine. Yohimbine (0.1 μM) increased the evoked tritium overflow by about 30%. ORG 20350 and (+)-mianserin (0.1 and 0.3 μM), but not the (−)-enantiomer (0.3 μM), were also effective. Prazosin and AR-C 239 were ineffective at a concentration of 0.3 μM. Desipramine (1 μM) increased the overflow of tritium by about 40%. The effects of 0.1 μM yohimbine and 1 μM desipramine appeared to be additive.

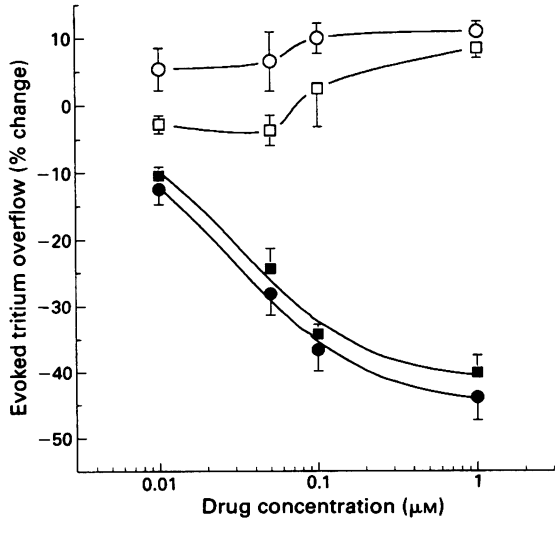

Figure 2 Effects of clonidine (●), oxymetazoline (■), phenylephrine (□) or methoxamine (○) on the efflux of tritium evoked by electrical stimulation. Starting from $t = 75$ min, increasing concentrations of the drugs were added to the superfusion medium at intervals of 20 min. Points represent the mean ± s.e.mean (vertical bars) of 3–14 experiments in duplicate.

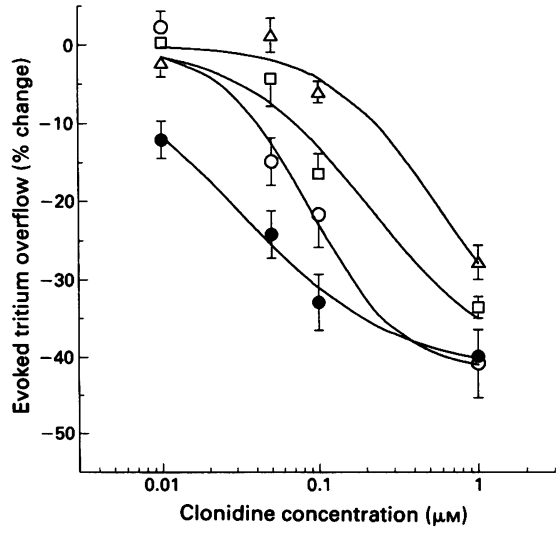

Figure 3 Effect of yohimbine on the clonidine-induced inhibition of the electrically-evoked overflow of tritium. When used, yohimbine was present throughout the superfusion. Points represent the mean ± s.e.mean (vertical bars) of 3–4 experiments in duplicate: (●) clonidine; (○) clonidine + 0.01 μM yohimbine; (□) clonidine + 0.03 μM yohimbine; (△) clonidine + 0.1 μM yohimbine.

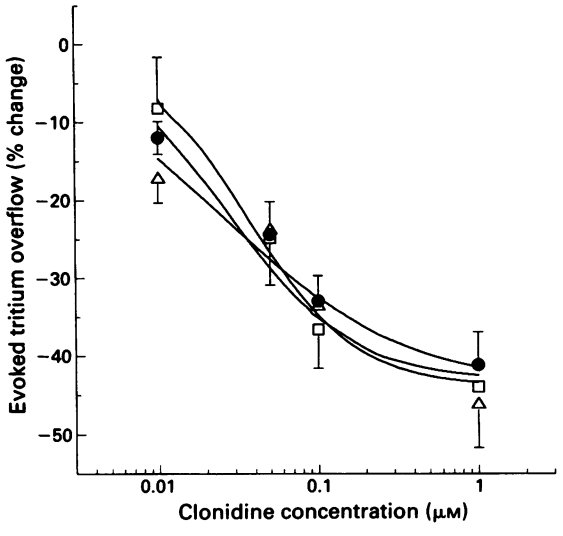

Figure 4 Effects of prazosin or AR-C 239 on the clonidine-induced inhibition of the electrically-evoked overflow of tritium. When used, prazosin or AR-C 239 was present throughout the superfusion. Means ± s.e.mean (vertical bars) of 3–5 experiments in duplicate are presented: (●) clonidine; (△) clonidine + 0.3 μM prazosin; (□) clonidine + 0.3 μM AR-C 239.

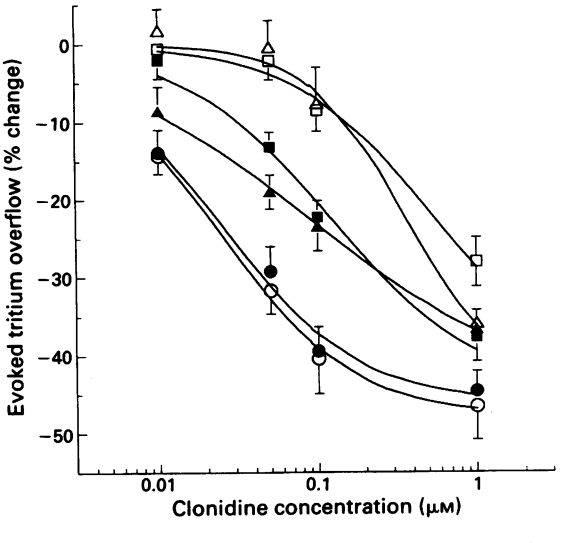

Figure 5 Effects of (−)-mianserin, (+)-mianserin or ORG 20350 on the clonidine-induced inhibition of the electrically-evoked overflow of tritium. When used, (−)-mianserin, (+)-mianserin or ORG 20350 was present throughout superfusion. Each point represents the mean ± s.e.mean (vertical bars) of 3–4 experiments in duplicate: (●) clonidine; (○) clonidine + 0.3 μM (−)-mianserin; (■) clonidine + 0.1 μM (+)-mianserin; (□) clonidine + 0.3 μM (+)-mianserin; (▲) clonidine + 0.1 μM ORG 20350; (△) clonidine + 0.3 μM ORG 20350.

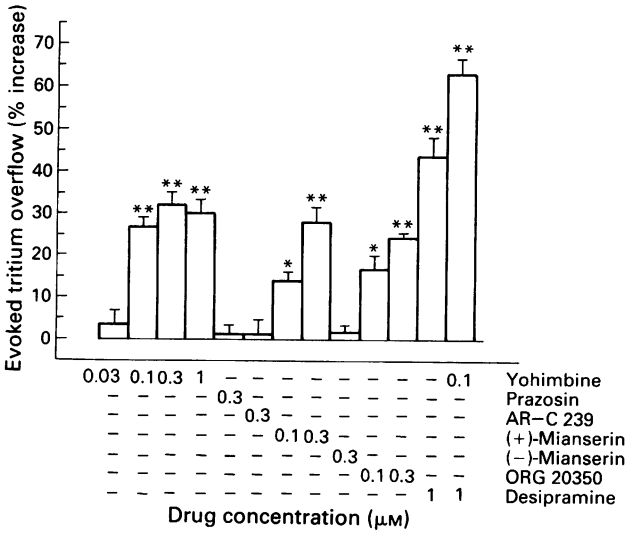

Figure 6 Effects of desipramine and of α_2-adrenoceptor antagonists on the electrically-evoked overflow of tritium. The effects of drugs were calculated by comparing the depolarization-evoked overflow at F_1 obtained in the presence of drugs and in standard medium (control conditions) as follows: $[(F_1\text{drug} - F_1\text{control})/F_1\text{control}] \times 100$. Means ± s.e.mean (vertical bars) of 3–5 experiments in duplicate are shown. Significance of changes versus the electrically-evoked overflow in standard medium was determined by Student's t test: $^*P<0.05$ and $^{**}P<0.001$.

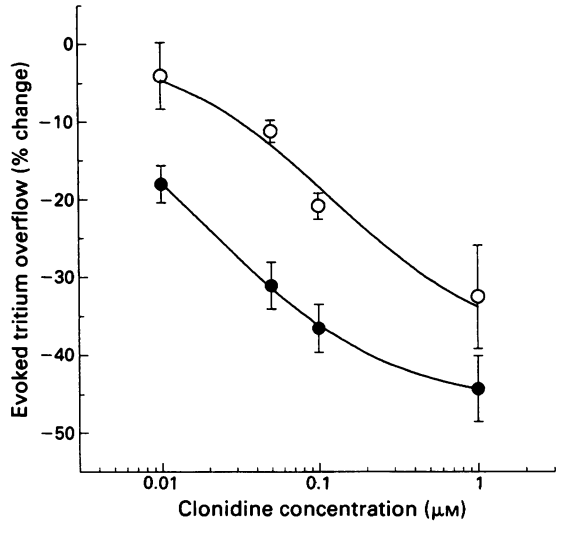

Figure 7 Effect of desipramine on the clonidine-induced inhibition of the electrically-evoked overflow of tritium. When used, desipramine was present throughout the superfusion. Points are mean ± s.e.mean (vertical bars) of 3 experiments in duplicate: (●) clonidine; (○) clonidine + 1 μM desipramine.

Interaction between clonidine and desipramine

The concentration-response curve of clonidine as an inhibitor of tritium overflow was shifted to the right when 1 μM desipramine was present in the superfusion medium (Figure 7).

Discussion

The electrically-evoked tritium overflow from slices of human neocortical tissue prelabelled with [^{3}H]-NA was almost totally Ca^{2+}-dependent and tetrodotoxin-sensitive, suggesting that the evoked overflow of radioactivity reflects a quasi-physiological, action-potential induced, exocytotic release of [^{3}H]-NA.

Studies in animals have shown that the overflow of NA produced by exposing brain slices of synaptosomes to different depolarizing stimuli can be modulated by autoreceptors classifed as α_2-adrenoceptors (see review by Starke, 1987). The results in Figure 2, showing that the electrically-evoked overflow of [^{3}H]-NA was inhibited by clonidine and oxymetazoline, two α_2-adrenoceptor agonists, but not by the α_1-adrenoceptor agonists, phenylephrine and methoxamine,

and the results of Figure 3, showing that the inhibitory effect of clonidine was attenuated by the α_2-adrenoceptor antagonist, yohimbine, indicate that an α_2-autoreceptor mechanism also exists in human brain.

Binding studies performed on various tissues and cell lines (reviewed by Docherty, 1989; see also Paris *et al.*, 1990; Blaxall *et al.*, 1991; Simonnneaux *et al.*, 1991; Remaury & Paris, 1992) as well as rat and human receptor gene products (Kobilka *et al.*, 1987; Regan *et al.*, 1988; Lorenz *et al.*, 1990; Lanier *et al.*, 1991; Harrison *et al.*, 1991; Lomasney *et al.*, 1991) have led to a subdivision of α_2-adrenoceptors into four pharmacologically distinct subtypes termed α_{2A}, α_{2B}, α_{2C} and α_{2D}.

According to the above studies, a number of drugs exist that are useful in discriminating between α_2-adrenoceptor subtypes because their affinities for the subtypes differ by more than 10 fold. The antagonists which seem most selective are prazosin and AR-C 239 which display low to moderate affinity for the α_{2A} and α_{2D} subtypes but a high affinity for α_{2B} and α_{2C} sites. Conversely, the α_2-adrenoceptor agonist, oxymetazoline, displays high affinity for α_{2A} and α_{2D} receptors and low affinity for the α_{2B} and α_{2C} subtypes. Our finding that oxymetazoline potently inhibited the [^{3}H]-NA overflow is compatible with an involvement of α_{2A} or α_{2D} receptors. On the other hand, prazosin and AR-C 239 did not change the inhibitory effect of clonidine at 0.3 μM, a concentration at least 30 fold higher than the K_i values obtained in binding studies (Bylund *et al.*, 1988; Michel *et al.*, 1989; Brown *et al.*, 1990; Lanier *et al.*, 1991; Simonneaux *et al.*, 1991; Uhlén & Wikberg, 1991; Remaury & Paris, 1992). Therefore the present results tend to exclude the involvement of receptors of the α_{2B} or α_{2C} subtype.

Using rat cerebral cortex synaptosomes we had found previously that the α_2-autoreceptors were stereoselectively blocked by the (+)-enantiomer of mianserin, while the α_2-adrenoceptors mediating inhibition of 5-hydroxytryptamine release were blocked with identical potency by the two enantiomers, suggesting the existence of α_2-adrenoceptor subtypes (Raiteri *et al.*, 1983; Maura *et al.*, 1985). The human α_2-autoreceptor also is sensitive to (+)-mianserin ($pA_2 = 7.74$), but not to the (−)-isomer used at 0.3 μM (Figure 5).

Recently, it was reported that the affinity of (−)-mianserin for α_{2A} sites was 10–20 times higher than that for α_{2D} sites for which a pK_i value of 6.0 was found (Simonneaux *et al.*, 1991). These data, together with the poor sensitivity of the autoreceptors towards (−)-mianserin (Figure 5), suggest a classification of the autoreceptors as α_{2D}. However, more convincing experimental evidence is clearly necessary to discriminate between two receptor subtypes which appear to be pharmacologically very similar.

The pA_2 value for (+)-mianserin in the human brain (7.74) is close to that found in the rat (7.80; Maura *et al.*, 1985) suggesting similarity between the autoreceptors in the two species. The pA_2 of yohimbine in human cortex was 8.29. This value is almost superimposable on those reported for yohimbine tested against clonidine, in rat or rabbit brain (see Starke, 1987; Limberger *et al.*, 1991). The presynaptic α_2-autoreceptors in rabbit or rat brain are known to be prazosin-insensitive (Hedler *et al.*, 1981; Reichenbacher *et al.*, 1982; Raiteri *et al.*, 1983; Gobbi *et al.*, 1990; Limberger *et al.*, 1991). Recently, the α_2-autoreceptors in rat brain cortex were found to be insensitive also to AR-C 239 (Maura *et al.*, 1992). Finally, the novel α_2-adrenoceptor blocker, ORG 20350, which blocked rat brain cortex autoreceptors ($pA_2 = 7.25$) but was ineffective at the α_2-heteroreceptors regulating 5-hydroxytryptamine release (Maura *et al.*, 1992), blocked the human α_2-autoreceptors with a pA_2 very close to that found in the rat. All together these data strongly suggest that the α_2-autoreceptors in the brain of humans, rats and probably rabbits are pharmacologically very similar.

The results obtained when adrenoceptor antagonists were added to electrically-stimulated human cerebrocortical slices in the absence of exogenous agonist indicate that, under the stimulation conditions used, the autoreceptors were in part activated by NA endogenously released and present in the receptor biophase (see Limberger *et al.*, 1989): yohimbine (but not prazosin or AR-C 239), (+)-mianserin (but not the (−)-enantiomer) and ORG 20350 increased the evoked [^{3}H]-NA overflow. These data (a) are in keeping with the receptor subclassification proposed above; (b) suggest that, under certain conditions, the autoreceptors can be activated by the synaptically released NA; (c) are compatible with the view that in human cerebral cortex, clonidine acts where endogenous NA acts.

The data of Figure 6 show, probably for the first time in man, that two antidepressants, desipramine and mianserin (actually its (+)-enantiomer) can enhance NA overflow, the former by preventing NA reuptake and the latter by preventing autoreceptor activation due to endogenous NA. The (apparent) additivity of the effects of desipramine and of yohimbine reflects the different mechanisms involved.

As shown in Figure 7, the presence of desipramine largely prevented the effect of clonidine on [^{3}H]-NA overflow. It has long been known that tricyclic antidepressants may inhibit the centrally-mediated antihypertensive effect of clonidine, although the mechanism of this interaction has not been clarified (Gerber & Nies, 1990). Our results with human brain tissue are compatible with the view that clonidine loses activity because it has to compete for α_2-adrenoceptors (either pre- or postsynaptically located) with endogenously released NA, the concentration of which in the receptor biophase is raised following reuptake inhibition by desipramine. This interpretation is in line with the previous finding that the interaction between clonidine and desipramine does not occur in superfused synaptosomes, i.e. in conditions in which the concentration of the released NA can not increase in the vicinity of the receptors (Maura *et al.*, 1984).

In conclusion, terminal α_2-autoreceptors which may be involved in a local negative feedback regulation of NA release exist in human cerebral cortex. The pharmacological characterization suggests that these autoreceptors are not α_{2B} or α_{2C}. According to the present classification, they belong either to the α_{2A} or to the α_{2D} subtype. Data obtained with a number of drugs, including the enantiomers of mianserin and ORG 20350, show that the noradrenaline autoreceptors in human and rat brain are pharmacologically very similar. Thus the rat brain is likely to represent a useful model to characterize better terminal noradrenaline autoreceptors in the central nervous system and to develop more selective α_2-adrenoceptor drugs.

This work was funded by grants from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) and Consiglio Nazionale delle Ricerche (CNR). We wish to thank Mrs Maura Agate for her assistance in preparing the manuscript.

References

AKERS, J., COATES, J., DREW, G.M. & SULLIVAN, A.T. (1991). α_2-Adrenoceptor blocking profile of SK&F 104078: further evidence for receptor subtypes. *Br. J. Pharmacol.*, **102**, 943–949.

ALABASTER, V.A., KEIR, R.F. & PETERS, C.J. (1986). Comparison of potency of α_2-adrenoceptor antagonists *in vitro*: evidence for heterogeneity of α_2-adrenoceptors. *Br. J. Pharmacol.*, **88**, 607–614.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BLAXALL, H.S., MURPHY, T.J., BAKER, J.C., RAY, C. & BYLUND, D.B. (1991). Characterization of the alpha-2C adrenergic receptor subtype in the opossum kidney and the OK cell line. *J. Pharmacol. Exp. Ther.*, **259**, 323–329.

BONANNO, G. & RAITERI, M. (1987). Interaction between 5-HT uptake inhibition and activation of 5-HT autoreceptors by exogenous agonists in rat cerebral cortex slices and synaptosomes. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **335**, 219–225.

BROWN, C.M., MACKINNON, A.C., MCGRATH, J.C., SPEDDING, M. & KILPATRICK, A.T. (1990). Heterogeneity of α_2-adrenoceptors in rat cortex but not human platelets can be defined by 8-OH-DPAT, RU 24969 and methysergide. *Br. J. Pharmacol.*, **99**, 481–486.

BYLUND, D.B. (1981). Comparison of [^{3}H]clonidine and [^{3}H]yohimbine binding: possible subtypes of the alpha$_2$-adrenergic receptors. *Pharmacologist*, **23**, 215–221.

BYLUND, D.B. (1985). Heterogeneity of alpha-2 adrenergic receptors. *Pharmacol. Biochem. Behav.*, **22**, 835–843.

BYLUND, D.B., RAY-PRENGER, C. & MURPHY, T.J. (1988). Alpha-2A and Alpha-2B adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype. *J. Pharmacol. Exp. Ther.*, **245**, 600–607.

CHEUNG, Y.-D., BARNETT, D.B. & NAHORSKI, S.R. (1982). [^{3}H]-rauwolscine and [^{3}H]-yohimbine binding to rat cerebral and human platelet membranes: possible heterogeneity of alpha$_2$-adrenoceptors. *Eur. J. Pharmacol.*, **84**, 79–85.

CONNAUGHTON, S. & DOCHERTY, J.R. (1990). Functional evidence for heterogeneity of peripheral prejunctional α_2-adrenoceptors. *Br. J. Pharmacol.*, **101**, 285–290.

DOCHERTY, J.R. (1989). The pharmacology of α_1- and α_2-adrenoceptors: evidence for and against a further subdivision. *Pharmacol. Ther.*, **44**, 241–284.

DOXEY, J.C. & EVERITT, J. (1977). Inhibitory effects of clonidine on responses to sympathetic nerve stimulation in the pithed rat. *Br. J. Pharmacol.*, **61**, 559–566.

DUBOCOVICH, M.L. (1979). Pharmacological differences between the alpha-presynaptic adrenoceptors in the peripheral and the central nervous systems. In *Presynaptic Receptors, Advances in the Biosciences*. ed. Langer, S.Z., Starke, K. & Dubocovich, M.L. pp. 29–36. Oxford: Pergamon Press.

FEUERSTEIN, T.J., DOOLEY, D.J. & SEEGER, W. (1990). Inhibition of norepinephrine and acetylcholine release from human neocortex by ω-conotoxin GVIA. *J. Pharmacol. Exp. Ther.*, **252**, 778–785.

FRANKHUYZEN, A.L. & MULDER, A.H. (1982). A cumulative dose-response technique for the characterization of presynaptic receptors modulating [^{3}H]noradrenaline release from rat brain slices. *Eur. J. Pharmacol.*, **78**, 91–97.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology. Catecholamines*, Vol. XXXIII. ed. Blaschko, H. & Muscholl, E. pp. 283–335, Berlin, Heidelberg, New York: Springer.

GERBER, J.G. & NIES, A.S. (1990). Antihypertensive agents and the drug therapy of hypertension. In *The Pharmacological Basis of Therapeutics*. ed. Goodman Gilman, A., Rall, T.W., Nies, A.S. & Taylor, P. pp. 784–813. New York: Pergamon Press.

GOBBI, M., FRITTOLI, E. & MENNINI, T. (1990). The modulation of [^{3}H]noradrenaline and [^{3}H]serotonin release from rat brain synaptosomes is not mediated by the α_{2B}-adrenoceptor subtype. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **342**, 382–386.

HARRISON, J.K., D'ANGELO, D.D., ZENG, D. & LYNCH, K.R. (1991). Pharmacological characterization of rat α_2-adrenergic receptors. *Mol. Pharmacol.*, **40**, 407–412.

HEDLER, L., STAMM, G., WIETZELL, R. & STARKE, K. (1981). Functional characterization of central α-adrenoceptors by yohimbine diastereomers. *Eur. J. Pharmacol.*, **70**, 43–52.

KAPOCSI, J., SOMOGYI, G.T., LUDVIG, N., SERFOZO, P., HARSING, L.G. Jr., WOODS, R.J. & VIZI, E.S. (1987). Neurochemical evidence for two types of presynaptic alpha$_2$-adrenoceptors. *Neurochem. Res.*, **12**, 141–147.

KOBILKA, B.K., MATSUI, H., KOBILKA, T.S., YANG-FENG, T.L., FRANCKE, U., CARON, M.G., LEFKOWITZ, R.J. & REGAN, J.W. (1987). Cloning, subsequencing, and expression of the gene coding for the human platelet α_2-adrenergic receptor. *Science*, **238**, 650–656.

LANIER, S.M., DOWNING, S., DUZIC, E. & HOMEY, C.J. (1991). Isolation of rat genomic clones encoding subtypes of the α_2-adrenergic receptor. Identification of a unique receptor subtype. *J. Biol. Chem.*, **266**, 10470–10478.

LIMBERGER, N., MAYER, A., ZIER, G., VALENTA, B., STARKE, K. & SINGER, E.A. (1989). Estimation of pA_2 values at presynaptic α_2-autoreceptors in rabbit and rat brain cortex in the absence of autoinhibition. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **340**, 639–647.

LIMBERGER, N., SPÄTH, L. & STARKE, K. (1991). Subclassification of the presynaptic α_2-autoreceptors in rabbit brain cortex. *Br. J. Pharmacol.*, **103**, 1251–1255.

LOMASNEY, J.W., COTECCHIA, S., LEFKOWITZ, R.J. & CARON, M.G. (1991). Molecular biology of alpha-adrenergic receptors: implications for receptor classification and for structure-function relationships. *Biochim. Biophys. Acta*, **1095**, 127–139.

LORENZ, W., LOMASNEY, J.W., COLLINS, S., REGAN, J.W., CARON, M.G. & LEFKOWITZ, R.J. (1990). Expression of three α_2-adrenergic receptor subtypes in rat tissues: implications for α_2-receptor classification. *Mol. Pharmacol.*, **38**, 599–603.

MAURA, G., PITTALUGA, A., RICCHETTI, A. & RAITERI, M. (1984). Noradrenaline uptake inhibitors do not reduce the presynaptic action of clonidine on ^{3}H-noradrenaline release in superfused synaptosomes. *Naunyn Schmiedebergs Arch. Pharmacol.*, **327**, 86–89.

MAURA, G., GEMIGNANI, A. & RAITERI, M. (1985). α_2-Adrenoceptors in rat hypothalamus and cerebral cortex: functional evidence for pharmacologically distinct subpopulations. *Eur. J. Pharmacol.*, **116**, 335–339.

MAURA, G., BONANNO, G. & RAITERI, M. (1992). Presynaptic α_2-adrenoceptors mediating inhibition of noradrenaline and 5-hydroxytryptamine release in rat cerebral cortex: further characterization as different α_2-adrenoceptor subtypes. *Naunyn-Schmiedebergs Arch Pharmacol.*, **345**, 410–416.

MICHEL, A.D., LOURY, D.N. & WHITING, R.L. (1989). Differences between the α_2-adrenoceptor in rat submaxillary gland and the α_{2A}- and α_{2B}-adrenoceptor subtypes. *Br. J. Pharmacol.*, **98**, 890–897.

PARIS, H., VOISIN, T., REMAURY, A., ROUYER-FESSARD, C., DAVIAUD, D., LANGIN, D. & LABURTHE, M. (1990). Alpha-2 adrenoceptor in rat jejunum epithelial cells: characterization with [^{3}H]RX821002 and distribution along the villus-crypt axis. *J. Pharmacol. Exp. Ther.*, **254**, 888–893.

RAITERI, M., MAURA, G. & VERSACE, P. (1983). Functional evidence for two stereochemically different alpa-2 adrenoceptors regulating central norepinephrine and serotonin release. *J. Pharmacol. Exp. Ther.*, **224**, 679–684.

REGAN, J.W., KOBILKA, T.S., YANG-FENG, T.L., CARON, M.G., LEFKOWITZ, R.J. & KOBILKA, B.K. (1988). Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 6301–6305.

REICHENBACHER, D., REIMANN, W. & STARKE, K. (1982). α-Adrenoceptor-mediated inhibition of noradrenaline release in rabbit brain cortex slices. Receptor properties and role of the biophase concentration of noradrenaline. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **319**, 71–77.

REMAURY, A. & PARIS, H. (1992). The insulin-secreting cell line, RINm5F, expresses an alpha-2D adrenoceptor and nonadrenergic idazoxan-binding sites. *J. Pharmacol. Exp. Ther.*, **260**, 417–426.

RICHARDS, M.H. (1985). Efflux of ^{3}H-5-hydroxytryptamine from rat hypothalamic slices by continuous electrical stimulation: frequency-dependent responses to serotonergic antagonists and 5-hydroxytryptamine. *Naunyn-Schmiedebergs Arch Pharmacol.*, **329**, 359–366.

RUFFOLO, R.R. Jr., SULPIZIO, A.C., NICHOLS, A.J., DEMARINIS, R.M. & HIEBLE, J.P. (1987). Pharmacologic differentiation between pre- and postjunctional α_2-adrenoceptors by SK&F 104078. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **336**, 415–418.

SIMONNEAUX, V., EBADI, M. & BYLUND, B. (1991). Identification and characterization of α_{2D}-adrenergic receptors in bovine pineal gland. *Mol. Pharmacol.*, **40**, 235–241.

STARKE, K. (1987). Presynaptic α-autoreceptors. *Rev. Physiol. Biochem. Pharmacol.*, **107**, 73–146.

UHLÉN, S. & WIKBERG, J.E.S. (1991). Delineation of three pharmacological subtypes of α_2-adrenoceptor in the rat kidney. *Br. J. Pharmacol.*, **104**, 657–664.

(Received March 16, 1992
Revised July 31, 1992
Accepted August 14, 1992)

Br. J. Pharmacol. (1992), **107**, 1152–1158

ATP activates P_{2x}-contracting and P_{2y}-relaxing purinoceptors in the smooth muscle of mouse vas deferens

[1]Benoit Boland, †Bernard Himpens, *M. Françoise Vincent, Jean-Marie Gillis & †Rik Casteels

Department of Physiology, U.C. Louvain, 1200 Brussels, Belgium, †Physiological Laboratory, Gasthuisberg, O/N, K.U. Leuven, 3000 Leuven, Belgium and *Laboratory of Physiological Chemistry, International Institute of Cellular and Molecular Pathology, 1200 Brussels, Belgium

1 The mechanism for the low potency of exogenous ATP in producing contraction at the P_{2x}-purinoceptors in the smooth muscle of the mouse vas deferens (VD) was examined.

2 The measure of the breakdown of ATP in contact with the VD showed that its degradation was limited and did not account for its weak contractile effect.

3 Externally applied, ATP induced a small and transient contraction but a marked and prolonged increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which suggests an efficient binding to the P_{2x}-purinoceptors. Such a calcium-force dissociation was not observed with β,γ-methylene ATP (β,γ-Me-ATP), a structural ATP analogue.

4 The force response of precontracted VD to ATP was biphasic, consisting of a small initial contraction followed by a sustained marked relaxation. In contrast, β,γ-Me-ATP elicited a pronounced contraction without ensuing relaxation.

5 ATP was more potent than adenosine in producing relaxation, and the relaxation was not antagonized by 8-phenyltheophylline, suggesting the activation of P_2-purinoceptors.

6 For this relaxation, the rank order of potency was 2-methyl-thio-ATP (2-MeSATP) > ATP > β,γ-Me-ATP, which is characteristic for the P_{2y}-purinoceptors.

7 Reactive Blue 2, a P_{2y}-purinoceptor antagonist, was found to reduce the relaxation mediated by ATP.

8 These results indicate that ATP acts in VD not only on contracting but also on relaxing P_2-purinoceptors, eliciting thereby overlapping opposite effects. In VD, the classical low potency of ATP for contraction is thus not explained by its low bioavailability or its low binding, but rather by its low specificity for the contracting P_{2x}-purinoceptors, leading to the activation of the relaxing P_{2y}-purinoceptors.

Keywords: ATP: P_2-purinoceptors; cytoplasmic Ca^{2+} concentration; smooth muscle, vas deferens

Introduction

In 1978, Burnstock introduced a classification of purinoceptors into P_1- and P_2-types. At the P_1-purinoceptors, adenosine acts with greater potency than adenosine 5'-triphosphate (ATP) and the response is inhibited by theophylline. At the P_2-purinoceptors, however, ATP is more potent than adenosine and not antagonized by theophylline. The P_2-purinoceptors have been further subdivided into major classes, the P_{2x}- and P_{2y}-subtypes (Burnstock & Kennedy, 1985), mediating in smooth muscles contracting and relaxing effects, respectively. These two subtypes are mainly distinguished by the rank order of potency for the contractile response of substituted analogues of ATP, i.e. β,γ-methylene-ATP (β,γ-Me-ATP) and 2-methyl-thio-ATP (2-MeSATP). At the contracting P_{2x}-purinoceptor, the characteristic rank order of potency is β,γ-Me-ATP > ATP ≥ 2-MeSATP. An inverse order is observed at the relaxing P_{2y}-purinoceptor. Other clues supporting the existence of P_2-purinoceptor subtypes (Kennedy, 1990) are the desensitization occurring at the P_{2x}- but not at the P_{2y}-purinoceptor, and the specific antagonism of Reactive Blue 2 for the P_{2y}-purinoceptor. The classification of smooth muscle P_1- and P_2-purinoceptors is mainly based on mechanical studies, the interpretation of which remains complex because of both the possible extracellular breakdown of nucleotides and the heterogeneity of purinoceptors present in a given preparation. This may lead to simultaneous interactions between various agonists and different receptors. A study of the post-receptor signal of P_{2x}-purinoceptors would clarify the biochemical effects of purines in the smooth muscles.

The contracting P_{2x}-purinoceptor was initially described in the vas deferens (Fedan *et al.*, 1982; Burnstock *et al.*, 1985) and is still considered to be the only type of postsynaptic purinoceptor activated by ATP in this tissue. However, the vas deferens contractile response to exogenous ATP is very weak (Fedan *et al.*, 1982; Hourani *et al.*, 1986; Wilkund & Gustafsson, 1988; von Kügelen *et al.*, 1990). We used the mouse vas deferens (VD) to examine three hypotheses which could explain the low potency of ATP at the contracting P_{2x}-purinoceptors; low bioavailability, low binding or low specificity. To clarify the question of the agonist bioavailability, we measured the breakdown of ATP in contact with VD. To test the binding of ATP to the P_{2x}-purinoceptors, we determined the amplitudes of its post-receptor cytosolic messenger, i.e. of the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). To investigate whether the low potency of ATP in producing contraction could result from its simultaneous binding to an unknown relaxing purinoceptor, we applied ATP to precontracted VD. The results reported here lead us to the conclusion that both P_{2x}- and P_{2y}-purinoceptor subtypes are present in the VD smooth muscle cells, and that the weak and transient contractile effect of ATP probably results from the opposite effects of its binding to contracting P_{2x}- and relaxing P_{2y}-purinoceptors.

[1] Author for correspondence.

Methods

Muscle tissues

Adult male albino mice (NMRI, 3–4 months old, 30–40 g) were killed by cervical dislocation after anaesthesia with ether. The vas deferens (VD) was removed and transferred to the oxygenated HEPES-buffered Krebs solution. The 10 mm end segment of its prostatic part was isolated, dissected free from surrounding tissues and opened longitudinally in order to remove its epithelial layer by gentle rubbing. Histological analysis of these VD strips showed that they consisted of a thick smooth muscular wall of about 20 cell layers, surrounded by poorly developed interstitial tissue. We also confirmed that the epithelial layer had been completely scraped away. Histological sections showed that smooth muscle represented more than 95% of the cells of the preparation.

Analysis of purine concentrations

The concentrations of adenosine, AMP, ADP and ATP generated at room temperature after application of purines on the VD muscle (4.82 ± 0.38 mg, $n = 12$) in the 1 ml chamber were measured by high performance liquid chromatography (h.p.l.c.). The stock solution of ATP contained 1.6% ADP, 0.2% AMP and no detectable adenosine. The stock solution of adenosine contained 0.05% AMP and no detectable ADP or ATP. VD strips were incubated for 60 min in 1 ml Krebs solution, as a control for possible spontaneous endogenous release of purines by the muscle. As control for non-enzymatic breakdown, ATP was incubated for 30 min in 1 ml HEPES-Krebs solution without any contact with VD. Adenosine was incubated for 15 min with VD to measure its possible uptake by the muscle. The breakdown of purines was determined by applying ATP and β,γ-Me-ATP to VD into the chamber, and collecting samples of the medium at the surface of the VD after 3 s, 30 s, 2 min, 5 min and 15 min incubation. Aliquots (100 µl) of the medium were transferred into 25 µl of ice-cold 10% (w/v) $HClO_4$. After 15 min at 4°C, the extracts were neutralized with 3 M-KOH/3 M-$KHCO_3$ and stored at −20°C until the assays were performed. Adenylic nucleotides (ATP, ADP and AMP) were measured by anion exchange h.p.l.c. on a 100 × 4.7 mm PartiSphere 5 SAX anion exhange column (Whatman, Maidstone, Kent) using a gradient from 0.01 M $NH_4H_2PO_4$ at pH 3.7 to 0.48 M $NH_4H_2PO_4$ at pH 3.7 over 30 min at a flow rate of 2 ml min^{-1}. The adenylic nucleoside (adenosine) was measured by reversed phase h.p.l.c. on a 110 × 4.7 mm PartiSphere 5 C_{18} column (Whatman, Maidstone, Kent) eluted over 15 min at a flow rate of 1.4 ml min^{-1} with 0.01 M NaH_2PO_4 at pH 5.5, and a 0 to 20% gradient of methanol/H_2O (1/1).

Simultaneous measurements of $[Ca^{2+}]_i$ and force

VD was loaded for 3 h with 2 µM fura-2AM, a fluorescent Ca^{2+}-indicator, in the normal HEPES-buffered Krebs solution at room temperature. This procedure did not affect either the amplitude or the kinetics of the force response to 140 mM K^+ or to purine agonists ($n = 6$). After loading, VD was rinsed for 30 min and mounted in a 1 ml chamber under isometric conditions. VD was stretched to a steady passive tension of 2 mN and allowed to equilibrate for 30 min before addition of drugs. VD was superfused at a constant flow rate of 4 ml min^{-1}. The mounting of VD in the chamber (Boland *et al.*, 1992) and the apparatus used for the simultaneous measurements of the fura-2 fluorescence and the force response in intact smooth muscle strips (Himpens & Somlyo, 1988) have been described. An internal calibration of the fluorescent signals was performed at the end of each experiment to determine the value of the minimal (R_{min}) and the maximal (R_{max}) ratio, using the procedure designed by Himpens *et al.* (1988). $[Ca^{2+}]_i$ values are routinely expressed in nM in the text and on the original traces. The force response is expressed either in mN or as a percentage of the maximal force level (100%) obtained in VD during initial stimulation with high K^+ solution, as usually used for the vas deferens (Fedan *et al.*, 1982; Burnstock *et al.*, 1985; Hourani *et al.*, 1986). The passive tension of 2 mN is used as the basal force reference (0%). For the study of the effect of the purine antagonists (see Figure 5), the relaxation by ATP was normalized to the raised-tone level elicited by prolonged stimulation with the 140 mM K^+ solution. Owing to the limited supply of the ATP analogues, perfusions with high concentrations could not be used to obtain maximal responses. Because of the marked desensitization occurring at the P_2-purinoceptors, only one concentration of one purine analogue was applied on each VD strip, except for the study of the antagonism of the purinoceptors where a second application of the agonist was performed in the presence of 8-phenyltheophylline (8PT) or Reactive blue 2 (RB2), 20 min after washing out the control agonist application. Neither ATP nor its analogues used here were autofluorescent. However, both 8PT and RB2 increased the fluorescent signals at 510 nm, especially after excitation with 340 nm, and thereby affected the 340/380 ratio values and the estimated $[Ca^{2+}]_i$. This interference made it impossible to determine $[Ca^{2+}]_i$ in their presence.

Solutions

The normal HEPES-Krebs solution contained (in mM): NaCl 135.5, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 11.6 and glucose 11.5. In the Ca^{2+}-free Krebs solution, $CaCl_2$ was omitted and 2 mM EGTA was added. Isotonic high K^+ solutions (70 and 140 mM $[K^+]_o$) were obtained by replacing external Na^+ by an equivalent amount of K^+. Sodium salt of adenosine triphosphate (ATP), sodium salt by β,γ-methylene ATP (β,γ-Me-ATP), hemisulphate salt of adenosine, phenylephrine and Reactive blue 2 (Cibacron blue 3GA, 55%) were obtained from Sigma. Tetrasodium salt of 2-methylthioadenosine triphosphate (2-MeSATP) was obtained from ICN Biochemicals (Cleaveland, Ohio, USA). Isoprenaline hydrochloride was from Winthrop Lab. (Brussels, Belgium). 8-phenyltheophylline and Ionomycin were from Calbiochem. Fura-2AM was from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were of analytical grade. All ATP analogues were the D-isomers. Drugs were dissolved in the HEPES-Krebs solution, except for 8PT which was dissolved in 80% methanol containing 0.2 M NaOH (Griffith *et al.*, 1981).

Statistics

The results are presented as means ± standard error of mean (s.e.mean), and n is the number of experiments. The data were evaluated for differences using Student's t test (paired two-tailed t test). A probability of less than 0.05 was considered significant.

Results

Extracellular metabolism of purines

Incubation of the VD strips in 1 ml HEPES-Krebs solution for 30 min did not lead to detectable levels of purines in the bathing medium ($n = 4$), indicating that the release of purines by VD was not significant. After 15 min of application of 1000 µM adenosine on the VD in the chamber, its concentration did not decrease ($n = 2$). This result excludes a significant lowering of the adenosine concentration due to nucleoside uptake by VD. Application of 100 µM β,γ-Me-ATP, a slowly degradable structural ATP analogue (Fedan *et al.*, 1982), did not result in the occurrence of any detectable

breakdown products ($n = 2$). Incubation of 100 μM ATP alone for 60 min in 1 ml HEPES-Krebs solution, without contact with VD, was not associated with purine degradation ($n = 3$). Exposure of VD to ATP was followed by a time-dependent accumulation of breakdown products, i.e. ADP, AMP and adenosine. Aliquots in contact with the VD were collected after 3 s, 30 s, 2 min, 5 min and 15 min of ATP application. After application of 100 μM ATP for 5 min ($n = 2$), the concentrations of ATP and ADP (P_2-agonists) were 75 and 12 μM, respectively. Under these conditions, AMP and adenosine (P_1-agonists) were 5 and 2 μM. As can be seen in Figure 1, after 5 min contact between VD and 1000 μM ATP, the concentrations of ATP and ADP were 953 and 56 μM, respectively, while the concentrations of AMP and adenosine were 15 and 13 μM, respectively. Preincubation of VD with 100 μM AOPCP, a specific inhibitor of the ecto 5'-nucleotidase (Burger & Lowenstein, 1975), which is the enzyme involved in the extracellular degradation of AMP to adenosine, modified the concentration changes in AMP and adenosine, which were respectively 19 and 6 μM after 5 min application of 1000 μM ATP ($n = 2$). Thus, whatever the ATP concentration, the concentration of P_1-agonists remained low, while the ATP concentration did not decrease to less than 75%. The bioavailability of ATP for the purinoceptors was therefore only slightly impaired.

Effects of ATP on $[Ca^{2+}]_i$ and force

VD does not present basal tonus or spontaneous contractile activity (Boland *et al.*, 1992). In the resting condition, $[Ca^{2+}]_i$ was 103 ± 5 nM ($n = 21$). Superfusing VD with a depolarizing solution containing 140 mM K^+ and 1.5 mM Ca^{2+} (Figure 2a) induced a maximal increase of $[Ca^{2+}]_i$ to 433 ± 44 nM ($n = 21$) and of force to 15 ± 1 mN. These values will be used further on as the 100% reference, for $[Ca^{2+}]_i$ and force, respectively. We studied the effects of superfusion for 5 min at 4 ml min^{-1} with purinoceptor agonists (100 μM) on $[Ca^{2+}]_i$ and force level in VD. ATP elicited an increase of $[Ca^{2+}]_i$ up to 361 ± 48 nM ($n = 9$) but force hardly rose (by 3 ± 1%, Figure 2a) and returned to its basal level within 1 min while $[Ca^{2+}]_i$ was still at 235 nM ($n = 9$). The amplitude of this ATP-induced $[Ca^{2+}]_i$ transient suggests that the transduction process worked efficiently in VD. When the superfusion with 100 μM ATP was repeated at 5 min intervals, the peak of $[Ca^{2+}]_i$ declined by 65 ± 3% at the second and the third stimulation (Figure 2b), while the force level remained at its basal level ($n = 4$). This desensitization was specific for the purinoceptors because 100 μM phenylephrine elicited, after 3 such successive ATP applications, an increase of $[Ca^{2+}]_i$ which was 92 ± 11% ($n = 4$) of the one it induced before the ATP applications ($n = 4$). These findings suggest that the involved purinoceptors displayed tachyphylaxis. However, the kinetics of this coupling could not be studied at the rather slow superfusion procedure. Therefore, we injected ATP (final concentration 100 μM) over a period of about 100 ms, close to the muscle (Figure 3). The latency period between the ATP injection and the $[Ca^{2+}]_i$ rise was 0.32 ± 0.8 s and $[Ca^{2+}]_i$ peaked after 4.5 ± 0.4 s, while contraction reached 13 ± 1% of the 140 K^+ reference. Finally, to study the dependence of the ATP-induced Ca^{2+} transient on the external Ca^{2+}, we applied ATP after 15 min perfusion with a Ca^{2+}-free Krebs solution containing 2 mM EGTA. Under these conditions, $[Ca^{2+}]_i$ decreased to 65 ± 8 nM, as previously reported (Boland *et al.*, 1992) and 100 μM ATP only increased $[Ca^{2+}]_i$ to 86 ± 18 nM ($n = 4$), without inducing any force (not shown).

The effects of ATP were compared to those induced by superfusion with its substituted analogue. Superfusion with 100 μM 2-MeSATP ($n = 4$) induced an increase of $[Ca^{2+}]_i$ and of force comparable to those elicited by ATP (not shown). However, β,γ-Me-ATP increased $[Ca^{2+}]_i$ to 187 ± 13 nM ($n = 7$) and force up to 27 ± 8% (Figure 2a). The two parameters progressively declined after 5 min stimulation

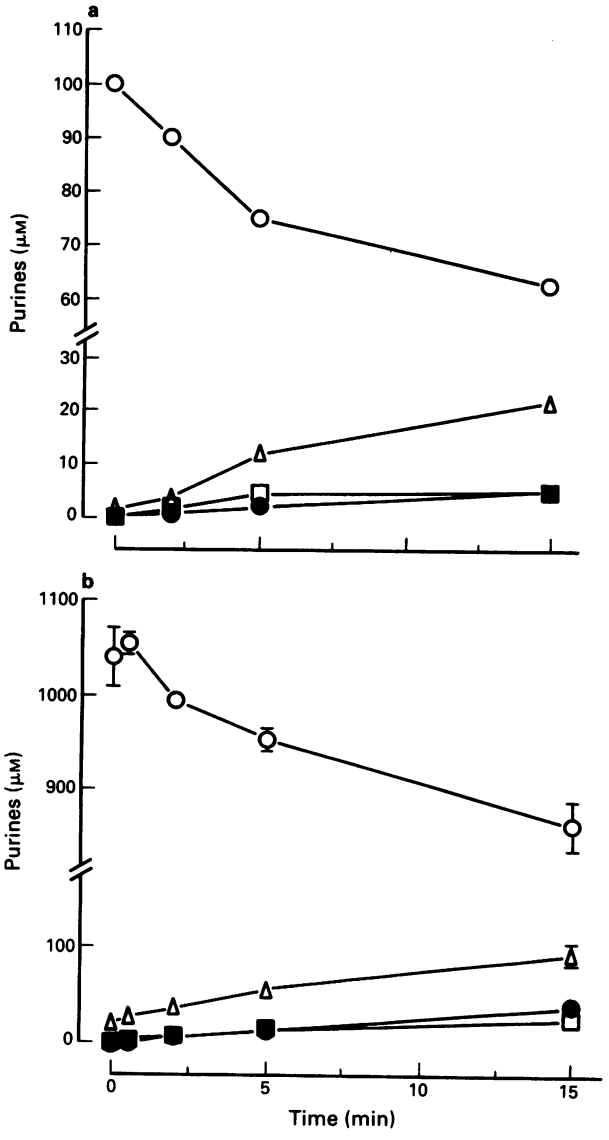

Figure 1 Time-course of the breakdown of ATP (○) into ADP (△), AMP (□) and adenosine (●) by the vas deferens (VD). ATP was applied to the mouse VD at a final concentration of (a) 100 μM ($n = 2$) and (b) 1000 μM ($n = 3$) in the chamber containing 1 ml HEPES-Krebs solution. Samples of the medium were taken at the VD surface at the times shown and further analysed by h.p.l.c.

with β,γ-Me-ATP to 130 ± 10 nM and 15 ± 5%, respectively. Adenosine 100 μM ($n = 4$) did not alter either $[Ca^{2+}]_i$ or force. This indicates that P_1-purinoceptors are not involved in the contractile response or in the Ca^{2+} changes in VD. So far, our results on the potency for contraction and on the tachyphylaxis indicate that P_{2x}-purinoceptors mediate the ATP-induced contraction in VD. The paradoxical dissociation, both in kinetics and amplitude, between the increases of $[Ca^{2+}]_i$ and of force is a puzzling finding. The respective evolutions of the prolonged and high $[Ca^{2+}]_i$ rise and the transient and small force contraction evoked by ATP suggest the presence of an unknown relaxing process. Because of the absence of basal tonus in VD, this relaxation could have been masked. We therefore applied purines on the precontracted VD to investigate this hypothesis.

Effects of ATP in the precontracted preparations

Non-purine agonists (noradrenaline, carbachol, histamine) in concentrations up to 1 mM failed to induce in VD a sustained

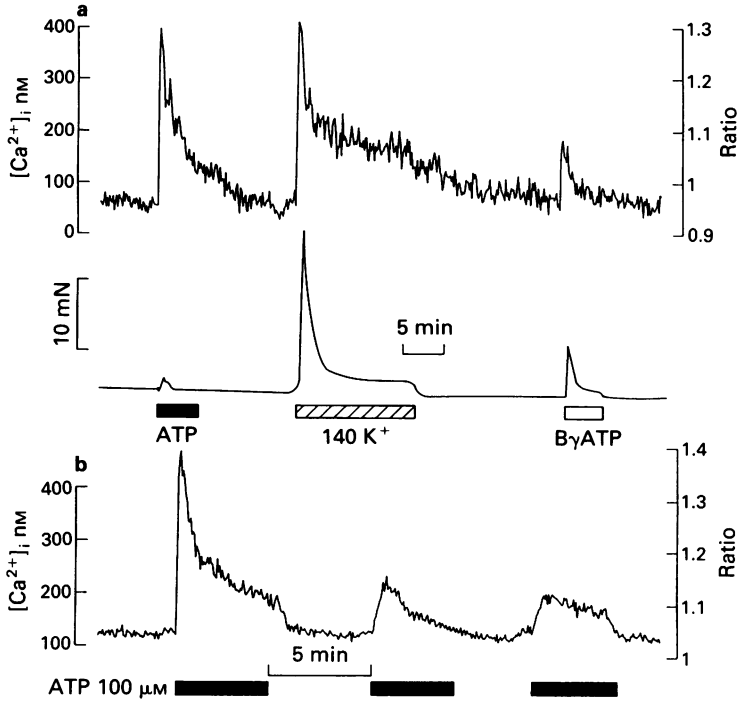

Figure 2 Panel (a) shows the changes in $[Ca^{2+}]_i$ (upper trace) and force (lower trace) in the vas deferens in response to superfusion with 100 μM ATP (solid bar) or 100 μM β,γ-methylene-ATP (β,γ-Me-ATP, open bar). Maximal increases (100%) of $[Ca^{2+}]_i$ and of force were obtained after 1 min depolarization with 140 mM K^+ (hatched bar). Results were independent of the sequence of perfusion with ATP, β,γ-Me-ATP and 140 K^+. In panel (b), for study of receptor tachyphylaxis, 100 μM ATP was superfused three times for 5 min at 5 min intervals.

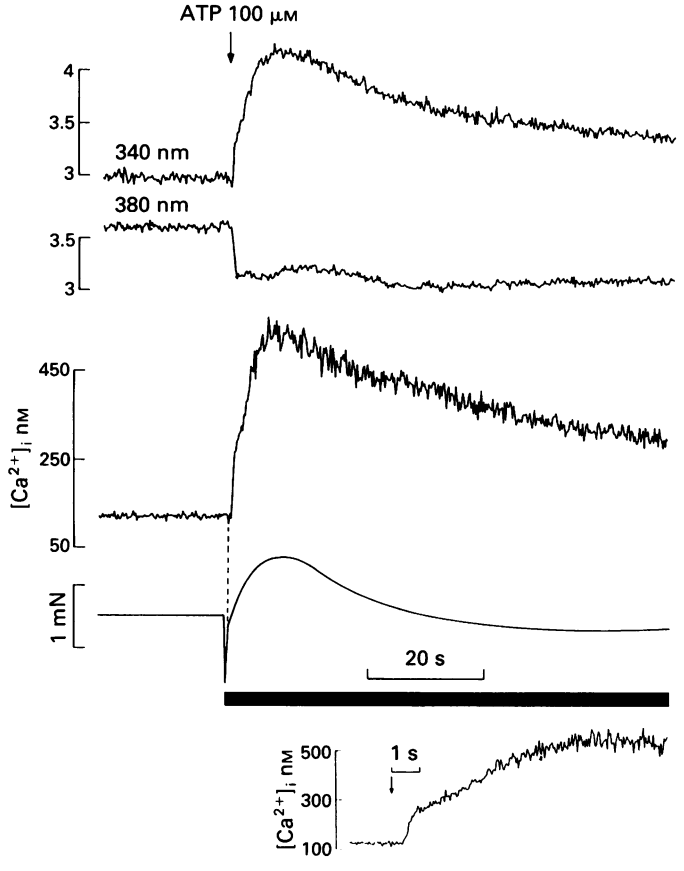

Figure 3 Effect of direct injection in the medium of 100 μM ATP on the fluorescent signals (upper traces), the $[Ca^{2+}]_i$ (middle trace) and the force (lower trace) of the vas deferens. The sampling frequency was 100 Hz. To obtain a precise time reference, a negative signal applied to the transducer was suddenly removed at the time of the ATP injection. The methodological imprecision was lower than 100 ms. In the inset, the same ATP stimulation is displayed at a higher time resolution, showing that the increase of $[Ca^{2+}]_i$ began within less than 500 ms after the ATP injection (arrow).

contraction. However, prolonged depolarization with 140 mM K^+ induced a sustained contraction (Figure 2a). After 15 min stimulation, $[Ca^{2+}]_i$ was still at 247 ± 15 nM, and force at 18 ± 2% of the initial peak reference value ($n = 21$). Under these conditions, no force change was induced by superfusion with 10 μM ATP or 10 μM adenosine, while 10 μM 2-Me-SATP produced a slight but significant relaxation to 16 ± 1%. Next, we superfused purines for 5 min at 100 μM concentration. ATP elicited a rise in $[Ca^{2+}]_i$ up to 417 ± 71 nM ($n = 6$) (Figure 4a). This parameter returned within 2 min to the previous level (257 ± 20 nM), while the force response presented after 30 s a relaxation to 14 ± 0.6%, following an initial variable and small contraction to 20 ± 0.7%. After washing out ATP, force recovered its previous level (18%). Changes in $[Ca^{2+}]_i$ and force induced by 2-MeSATP (Figure 4a) were comparable to those obtained by ATP ($n = 3$). β,γ-Me-ATP (Figure 4a) increased $[Ca^{2+}]_i$ to 320 ± 30 nM ($n = 6$) and of force to 31 ± 2%, and within 2 min, both $[Ca^{2+}]_i$ and force recovered their previous levels. No relaxation below the precontracted force level was thus observed. Adenosine 100 μM did not significantly alter either $[Ca^{2+}]_i$ or force ($n = 5$), except in 2 preparations in which force decreased slightly to 17%.

The biphasic pattern of the ATP-induced force response became more pronounced at 1000 μM ATP. Force initially rose to 23.2 ± 3% and thereafter relaxed to 6.3 ± 1% ($n = 14$) (Figure 4b). In order to study the purinoceptor tachyphylaxis, we also superfused the tissue with 1000 μM ATP twice at 5 min intervals ($n = 4$). In this condition, we could not observe the initial contraction during the second ATP application (Figure 4b), while the relaxation was similar to that obtained during the first ATP stimulation. Superfusion of VD with a mixture of the concentrations of the P_1-agonists as produced by the breakdown of 1000 μM ATP, i.e. with 15 μM AMP and adenosine (see Figure 1), induced only a relaxation to 17 ± 0.5% ($n = 4$). Adenosine, 1000 μM relaxed VD to 10 ± 1%; isoprenaline, 100 μM, ($n = 5$) and papaverine, 1000 μM, ($n = 4$) produced relaxation to 9 ± 1 and to 6 ± 2%, respectively. The relaxing effect of ATP is not species specific: in the rat ($n = 6$) and the guinea-pig ($n = 6$) vas deferens, 1000 μM ATP also induced a biphasic force response consisting of a small initial contraction followed by a steady relaxation (data not shown).

To characterize further the receptor involved in the relaxation induced by ATP, we studied the effect of 8PT, a potent P_1-antagonist (Griffith *et al.*, 1981) and of RB2, a selective (Burnstock & Warland, 1987) but weak P_{2y}-antagonist, during non-cumulative applications for 5 min of 100, 250, 500 or 1000 μM ATP. The results are presented in Figure 5 and the relaxation is expressed in these experiments as a percentage of the steady raised-tone induced by 140 mM K^+. The concentration-response curve in the absence of the antagonist is represented by circles. A preceding incubation of VD for 15 min with 10 μM 8PT (triangles) did not significantly modify the relaxation by ATP up to 1000 μM. This 8PT concentration is reported to inhibit by about 50% the relaxation induced by 1000 μM ATP in the histamine-contracted rabbit central ear artery (Kennedy & Burnstock, 1985), in which the relaxation by ATP is P_1-dependent. We observed an inhibition of 60% of the ATP-induced relaxation in this rabbit preparation ($n = 5$), indicating that our 8PT solution was effective. Also 100 μM 8PT did not inhibit the ATP-induced relaxation in VD, but it should be mentioned that at this concentration, not all 8PT was dissolved. Incubation of VD for 15 min with 200 μM RB2 did not produce by itself any force decline. RB2 slightly but significantly inhibited the relaxation by ATP (squares), but not that caused by 100 μM isoprenaline ($n = 4$) or by 1000 μM papaverine ($n = 3$). RB2 200 μM did not affect the force response to ATP in 25% of preparations. In the precontracted VD incubated with 200 μM RB2, 1000 μM ATP elicited an initial contraction to 29 ± 2%, which is higher than the value induced by the same ATP solution in the absence of RB2 (see above).

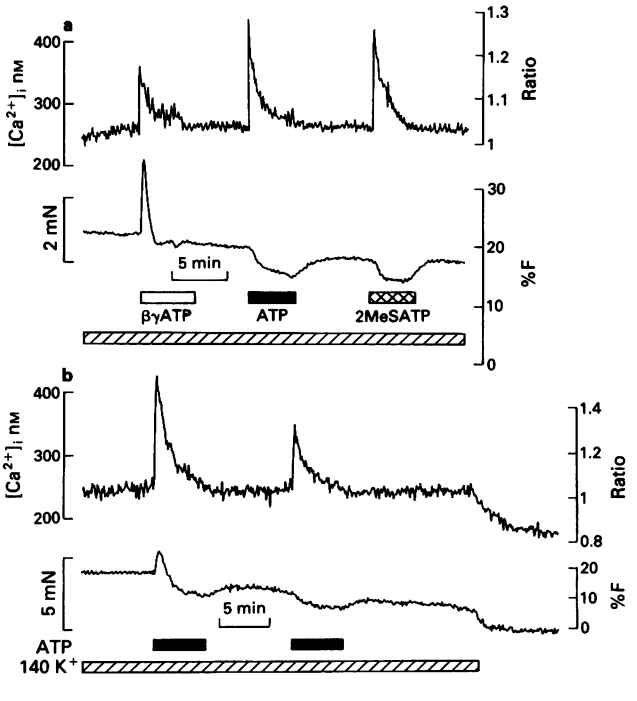

Figure 4 (a) Comparison of the effect of β,γ-methylene-ATP (β,γ-Me-ATP) (open bar), ATP (solid bar) and 2-methyl-thio-ATP (2-MeSATP) (cross hatched bar) on $[Ca^{2+}]_i$ (upper trace) and on force (lower trace) in the K^+-precontracted vas deferens (VD). β,γ-Me-ATP induced a contraction, while ATP and 2-MeSATP elicited marked relaxation after a small and initial contraction. Here, the stimulation with β,γ-Me-ATP produced a P_{2x}-purinoceptor tachyphylaxis so that the initial contraction during the following ATP application was absent. (b) Shows the effect of 1000 μM ATP on $[Ca^{2+}]_i$ (upper trace) and the force (lower trace) in K^+-precontracted VD. The first ATP application always triggered a pronounced and transient rise in $[Ca^{2+}]_i$ and a biphasic force pattern consisting of an initial contraction followed by a steady relaxation. A second ATP application induced a lower rise of $[Ca^{2+}]_i$ and a loss of the early contraction, suggesting tachyphylaxis of the P_{2x}-purinoceptors. The relaxation was reproducible and was not associated with a detectable decline in $[Ca^{2+}]_i$.

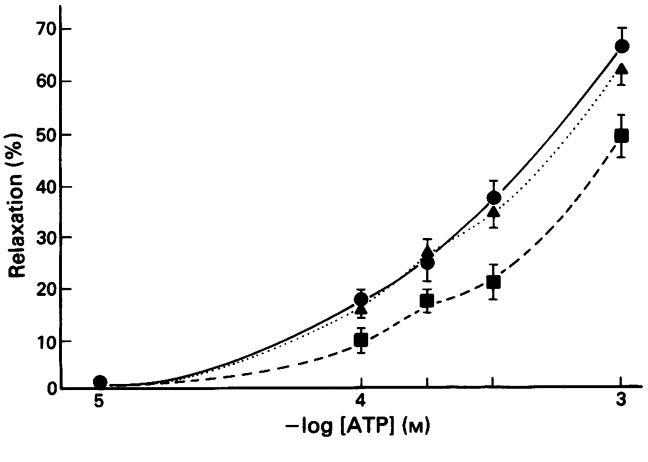

Figure 5 Effects of the antagonist of P_1-purinoceptor, 8-phenyltheophylline (10 μM) (▲), and of P_{2y}-purinoceptor, Reactive blue 2 (200 μM) (■), on the relaxation by ATP. Relaxation is expressed as a percentage of the maintained tone obtained in the vas deferens by a prolonged stimulation with 140 mM K^+; (●) represent the controls.

Discussion

We describe here the first study of the simultaneous measurements of the Ca^{2+}-transients and the force responses induced by ATP and related nucleotides in the intact vas deferens. It is generally accepted that ATP is less potent than β,γ-Me-ATP in activating the contracting P_{2x}-purinoceptors of the vas deferens, because ATP is enzymatically broken down or because it presents a low binding to its receptor. Although the breakdown observed at the tissue surface might be an underestimate of the actual breakdown occurring at the receptor level, our measurements rule out a significant enzymic degradation of ATP in the VD. Similar findings were reported in the taenia coli in which no correlation was found either between the ATP analogue potency for contraction and its rate of degradation (Welford *et al.*, 1986). However, we observed a very large ATP-induced rise in $[Ca^{2+}]_i$.

In the vas deferens smooth muscle, the P_{2x}-purinoceptors trigger the opening of non-selective ion channels associated to membrane depolarization and early Ca^{2+} influx (Friel, 1988). Thus an ATP-induced Ca^{2+} signal mediated through the P_{2x}-purinoceptors is expected to be strongly dependent on the external Ca^{2+} and to occur relatively fast. The activation of the P_{2y}-purinoceptors in endothelial cells (Carter *et al.*, 1988) and in erythrocytes (Boyer *et al.*, 1989; Cooper *et al.*, 1989), can also lead to an elevation of $[Ca^{2+}]_i$, but through an IP_3-induced Ca^{2+}-release independent of the extracellular Ca^{2+} and delayed by several seconds (Boeynaems & Pearson, 1990). Here, we describe the marked inhibition of the ATP-induced Ca^{2+} signal in the Ca^{2+}-free solution and its short delay (320 ms). These characteristics make the participation of P_{2y}-purinoceptors in the Ca^{2+} signal triggered by ATP less likely, and suggest that the ATP-induced Ca^{2+} signal was mainly due to the activation of P_{2x}-purinoceptors. Moreover, the P_{2y}-induced Ca^{2+}-dependent relaxation observed in digestive smooth muscle is caused by membraneous hyperpolarization (Crist *et al.*, 1992). The finding that ATP relaxed K^+-depolarized preparations in our experiments suggests that another pathway is involved in the VD and provides an additional argument against the P_{2y} participation in the ATP-induced rise of Ca^{2+}. As indicated by electrophysiological measurements of the induced inward current (Friel, 1988), the potency of ATP is much higher than that of β,γ-Me-ATP at the P_{2x}-purinoceptor. The resulting membranous depolarization activates Ca^{2+}-influx (Kennedy, 1990). Our measurement of the rise of $[Ca^{2+}]_i$ confirms that ATP is more potent than β,γ-Me-ATP at the P_{2x}-purinoceptors. Taken together, these findings suggest that the difference in Ca^{2+} rise observed between ATP and β,γ-Me-ATP is mainly due to different potencies at the P_{2x}-purinoceptors. It is therefore not necessary to invoke another mechanism such as a P_{2y}-associated rise of Ca^{2+} to explain the observed rank order of potency for the Ca^{2+}-increase (ATP = 2-MeSATP > β,γ-Me-ATP).

We applied ATP to precontracted VD to unmask a possible relaxant effect and we observed a superimposed biphasic force response consisting of a small and variable initial contraction followed by a maintained relaxation. The small initial contraction showed marked tachyphylaxis and a rank order of potency (β,γ-Me-ATP > ATP ≥ 2-MeSATP), which is typical for the P_{2x}-purinoceptors (Kennedy, 1990). The relaxation by ATP was not inhibited by 8PT and was more pronounced than that due to adenosine, indicating that the relaxation was P_2-dependent. The breakdown products of ATP which are P_1-agonists induced less than 10% of the relaxation produced by ATP. Therefore, ATP must act mainly by itself on VD P_2-purinoceptors. The P_{2y}-mediation of the relaxation by ATP was supported by the observed rank order of potency for relaxation (2-MeSATP ≥ ATP > β,γ-Me-ATP) and by the specific antagonism by RB2 (Manzini *et al.*, 1985; Burnstock & Warland, 1987; Lefebvre & Burnstock, 1990). The findings that successive ATP applications led to tachyphylaxis of the contractile response but not of the relaxation (Figure 4b) and that RB2 induced in the precontracted VD both an increase of the contraction and an inhibition of the relaxation in response to ATP, suggest that the contracting and the relaxing effects are mediated through different receptors. Such a biphasic force response to ATP has previously been reported in the guinea-pig trachea (Brown & Burnstock, 1981) and in contracted vascular preparations (Ralevic & Burnstock, 1991). The force response to ATP thus seems to be the result of two opposite effects in preparations expressing both P_{2x}- and P_{2y}-purinoceptors, while that caused by β,γ-Me-ATP depends mainly on P_{2x}-activation. Substitution of a methylene bridge in the polyphosphate chain of ATP indeed increases the specificity for P_{2x}-purinoceptors and reduces its potency in activating the relaxing P_{2y}-purinoceptors (Gordon, 1986): in precontracted VD, β,γ-Me-ATP produced a large transient contraction but did not evoke a subsequent relaxation, as previously observed in guinea-pig smooth muscle preparations containing P_{2y}-purinoceptors, i.e. trachea (Brown & Burnstock, 1981), taenia coli and aorta (Hourani *et al.*, 1985).

It can therefore be proposed that in the VD, ATP acts also on P_{2y}-purinoceptors, inducing a relaxation that can overcome the contractile effect mediated by the P_{2x}-purinoceptors. Binding of ATP at the activating P_{2x}-purinoceptors indeed directly activates ion channels (Friel, 1988), while binding at the inhibiting P_{2y}-purinoceptors elicits a slower G protein-dependent enzymic activation (Kennedy, 1990). This might lead to a difference in time-course between the opposite influences of activating and inhibiting processes. The fast transduction through P_{2x}-purinoceptors would first trigger muscle activation, but the combined effect of the P_{2x}-desensitization and the activation of the inhibiting P_{2y}-purinoceptors (which do not desensitize) eventually turns the balance in favour of relaxation. In contrast, ATP induces in the digestive and the respiratory smooth muscles an initial transient relaxation followed by a sustained contraction. The delay of this latter P_{2x}-dependent contraction might be explained by its mediation through the rather slow prostaglandin pathway, as indicated by its abolition by indomethacin (Burnstock *et al.*, 1978; Brown & Burnstock, 1981; Manzini *et al.*, 1985; Lefebvre & Burnstock, 1990). In VD, indomethacin did not modify the force response to ATP, as previously reported (Fedan *et al.*, 1982; Wilkund & Gustafsson, 1988). The release of ATP from purinergic nerve terminals *in situ* will minimize the diffusion delay and favour the initial contraction. This might explain why transmural electrical stimulation produces contractions up to 60% of the reference peak K^+ stimulation in VD incubated in the presence of phentolamine (Boland *et al.*, 1992). Finally, our hypothesis can also readily explain the difference in potency of ATP analogues on the $[Ca^{2+}]_i$ rise and on force development. Even if β,γ-Me-ATP binds less tightly than ATP to the P_{2x}-purinoceptors, producing thereby a smaller rise of $[Ca^{2+}]_i$ (Figure 2a), its preferential binding to P_{2x}-purinoceptors has to result in a higher force response because the inhibitory P_{2y}-effect comes into play very little.

Our simultaneous study of $[Ca^{2+}]_i$ and force clearly shows that the binding of ATP to P_{2x}- and to P_{2y}-purinoceptors does not result in a competition at the level of the same messenger because ATP produced a large increase of $[Ca^{2+}]_i$ but a limited force development. The effect of the elevated $[Ca^{2+}]_i$ on the force is thus somehow antagonized by the activation of the P_{2y}-purinoceptors through a still unknown mechanism which might affect either the level of the myosin phosphorylation or the activity of proteins involved in the actin-myosin interaction, like caldesmon or calponin.

These results also indicate that the order of potency for VD contraction by the ATP analogues, i.e. the essential criteria for the P_2-purinoceptor subclassification, depends mainly on their specificity for the P_{2x}-purinoceptors. The unexpected purinoceptor heterogeneity observed in the vas deferens suggests that the order of potency could appreciably vary among smooth muscles according to differences in the

expression of the P_2-purinoceptor-subtype(s), and it could explain, at least partly, the increasing discrepancy reported in the classification of P_2-purinoceptors.

B.B. is a research assistant of the FNRS (Belgium). The assistance of Mrs I. Willems and of Mr R. Verbist, M. Coenen & V. Trappeniers is gratefully acknowledged. We thank Prof. G. vanden Berghe for helpful discussions.

References

BOEYMAENS, J.M. & PEARSON, J.D. (1990). P_2-purinoceptors on vascular endothelial cells: physiological significance and transduction mechanisms. *Trends Pharmacol. Sci.*, **11**, 34–37.

BOLAND, B., HIMPENS, B., GILLIS, J.M. & CASTEELS, R. (1992). Force-Ca^{2+} relationship in anococcygeal and vas deferens smooth muscle cells of mouse. *Pflügers Arch., Eur. J. Physiol.*, **421**, 43–51.

BOYER, J.L., DOWNES, C.P. & HARDEN, T.K. (1989). Kinetics of activation of phospholipase C by P_{2y} purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.*, **264**, 884–890.

BROWN, C.M. & BURNSTOCK, G. (1981). The structural conformation of the polyphosphate chain of the ATP molecule is critical for its promotion of prostaglandin biosynthesis. *Eur. J. Pharmacol.*, **69**, 81–86.

BURGER, R.M. & LOWENSTEIN, J.M. (1975). 5'-Nucleotidase from smooth muscle of small intestine and from brain. Inhibition by nucleotides. *Biochemistry*, **14**, 2362–2366.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach.* ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G., COCKS, T. & RAHIMA CROWE (1978). Evidence for purinergic innervation of the anococcygeus muscle. *Br. J. Pharmacol.*, **64**, 13–20.

BURNSTOCK, G., CUSACK, N.J. & MELDRUM, L.A. (1985). Studies on the stereoselectivity of the P_2-purinoceptor on the guinea-pig vas deferens. *Br. J. Pharmacol.*, **84**, 431–434.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **5**, 433–440.

BURNSTOCK, G. & WARLAND, J.J.I. (1987). P_2-purinoceptors of two subtypes in the rabbit mesentery artery: reactive blue 2 selectively inhibits responses mediated via the P_{2y}- but not the P_{2x}-purinoceptor. *Br. J. Pharmacol.*, **90**, 383–391.

CARTER, T.D., HALLAM, T.J., CUSACK, N.J. & PEARSON, J.D. (1988). Regulation of P_{2y}-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br. J. Pharmacol.*, **95**, 1181–1190.

COOPER, C.L., MORRIS, A.J. & HARDEN, T.K. (1989). Guanine nucleotide-sensitive interaction of a radiolabelled agonist with a phospholipase C-linked P_{2y}-purinergic receptor. *J. Biol. Chem.*, **265**, 6202–6206.

CRIST, J.R., XUE, D., HE & GOYAL, R.K. (1992). Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. *J. Physiol.*, **447**, 119–131.

FEDAN, J.S., HOGABOOM, G.K., WESTFALL, D.P. & O'DONNELL, J.P. (1982). Comparison of contractions of the smooth muscle of the guinea-pig vas deferens induced by ATP and related nucleotides. *Eur. J. Pharmacol.*, **81**, 193–204.

FRIEL, D.D. (1988). An ATP-sensitive conductance in single smooth muscle cells from the rat vas deferens. *J. Physiol.*, **401**, 361–380.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

GRIFFITH, S.G., MEGHJI, P., MOODY, C.J. & BURNSTOCK, G. (1981). 8-Phenyltheophylline: a potent P_1-purinoceptor antagonist. *Eur. J. Pharmacol.*, **75**, 61–64.

HIMPENS, B. & SOMLYO, A.P. (1988). Free calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J. Physiol.*, **395**, 507–530.

HIMPENS, B., MATTIJS, G., SOMLYO, A.V., BUTLER, T.M. & SOMLYO, A.P. (1988). Cytoplasmic free calcium, myosin light chain phosphorylation and force in phasic and tonic smooth muscle. *J. Gen. Physiol.*, **92**, 713–729.

HOURANI, S.M.O., LOIZOU, G.D. & CUSACK, N.J. (1986). Pharmacological effects of L-AMP-PC P on ATP receptors in smooth muscle. *Eur. J. Pharmacol.*, **131**, 99–103.

KENNEDY, C. & BURNSTOCK, G. (1985). ATP produces vasodilatation via P_1-purinoceptors and vasoconstriction via P_2-purinoceptors in the isolated rabbit central ear artery. *Blood Vessels*, **22**, 145–155.

KENNEDY, C. (1990). P_1- and P_2-purinoceptor subtypes – an update. *Arch. Int. Pharmacodyn.*, **303**, 30–50.

LEFEBVRE, R.A. & BURNSTOCK, G. (1990). Effect of adenosine triphosphate and related purines in the rat gastric fundus. *Arch. Int. Pharmacodyn.*, **303**, 199–215.

MANZINI, S., MAGGI, C.A. & MELI, A. (1985). Further evidence for involvement of adenosine-5'-triphosphate in non-adrenergic non-cholinergic relaxation of the isolated rat duodenum. *Eur. J. Pharmacol.*, **113**, 399–408.

RALEVIC, V. & BURNSTOCK, G. (1991). Roles of P_2-purinoceptors in the cardiovascular system. *Circ.*, **84**, 1–14.

VON KÜGELGEN, I., BÜLTMAN, R. & STARKE, K. (1990). Interaction of adenine nucleotides, UTP and suramine in mouse vas deferens: suramin-sensitive and suramin-insensitive components in the contractile effect of ATP. *Naunyn. Schmiedebergs. Arch. Pharmacol.*, **342**, 198–205.

WELFORD, L.A., CUSACK, N.J. & HOURANI, S.M.O. (1986). ATP analogues and the guinea-pig taenia coli: a comparison of the structure-activity relationships of ectonucleotidases with those of the P_2-purinoceptor. *Eur. J. Pharmacol.*, **129**, 217–224.

WILKUND, N.P. & GUSTAFSSON, L.E. (1988). Indications for P_2-purinoceptor subtypes in guinea-pig smooth muscle. *Eur. J. Pharmacol.*, **148**, 361–370.

(Received May 18, 1992
Revised August 13, 1992
Accepted August 14, 1992)

Br. J. Pharmacol. (1992), **107**, 1159–1162

Protective effects of inhibitors of nitric oxide synthase in immune complex-induced vasculitis

M.S. Mulligan, [1,]*S. Moncada & P.A. Ward

Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109, U.S.A. and *Wellcome Research Laboratories, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS

1 The ability of analogues of L-arginine (N-iminoethyl-L-ornithine (L-NIO), N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NNA)) to protect against inflammatory injury induced by activated neutrophils was investigated in rats following intradermal or intrapulmonary deposition of immune complexes.

2 The descending order of potency for protective effects of these analogues was: L-NIO > L-NMMA > L-NNA = L-NAME. The approximate IC_{50} value for L-NIO in the dermal vasculitis model was 65 μM. For all other compounds, the IC_{50} values were > 5 mM.

3 The protective effect of L-NIO in the skin was reversed in a dose-dependent manner by the presence of L-arginine, but not by D-arginine. L-Arginine also reversed the protective effects of L-NIO in immune complex-induced lung injury.

4 The protective effects of L-NIO were not associated with reductions in neutrophil accumulation, as measured by extraction from tissues of myeloperoxidase.

5 These data demonstrate that L-NIO has the most potent protective effects against immune complex-induced vascular injury induced by activated macrophages. Furthermore, they indicate that this injury is dependent upon the generation of nitric oxide.

Keywords: Nitric oxide synthase inhibitors; immune complex-induced vasculitis; lung injury

Introduction

Phagocytic cells, including neutrophils as well as macrophages, generate nitric oxide (NO) via an NO synthase that is inducible by immunological stimuli such as endotoxin (LPS) and various cytokines (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; McCall *et al.*, 1989; Schmidt *et al.*, 1989; Stuehr & Nathan, 1989; Wright *et al.*, 1989; Billiar *et al.*, 1990; Curran *et al.*, 1990). Generation of NO by macrophages has been showed to kill tumour cells due to the inactivation of iron-sulphur centres of mitochondrial enzymes (Drapier & Hibbs, 1986; Hibbs *et al.*, 1987; 1988). The broader role of NO in the inflammatory response is not well established, although the reactivity of NO or its potential conversion product, peroxynitrite anion, with sulphydryl groups indicates the possibility of cellular biochemical targets whose alteration would put tissue at risk of injury.

Recently, it has been demonstrated that IgG immune complex-initiated injury of rat lung is greatly attenuated by the presence of the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA) (Mulligan *et al.*, 1991). The protective effects of L-NMMA were not associated with a reduced influx of neutrophils into lungs, suggesting that toxic metabolites of L-arginine may be responsible for the inflammatory injury, which is known to depend upon the recruitment and participation of neutrophils (Johnson & Ward, 1979). Since N-iminoethyl-L-ornithine (L-NIO) has recently been described as a highly potent inhibitor of phagocytic cell NO synthase (McCall *et al.*, 1991), this compound was tested for its ability to protect against immune complex-induced vascular injury in rats. In addition, the effects of L-NIO were compared with those of other L-arginine analogues, namely, L-NMMA, N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME).

[1] Author for correspondence.

Methods

Models of immune complex-induced alveolitis and dermal vasculitis

With adult male (300 g) specific pathogen-free Long-Evans rats (Charles River Breeding Laboratories), immune complex reactions were induced in lung and skin by the intratracheal (2.5 mg of antibody in 300 μl) or intradermal (0.42 mg of antibody in 50 μl) injection of polyclonal rabbit IgG antibody to bovine serum albumen (anti-BSA) followed by the intravenous injection of 10 mg BSA containing trace amounts of [^{125}I]-BSA. Tissue injury was assessed at 4 h by the increase in vascular permeability. Permeability values were defined as the ratio of ^{125}I-labelled BSA in lung or skin sites to the amount present in 1.0 ml blood obtained from the inferior vena cava at the time of death. Previously, we reported that BSA-anti-BSA immune complexes produced in untreated positive control rats lung and dermal vascular permeability values of approximately 0.46 (Mulligan *et al.*, 1991). In the current studies a new batch of anti-BSA was employed, giving the indicated permeability values which were approximately double those recorded early. Accordingly, computed percentages of protein were different from those observed earlier with the different batch of antibody which was less injurious.

Tissue myeloperoxidase (MPO) content

A standard reference curve was first established by measuring MPO in lungs and skin sites that had been injected with known numbers of neutrophils. Lung and skin sites were extracted by homogenization and sonication procedures that have been previously described (Mulligan *et al.*, 1991). MPO activity in supernatant fluids was measured by the change in optical density (at 460 nm) resulting from decomposition of H_2O_2 in the presence of *o*-dianisidine.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA). Additional comparisons were made between individual groups by the use of paired or unpaired Student's t tests. All values were expressed as mean ± s.e.mean unless otherwise indicated. Statistical significance was defined as $P<0.05$.

Materials

L-NIO and L-NNA were provided by Wellcome Research Laboratories (Beckenham, Kent) while L-NAME and L-NMMA were from Cal Biochem (La Jolla, California, U.S.A.). Rabbit polyclonal IgG antibody to bovine serum albumin (anti-BSA) was from Organon Teknicka (Westchester, PA, U.S.A.).

Results

Protective effects of L-arginine analogues in dermal vascular injury

Immune complex deposition was induced by the intra-dermal injection of anti-BSA and the intravenous injection of BSA. The resulting vascular injury was measured 4 h later by leakage of [^{125}I]-albumen into skin sites. In the negative controls (omission of intravenously injected BSA) and positive controls, the permeability values were 0.07 ± 0.01 and 0.81 ± 0.03, respectively. When used, the L-arginine analogues were mixed with the anti-BSA preparations immediately prior to intradermal injection. Concentrations of the analogues employed were: 0.01, 0.1, 0.5, 1.0 and 5.0 mM. The data in Figure 1 show the comparative abilities of the four analogues of L-arginine to reduce immune complex-induced vascular injury, as measured by changes in vascular permeability. The most effective compound was L-NIO, which caused a marked reduction in the increase in vascular permeability. L-NIO had an estimated IC_{50} of 65 μM. At 1.0 mM, L-NMMA reduced the permeability change by 32%, but this was not further reduced with 5 mM L-NMMA. L-NNA and L-NAME were much less potent; at 5 mM, the protective effects were only 19% and 16%, respectively. Thus, the rank order of protective effects for these L-arginine analogues in immune complex-induced dermal vascular injury is: L-NIO > L-NMMA > L-NNA = L-NAME.

Ability of L-arginine to reverse protective effects of L-NIO

In the same model of immune complex-induced dermal vascular damage, the presence of 1.0 mM L-NIO in the anti-BSA preparation resulted in a fall in the permeability index from the positive control (absence of L-NIO), from 0.81 ± 0.03 to 0.46 ± 0.02, a 47% reduction ($P<0.001$) in the permeability index. As L-arginine was added in increasing concentrations to anti-BSA preparations which contained 1.0 mM L-NIO, the reductions (reversals) in vascular permeability reflecting the protective effects of L-NIO were correspondingly reversed: with 0.1, 0.5, 1.0, 5.0 and 10 mM L-arginine, the permeability values were: 0.55 ± 0.03 (35% reduction, $P=0.002$); 0.66 ± 0.04 (20% reduction, $P=0.049$); 0.74 ± 0.02 (9% reduction, P = NS); 0.81 ± 0.02 (0% reduction, P = NS), and 0.93 ± 0.02 (16% increase in permeability compared to the positive control, P = NS), respectively. The presence of 1.0 mM D-arginine with 1.0 mM L-NIO did not reverse the protective effects of the latter (data not shown).

Protective effects of L-NIO in immune complex induced-dermal vascular injury: effects on tissue MPO content

These experiments were carried out with positive and negative (omission of intravenously injected BSA) controls and extraction of skin sites at 4 h for MPO following deposition of immune complexes. In a comparison series of skin sites injected with anti-BSA containing 1.0 mM L-NIO alone or in combination with 1 mM D-arginine, or with 0.1, 0.5, 1.0, 5.0 or 10 mM L-arginine, a protocol similar to that described in Figure 2 was employed. The negative and positive controls showed an MPO content of 0.08 ± 0.01 and 0.39 ± 0.02, respectively. The range of MPO content in all L-NIO-injected skin sites was 0.37 ± 0.02 to 0.41 ± 0.02, none of which was statistically significantly different from the positive reference control value (data not shown). Thus, the protective effects of L-NIO in immune complex-induced vasculitis are *not* associated with a blocking of the recruitment of neutrophils into the sites containing the immune complexes.

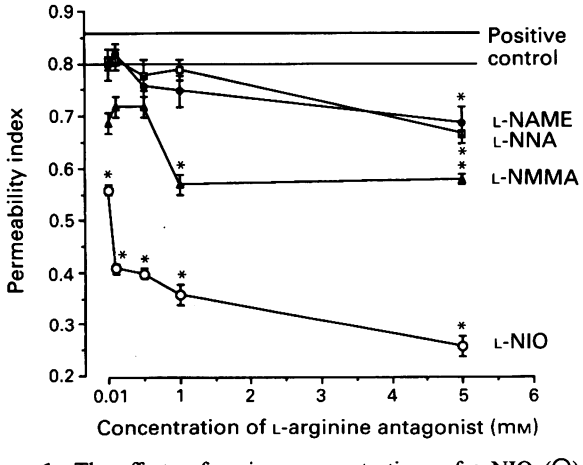

Figure 1 The effects of various concentrations of L-NIO (○), L-NMMA (▲), L-NNA (□), and L-NAME (●) on vascular injury (measured by leakage of [^{125}I]-albumen) in dermal skin sites containing IgG immune complexes. Compounds were added to the anti-BSA immediately prior to intradermal injection. All reactions were assessed 4 h after initiation of immune complex deposition. For each data point, $n=8$. For statistical purposes, comparisons were made with skin sites containing immune complexes in the absence of an L-arginine analogue. Vertical bars represent s.e.mean. For abbreviations, see text.

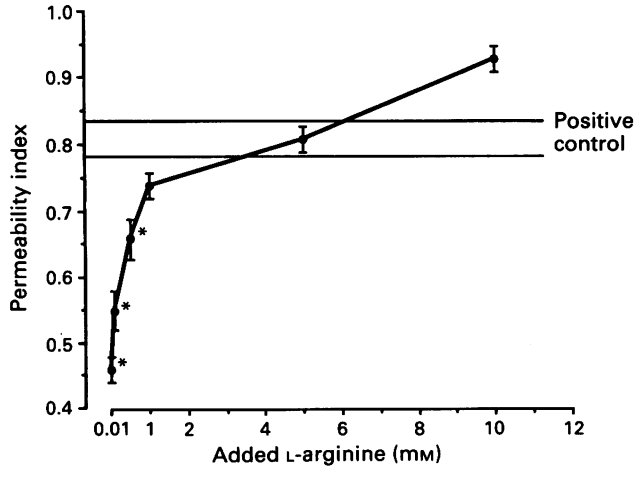

Figure 2 Reversal of protective effects of 1.0 nM L-NIO by copresence of L-arginine (●) which was added in a range of concentrations (0.01–10 nM) to the anti-BSA prior to intradermal injection. The reference positive value (for vascular permeability) was 0.81 ± 0.03 while the negative control (absence of intravenously injected BSA) was 0.07 ± 0.01. For each data point, $n=4$. For abbreviations, see text.

Protective effects of L-NIO in immune complex-induced alveolitis

IgG immune complex deposition was also induced in rat lung by the intratracheal instillation of anti-BSA (1.5 mg in a total volume of 300 μl) in the presence or absence of 5.0 mM L-NMMA or 5.0 mM L-NIO and the effects on vascular injury assessed according to the increase in lung vascular permeability. This lower dose of antibody was employed in order to accentuate differences in the protective effects of the L-arginine antagonists. For the experiments shown in Figure 3, the values for the negative (omission of intravenously injected BSA) and positive control groups were 0.16 ± 0.01 and 0.52 ± 0.02, respectively. When the negative control value was subtracted from the values of the positive control groups (treated or untreated with L-NMMA or L-NIO) and the ratios computed, 5.0 mM L-NMMA caused a 50% reduction ($P = 0.009$) in intensity of lung injury as reflected by change in vascular permeability while L-NIO caused an 82% reduction ($P = 0.002$) in lung injury (a fall in the permeability to 0.22 ± 0.01). L-NIO had statistically greater protective effects when compared to L-NMMA ($P = 0.021$), in which case the permeability value was 0.33 ± 0.01.

Additional studies on the effects of L-arginine analogues on the intensity of lung injury (as measured by permeability changes) were performed, as shown in Figure 4. For these experiments a concentration of 2.5 mg anti-BSA was employed. The negative and positive control values were 0.16 ± 0.01 and 0.75 ± 0.03, respectively. All results were compared to the positive control values (anti-BSA in the absence of any analogue of L-arginine). The presence of 5.0 mM L-NMMA, L-NAME, L-NIO, L-NIO + D-arginine, and L-NIO + L-arginine was associated with permeability values of 0.46 ± 0.02 (reduction of 49%, $P = 0.002$); 0.60 ± 0.03 (reduction of 25%, $P = 0.027$); 0.43 ± 0.02 (reduction of 54%, $P = 0.001$); 0.44 ± 0.01 (reduction of 53%, $P = 0.001$); and 0.84 ± 0.02 (intensification of injury by 15%, $P =$ NS), respectively. Thus, L-NIO is a potent inhibitor of vascular injury developing in lung following deposition of IgG immune complexes and its protective effects are reversed by L-arginine but not by D-arginine. In this experiment there are not sufficient data to compare the protective effects of L-NIO with the other analogues of L-arginine.

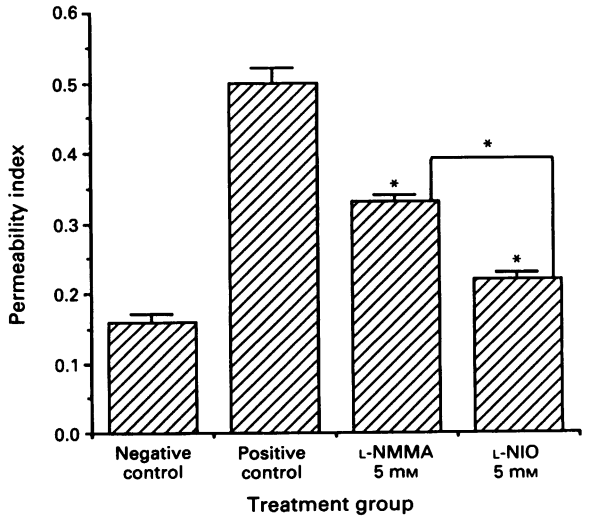

Figure 3 Protective effects (assessed by changes in vascular permeability) of L-NIO and L-NMMA on immune complex-induced pulmonary vascular injury. When employed, 5 mM L-NMMA or L-NIO was added to the anti-BSA preparation prior to its intratracheal administration. Reactions were measured 4 h after deposition of IgG immune complexes. All comparisons were to the positive control group (for each vertical column, $n = 4$). For abbreviations, see text.

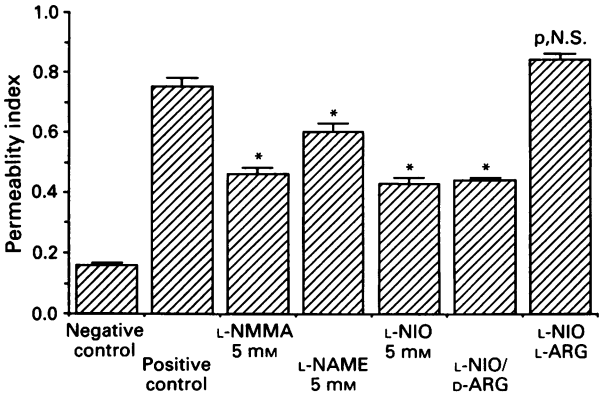

Figure 4 Ability of various analogues of L-arginine to reduce lung vascular injury (as assessed by increased vascular permeability) following intrapulmonary deposition of IgG immune complexes. All compounds employed were used at 5 nM concentrations. Statistical comparisions were made with the positive control group and represent significance of reductions in vascular permeability for each vertical column, $n = 4$. For abbreviations, see text.

When lungs from animals undergoing immune complex deposition were compared for MPO content to those lungs in which anti-BSA also contained L-NIO or L-NMMA, no difference was found in the lung content of MPO (data not shown), confirming the pattern found for the protective effects of analogues of L-arginine in the skin (described above).

Discussion

The ability of L-arginine analogues to reduce evidence of IgG immune complex-induced dermal vascular injury in rats reveals the rank order of potency: L-NIO > L-NMMA > L-NNA = L-NAME, which is in remarkable agreement with the *in vitro* effects of these compounds on their ability to block NO synthase in rat peritoneal neutrophils or in J774 cells, whether measured by interference with platelet aggregation or by direct measurement of NO formation in cytosolic fractions of cells (McCall *et al.*, 1991). The estimated IC_{50} value (65 μM) for the *in vivo* protective effects of L-NIO in skin is much higher than the value obtained *in vitro* (0.8 μM), but this is readily explained by the fact that the total volume of intradermally injected material is 50 μl, which becomes rapidly diluted as the permeability changes begin to occur in the developing acute inflammatory reaction. Since the minimal volume increase in the positive control sites is nearly 16 fold (to 800 μl) above the negative control value and the outflow, chiefly through efferent lymphatic channels, has not been measured, the effective local *in vivo* concentration cannot be accurately computed but would probably be reduced many fold below the initial concentration, perhaps to < 3 μM. A key factor in the efficacy of L-NIO when compared to the other analogues of L-arginine appears to be the more rapid onset in its inhibitory effects when added to the phagocytic cells (10 min versus 20–60 min) and the irreversible effect of L-NIO as compared to the other analogues (McCall *et al.*, 1991).

These data indicate that L-arginine analogues such as L-NIO may be useful in preventing tissue damage which is generated by toxic products of activated neutrophils (and macrophages). In the rat dermal and lung models of immune complex-induced vascular injury, neutrophils have long been shown to be key participants in the events leading to injury (Johnson & Ward, 1979; Warren *et al.*, 1990). The protective effects of antioxidants such as superoxide dismutase (SOD) and catalase have led to the suggestion that toxic metabolites

of oxygen may be key initiators of injury (Johnson & Ward, 1981). The protective effects of SOD are complicated by the fact that this compound has time-limited protective effects (2 h) in the immune complex models of injury (Johnson & Ward, 1981), which may be related to the generation of O_2-dependent chemotactic lipids (Von Zabern *et al.*, 1987; Vogt *et al.*, 1989). The recent finding that L-NMMA has significant protective effects in immune complex-induced injury in rat lung and skin (Mulligan *et al.*, 1991) has caused a re-evaluation of the pathways leading to injury and has emphasized that L-arginine, or presumably its metabolic products, are injurious, perhaps through the generation of peroxynitrite anion or the hydroxyl radical (Beckman *et al.*, 1990). The most remarkable finding in the earlier study (Mulligan *et al.*, 1991) and in the current one is that the protective effects are *not* associated with a reduced tissue accumulation of neutrophils, implying that the protective effects of L-NIO and L-NMMA are not attributable to an interference with neutrophil emigration from the vasculature. The current studies indicate that compounds capable of blocking NO formation may have significant protective effects against injury in a variety of human inflammatory diseases.

References

BECKMAN, J.S., BECKMAN, T.W., CHEN, J., MARSHALL, P.A. & FREEMAN, B.A. (1990). Apparent hydroxyl radical production from peroxynitrite: implications for endothelial cell injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1620–1624.

BILLIAR, T.R., CURRAN, R.D., FERRARI, F.K., WILLIAMS, D.L. & SIMMONS, R.L. (1990). Kupffer cell: hepatocyte cocultures release nitric oxide in response to bacterial endotoxin. *Surg. Res.*, **48**, 349–353.

CURRAN, R.D., BILLIAR, T.R., STUEHR, D.J., OCHOA, J.B., HARBRECHT, B.G., FLINT, S.G. & SIMMONS, R.L. (1990). Multiple cytokines are required to induce nitric oxide production and inhibit total protein synthesis. *Ann. Surg.*, **212**, 462–469.

DRAPIER, J.C. & HIBBS, J.B. Jr. (1986). Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron sulfur prosthetic group and is reversible. *J. Clin. Invest.*, **78**, 790–797.

HIBBS, J.B. Jr., VAVRIN, Z. & TAINTOR, R.R. (1987). L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.*, **138**, 550–565.

HIBBS, J.B. Jr., TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87–94.

JOHNSON, K.J. & WARD, P.A. (1979). Acute immunologic pulmonary alveolitis. *J. Clin. Invest.*, **54**, 349–357.

JOHNSON, K.J. & WARD, P.A. (1981). Role of oxygen metabolites in immune complex injury of lung. *J. Immunol.*, **126**, 2365–2369.

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochem.*, **27**, 8706–8711.

MCCALL, T.B., BOUGHTON-SMITH, N.K., PALMER, R.M.J., WHITTLE, B.J.R. & MONCADA, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem. J.*, **261**, 293–296.

MCCALL, T.B., FEELISCH, M., PALMER, R.M.J. & MONCADA, S. (1991). Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br. J. Pharmacol.*, **102**, 234–238.

MULLIGAN, M.S., HEVEL, J.M., MARLETTA, M.A. & WARD, P.A. (1991). Tissue injury caused by deposition of immune complexes is L-arginine dependent. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6338–6342.

SCHMIDT, H.H., SEIFERT, R. & BOHME, E. (1989). Formation and release of nitric oxide from human neutrophils and HL-60 cells induced by a chemotactic peptide, platelet activating factor and leukotriene B_4. *FEBS Lett.*, **244**, 357–360.

STUEHR, D.J. & NATHAN, C.F. (1989). Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Exp. Med.*, **169**, 1543–1555.

VOGT, W., DAMERAN, B., VON ZABERN, I., NOLTE, R. & BRUNAHL, D. (1989). Non-enzymatic activation of the fifth component of human complement, by oxygen radicals. Some properties of the activation product, C5b-like C5. *Mol. Immunol.*, **26**, 1133–1142.

VON ZABERN, W.V., HESSE, D., NOLTE, R. & HALLER, Y. (1987). Generation of an activated form of human C5 (C5b-like C5) by oxygen radicals. *Immunol. Lett.*, **14**, 209–215.

WARREN, J.S., YABROFF, K.R., MANDELL, D.M., JOHNSON, K.J. & WARD, P.A. (1990). Role of O_2^- in neutrophil recruitment into sites of dermal and pulmonary vasculitis. *Free Rad. Biol. Med.*, **8**, 163.

WRIGHT, C.D., MULSCH, A., BUSSE, R. & OSSWALD, H. (1989). Generation of nitric oxide by human neutrophils. *Biochem. Biophys. Res. Commun.*, **160**, 813–819.

(Received April 6, 1992
Revised August 12, 1992
Accepted August 17, 1992)

Br. J. Pharmacol. (1992), **107**, 1163–1172

Inflammatory mechanisms in the passive cutaneous anaphylactic reaction in the rabbit: evidence that novel mediators are involved

[1]Paul G. Hellewell, Peter J. Jose & Timothy J. Williams

Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

1 We have examined the mechanisms of local oedema formation in the passive cutaneous anaphylactic (PCA) reaction in the rabbit.

2 IgE-containing antiserum was injected i.d. and allowed to sensitize skin sites for periods up to 240 h. Antigen (bovine gamma globulin) was injected i.d. or i.v. and local oedema formation assessed by the accumulation of i.v. injected ^{125}I-labelled rabbit serum albumin. Potential inhibitors were mixed with antigen prior to i.d. injection or were administered i.v.

3 Maximum oedema formation was observed when a sensitization period of 48–72 h was used. Oedema formation in the PCA reaction was of short duration with a $t_{1/2}$ of approximately 15 min. No evidence of late oedema formation (up to 6 h) was found.

4 Local oedema formation in the PCA was reduced by indomethacin suggesting that vasodilator, oedema-potentiating prostaglandins were released. However, it was likely that other vasodilators were also generated.

5 Antihistamines were poor inhibitors of oedema formation as were PAF antagonists, a 5-lipoxygenase inhibitor, a kallikrein inhibitor, a bradykinin antagonist and anti-C5a antibody.

6 Local oedema formation in the PCA was partially reduced by neutrophil depletion and colchicine suggesting that neutrophil-dependent mediators were involved.

7 Exudate fluid from anaphylactic reactions in the rabbit peritoneal cavity contained permeability-increasing activity when injected into rabbit skin. This activity is now being characterized.

8 A vasodilator prostaglandin appears to be released in the rabbit PCA reaction but none of the established permeability-increasing mediators appears to be involved. Thus, there may be novel inflammatory mediators generated in this reaction which may have relevance for human allergic skin diseases.

Keywords: Cutaneous anaphylaxis; oedema formation; IgE; peritoneal anaphylaxis; rabbit skin; prostaglandins; PAF; histamine; neutrophil-dependent oedema

Introduction

One of the characteristic features of allergic inflammation is a local increase in microvascular permeability in response to locally generated mediators (Burke & Miles, 1958). Oedema formation in skin is dependent on a synergism between increased vascular permeability and increased blood flow (Williams and Morley, 1973; Williams & Peck, 1977). We have previously investigated the mediators responsible for the oedema formation in rabbit skin observed in the reversed passive Arthus reaction (Hellewell & Williams, 1986; Williams *et al.*, 1986; Hellewell *et al.*, 1988) and in response to intradermal injection of yeast cell walls (zymosan) (Wedmore & Williams, 1981; Williams & Jose, 1981) and synthetic cationic proteins (Needham *et al.*, 1988). In all these reactions, there was evidence for vasodilator prostaglandin production as indicated by reduced oedema formation in the presence of indomethacin. The complement activation fragment C5a, or its des Arg metabolite, is a major permeability-increasing agent in the Arthus reaction and in the response to zymosan but anaphylatoxic histamine release by C5a is relatively unimportant in these reactions (Williams & Jose, 1981; Williams *et al.*, 1986; Hellewell *et al.*, 1988). Platelet activating factor (PAF) also plays a significant role in oedema formation in the Arthus reaction (Hellewell & Williams, 1986; Warren *et al.*, 1989; Hellewell, 1990).

The passive cutaneous anaphylactic (PCA) reaction is a model of allergic inflammation characterized by local oedema formation in a number of species including the rabbit (Zvaifler & Becker, 1966; Watanabe & Ovary, 1977). The antibody responsible for inducing PCA reactions in the rabbit has been identified as IgE and shows similar properties to human IgE (Zvaifler & Becker, 1966; Lindqvist, 1968; Revoltella & Ovary, 1969; Zvaifler & Robinson, 1969; Ishizaka *et al.*, 1970). In rabbits, IgE antibody can be raised by immunisation with a low dose (μg quantities) of antigen mixed with aluminium hydroxide. This procedure produces maximum IgE titres after 120 days (Kravis & Zvaifler, 1974a) and a detailed study of the antibody was made by Stux & Ovary (1976). However, in this and other studies, no attempts were made to investigate which mediators were generated following antigen challenge, other than establishing that antihistamines were weak inhibitors.

In this paper, we have investigated the inflammatory response in the IgE-dependent PCA reaction in the rabbit to determine which mediators are released following antigen challenge. We have measured oedema formation in the PCA and used pharmacological agents to dissect the response. We also assessed the contribution of neutrophils to oedema formation. Apart from the involvement of vasodilator prostaglandins, none of the established mediators appear to contribute to inflammation in the PCA reaction suggesting the existence of novel mediators. We have also detected inflammatory activity in exudates obtained from passive anaphylactic reactions in the peritoneal cavity.

[1] Author for correspondence.

Methods

Animals

Male New Zealand White specific pathogen-free rabbits (2.5–3.5 kg) were purchased from Froxfield Farm, Hampshire and Hacking and Churchill, Huntingdon, Cambridgeshire.

Generation of antiserum for PCA reactions

Antiserum for PCA reactions was raised in rabbits according to a schedule which stimulates preferential production of IgE antibodies (Kravis & Zvaifler, 1974a). The antigen (bovine gamma globulin (BGG), 1.5 μg per rabbit) was dissolved in saline, mixed with two volumes of aluminium hydroxide, washed twice and injected subcutaneously in a volume of 1 ml. The procedure was repeated three times at monthly intervals and after 4 months the rabbits were bled by carotid cannulation. Serum was prepared and those sera containing skin fixing anti-BGG antibodies (when tested at 1:10 dilution, see below), were pooled and passed through a column of Protein-A Sepharose to remove the major part of the IgG present. Aliquots of the IgG-depleted antiserum (referred to as anti-BGG IgE) were stored at −25°C. Non-immune rabbit serum depleted of IgG was used as a control for PCA reactions.

Generation of goat anti-rabbit C5a IgG

Rabbit C5a was purified, as the des Arg metabolite, from zymosan-activated serum by the successive use of batch-wise cation exchange, ethanol precipitation of unwanted proteins, gel filtration and cation-exchange high performance liquid chromatographyl (h.p.l.c.) as described previously (Williams & Jose, 1981; Jose *et al.*, 1983; Haslett *et al.*, 1989). A goat was immunized (20 × 0.1 ml i.m. injections in the hind quarters and 4 × 0.25 ml i.m. injections in the shoulder region) with the purified material (50 μg) emulsified in Freund's complete adjuvant on days 0 and 21. Boost injections in Freund's incomplete adjuvant were given every 21 days. Test bleeds taken 7–10 days after each booster injection showed similar antibody titres. The serum used in this paper was prepared from blood taken 10 days after the final antigen injection. For use as a neutralizing agent in the rabbit skin assay, the IgG fraction of the anti-serum was prepared by use of n-octanoic acid to precipitate unwanted proteins (Steinbuch & Audran, 1969). The IgG fraction was then precipitated with ammonium sulphate, resuspended in a volume equal to that of the original serum, dialysed against saline, filtered (0.2 μm) and stored in aliquots at −25°C. The antibody was shown to bind both C5a and C5a des Arg.

Radiolabelling of rabbit serum albumin

Preliminary studies revealed that the anti-BGG IgE used to sensitize skin sites for the PCA reaction recognised ^{125}I-labelled human serum albumin which is normally employed as the plasma marker in our studies. No cross-reactivity was found with rabbit serum albumin (RSA), therefore radioiodinated [^{125}I]-RSA was prepared according to the chloramine-T procedure (Hunter & Greenwood, 1962). Free iodine was removed with Sephadex-G25M. Sterile, pyrogen-free reagents and receptacles were used because the radiolabelled protein was for intravenous administration. The iodinated albumin (approx. 30 μCi mg^{-1}) was stored at 4°C in sterile saline containing 0.9% benzyl alcohol as preservative.

Neutrophil depletion

Rabbits were rendered neutropenic by a single i.v. injection of nitrogen mustard (freshly prepared in sterile saline) at a dose of 1.75 mg kg^{-1} three days prior to the experiment (Stetson, 1951; Wedmore & Williams, 1981). Under these conditions, the circulating neutrophil count three days later was approximately 1% of the pretreatment value.

For total and differential circulating leukocyte counts, samples of peripheral blood were taken from a central ear artery. Total leukocytes were determined in a Coulter counter (type ZBI) and May-Grunwald-Giemsa stain was used for differential counts on at least 200 cells.

Measurement of local oedema formation in rabbit skin

Oedema formation was measured as the local accumulation of intravenously injected [^{125}I]-RSA. Rabbits were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}), the fur on the dorsal skin shaved and [^{125}I]-RSA (1.5 μCi kg^{-1}) mixed with a solution of Evans blue dye (10 mg kg^{-1}) in sterile saline was injected intravenously. Five minutes later agents under test, freshly prepared in sterile saline from stock solutions, were injected intradermally (0.1 ml volumes) in six replicates per treatment according to a balanced site injection plan. After 30 min, a 10 ml cardiac blood sample was taken into heparin for the preparation of plasma. The animal was then killed with an overdose of sodium pentobarbitone and the dorsal skin removed. Injection sites were punched out with a 17 mm diameter punch and counted together with three 1 ml plasma samples in a 12 head gamma counter (LKB, Wallac 1260 Multigamma). The amount of plasma protein that had accumulated in each skin site was calculated in terms of μl plasma by dividing the ^{125}I-count in each skin site by the ^{125}I-count in 1 μl plasma.

In the experiments designed to observe the effects of systemic administration of colchicine on oedema formation, the procedure was designed so that each animal served as its own control (Hellewell *et al.*, 1989). Test agents (antigen, FMLP + PGE_2 and bradykinin + PGE_2) were injected intradermally after i.v. administration of ^{125}I-RSA and Evans blue dye. Plasma protein accumulation was allowed to proceed for 30 min. Colchicine (1 mg kg^{-1}, or saline, 1 ml kg^{-1}, for the control; Hellewell *et al.*, 1989) was then injected intravenously and 5 min later the remaining skin sites were injected with the same combination of test agents. After a further 30 min, the animals were killed and local oedema formation was assessed as described above.

Passive cutaneous anaphylaxis (PCA)

Anti-BGG IgE (diluted 1:3, 1:10, 1:30 or 1:100 with sterile saline) was injected intradermally 48–72 h prior to antigen challenge. This was the optimum period required for IgE fixation (see Figure 2). After the fixation period, [^{125}I]-RSA was injected intravenously followed 5 min later by antigen challenge. BGG was administered either intravenously (5 mg kg^{-1}) or locally (1 μg 0.1 ml^{-1}) and local oedema formation was assessed as described above.

When drugs were tested intradermally, they were mixed with antigen or with combinations of inflammatory agents, prior to injection. One drug, L-659,989, was also tested intravenously as a 5 min pretreatment before the intradermal injection of test agents: these experiments were performed with pairs of animals, one rabbit receiving the drug, the other receiving vehicle.

Passive peritoneal anaphylaxis (PPA)

IgE-containing antiserum (2 ml, undiluted) or IgG-depleted non-immune serum (control) was injected into the peritoneal cavity of anaesthetized rabbits, followed by brief massage of the abdomen. After a 72 h fixation period, the animals were re-anaesthetized and 20 ml sterile saline was injected intraperitoneally followed by i.v. antigen challenge (5 mg kg^{-1}). At 90 min, the rabbits were killed with an anaesthetic overdose and the total available peritoneal exudate was collected into heparinized tubes on ice. After centrifugation (300 g) to

remove cells and debris, samples were analysed for inflammatory activity in the skin oedema assay using naive recipient rabbits. The time course of the peritoneal reaction was measured by removing 2 ml aliquots of exudate, at various times (30, 60, 120, 240 min) after antigen challenge, for testing in the skin oedema assay.

Drugs and materials

Arachidonic acid, colchicine, prostaglandin E_2 (PGE_2), bradykinin, indomethacin, N-formyl-methionyl-leucyl-phenylalanine (FMLP), phenylmethylsulphonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), rabbit serum albumin, zymosan and Freund's adjuvants were purchased from Sigma Chemical Co., Poole, Dorset; bovine gamma globulin (BGG), histamine and Evans blue dye were from British Drug Houses, Poole, Dorset; tissue kallikrein (Glumorin) and trasylol (Aprotinin) were from Bayer U.K. Ltd., Haywards Heath, West Sussex; PAF was from Bachem, Saffron Walden, Essex; aluminium hydroxide (4% in water; Aludrox) was from Wyeth Laboratories, Taplow, Berks; sodium pentobarbitone was from May and Baker, Dagenham, Essex; nitrogen mustard (Mustine HCl) and sterile 0.9% saline (Steriflex) was from Boots Company, Nottingham, Nottinghamshire; $Na^{125}I$ was from Amersham International, Amersham, Buckinghamshire; Protein-A Sepharose and Sephadex G-25M were from Pharmacia Fine Chemicals, Hounslow, Middlesex.

Zymosan-activated plasma (ZAP) was used as a source of C5a des Arg (approximately $3-5 \times 10^{-7}$ M). Briefly, rabbit heparinized (10 u ml^{-1}) plasma was incubated with zymosan (5 mg ml^{-1}) for 30 min at 37°C followed by centrifugation (2×10 min, 3000 *g*) to remove the zymosan. Aliquots of ZAP were stored at −25°C.

The following were obtained as gifts: mepyramine maleate was from May and Baker, Dagenham, Essex; cimetidine was from Smith Kline Beecham, Welwyn Garden City, Herts; L-659,989 (*trans*-2-(3-methoxy-5-methylsulphonyl-4-propoxy-phenyl)-5- (3,4,5-trimethoxy-phenyl) tetrahydrofuran) was from Dr J.C. Chabala, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey, USA; 48740 RP (3-(3-pyridyl)-1H,3H-pyrollo [1,2-c] thiazole-7-carboxamide) was from Dr P. Sedivy, Rhone-Poulenc Sante, Vitry sur Seine, France; WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno(2,3-f)(1,2,4)-triazolo-(4,3-a) (1,4)-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone) was from Dr H. Heuer, Boehringer Ingelheim KG, Ingleheim am Rhein, Federal Republic of Germany; REV 5901 (α-pentyl-3-(2-quinolinylmethoxy)-benzene-methanol) was from the Rorer Group Inc., Fort Washington, Pennsylvania, USA; B-3824, a synthetic bradykinin antagonist, was from Dr J.M. Stewart, University of Colorado School of Medicine, Denver, Colorado, USA.

Statistical analysis

Data are presented as the mean ± s.e.mean and have been analysed with Student's paired *t* test for within animal treatment, and with Student's unpaired *t* test for between animal treatments. A P value of <0.05 was considered statistically significant. Before calculating percentage inhibition of a response, the background value for i.d. injection of serum, saline or PGE_2 was subtracted from the data.

Results

Effect of heat treatment on IgE antibody

The effect of heating the anti-BGG IgE at 56°C for 30 min or 4 h on its ability to sensitize skin sites is shown in Figure 1. There was a heat-resistant component which is consistent with previous observations made with rabbit IgE (Revoltella & Ovary, 1969; Kravis & Zvaifler, 1974a; Stux & Ovary, 1976).

Time course of IgE fixation in skin

In order to investigate the kinetics of the sensitization period, IgE-containing antiserum was injected intradermally 240, 144, 72, 48, 24, 6 h and 1 h before an intravenous injection of ^{125}I-albumin and antigen. Oedema formation was measured over 30 min. The results shown in Figure 2 demonstrate that

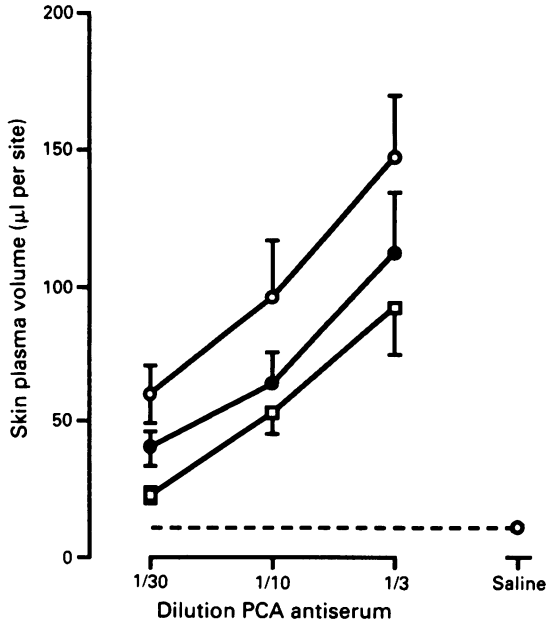

Figure 1 Effect of heat treatment of IgE-containing antiserum on its ability to sensitize skin for passive cutaneous anaphylactic (PCA) reactions. Undiluted antiserum was heated at 56°C for 30 min (●), or 4 h (□), or was untreated (○) before dilution with saline and injection into skin. After a fixation period of 72 h, antigen (BGG, 5 mg kg^{-1}) and [^{125}I]-albumin were injected intravenously and oedema formation measured as the 30 min accumulation of [^{125}I]-albumin in skin sites. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 6 replicate injections in one rabbit.

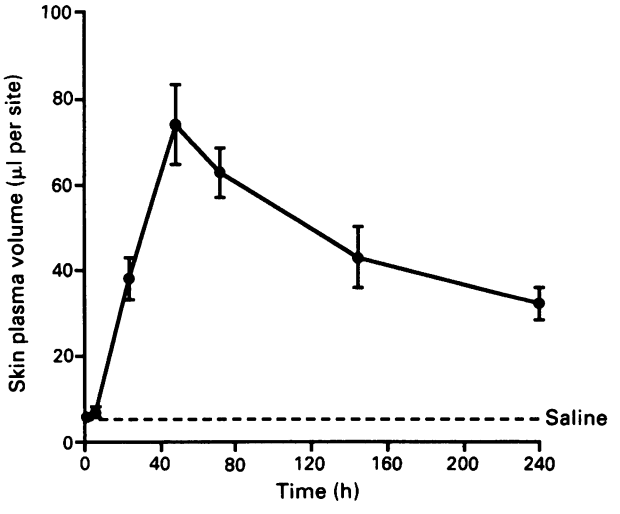

Figure 2 The kinetics of sensitization of rabbit skin by IgE. Antiserum diluted 1:10 with saline was injected at different intervals before the intravenous injection of antigen (BGG, 5 mg kg^{-1}) and measurement of oedema formation. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 6 replicate injections in one rabbit.

maximal oedema formation was obtained with a sensitization period of 48–72 h, although IgE persisted in skin for at least 10 days. Therefore, in all subsequent experiments a sensitization period of 48 or 72 h was used.

Duration of oedema formation in the PCA reaction

To establish the duration of oedema formation in the PCA following antigen challenge, the following experiment was performed. Antigen was injected locally into sensitized (72 h) skin sites at intervals before the i.v. injection of [^{125}I]-albumin at $t = 0$. Oedema formation was measured over a 30 min period. In this manner, PCA sites had been exposed to antigen for different time periods before the measurement of plasma leakage. As shown in Figure 3, oedema formation was short-lived, in this experiment a half-life of 15 min was obtained. In a total of three rabbits, the half-life value for oedema formation was 17 ± 3 min. We found no evidence for a late phase reaction in terms of oedema formation measured at 6 h post antigen challenge (data not shown). The latter experiments were performed in conscious rabbits, since anaesthesia had been demonstrated to interfere with late phase reactions in this species (Behrens *et al.*, 1987).

Effect of indomethacin on oedema formation in the PCA reaction

Figure 4 shows the effect of local administration of indomethacin on oedema formation in the PCA reaction. Indomethacin (10^{-8} mol), mixed with antigen prior to i.d. injection, reduced the PCA reaction by 41 ± 3% ($n = 7$ rabbits, $P < 0.002$). To confirm the cyclo-oxygenase inhibitory activity of indomethacin in the same group of rabbits, we examined the effect of the drug on oedema formation induced by i.d. injection of bradykinin mixed with arachidonic acid. The conversation of arachidonate to vasodilator prostaglandins in skin increases local blood flow and acts synergistically with the permeability-increasing activity of bradykinin to produce marked oedema formation (Williams & Peck, 1977). Indomethacin inhibited this response by 86 ± 7% ($P < 0.002$). i.e. reduced the response almost to that seen with bradykinin alone. Prostaglandin E_2 (PG E_2) and PGI_2 are potent vasodilators produced from arachidonate via the cyclo-oxygenase pathway, whereas none

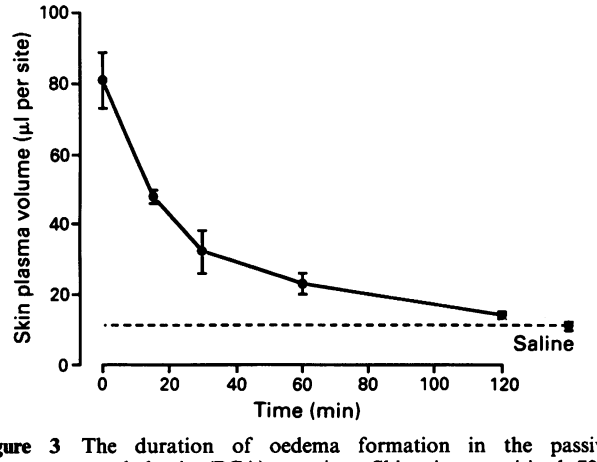

Figure 3 The duration of oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Skin sites sensitized 72 h previously with anti-BGG IgE (1:10 dilution) were injected with antigen (1 µg) at various time periods before the intravenous injection of [^{125}I]-albumin and measurement of oedema formation over a 30 min period. The interval between antigen challenge and albumin injection is shown on the abscissa scale. In this experiment the half-life for duration of oedema was approximately 15 min. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 6 replicate injections in one rabbit and are representative of three experiments.

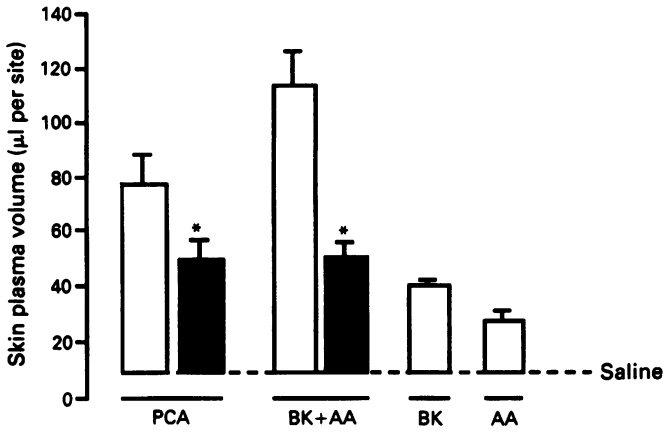

Figure 4 Effect of local administration of indomethacin on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Plasma leakage was induced by i.d. injection of BGG (1 µg) into PCA sites (1:3 dilution of IgE antiserum) and i.d. injection of bradykinin (BK; 10^{-10} mol) mixed with arachidonic acid (AA; 3×10^{-9} mol) into naive sites. Co-injection of indomethacin (10^{-8} mol) is indicated by the solid columns. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 7 rabbits.
*$P < 0.002$.

of known metabolites produced via this pathway increases microvascular permeability (Williams, 1979). Thus, it would appear that there is an endogenous vasodilator prostaglandin component in the PCA reaction, although it is likely that other vasodilators are also involved.

Effect of antihistamines on oedema formation in the PCA reaction

Figure 5 shows the effects of local administration of H_1 (mepyramine) and H_2 (cimetidine) antihistaminic drugs, alone and in combination, on the PCA reaction. Mepyramine (3×10^{-9} mol) inhibited the leakage response to i.d. injection of histamine mixed with PGE_2 by 92 ± 3% ($P < 0.01$, $n = 6$ rabbits). Cimetidine (3×10^{-7} mol) produced a partial but significant ($P < 0.02$) suppression of the response to histamine + PGE_2 (42 ± 7% inhibition) and did not further reduce the inhibition seen with mepyramine alone (94 ± 2%). Local administration of mepyramine in the PCA produced no significant suppression of the leakage response: 13 ± 5% inhibition ($P > 0.05$) and 20 ± 8% inhibition ($P > 0.05$) at 1:3 and 1:10 dilutions of the anti-BGG IgE respectively. Cimetidine alone produced no inhibition of the PCA, although when combined with mepyramine did produce significant inhibition (27 ± 8% inhibition, $P < 0.05$) of the response at 1:10 dilution of the sensitizing antibody. Thus histamine appears to play a minor role in mediating oedema formation in the PCA reaction.

Effect of PAF antagonists on the PCA reaction

The effect of local administration of two potent PAF antagonists, WEB 2086 (Casals-Stenzel *et al.*, 1987) and L-659,989 (Ponpipom *et al.*, 1988), on oedema formation in the PCA reaction is shown in Figure 6a. WEB 2086 (10^{-7} mol) and L-659,989 (5×10^{-8} mol) inhibited the leakage response to i.d. injection of PAF + PGE_2 by 94 ± 2% and 88 ± 5% respectively ($n = 4$ rabbits) in concordance with our earlier published data (Hellewell & Williams, 1989a). In the same group of animals, neither antagonist significantly affected oedema formation in the PCA reaction (Figure 6a). We also tested another selective, but less potent PAF antagonist, 48740 RP (Sedivy *et al.*, 1985; Hellewell & Williams, 1989a). 48740 RP (10^{-6} mol) inhibited the oedema response to PAF-

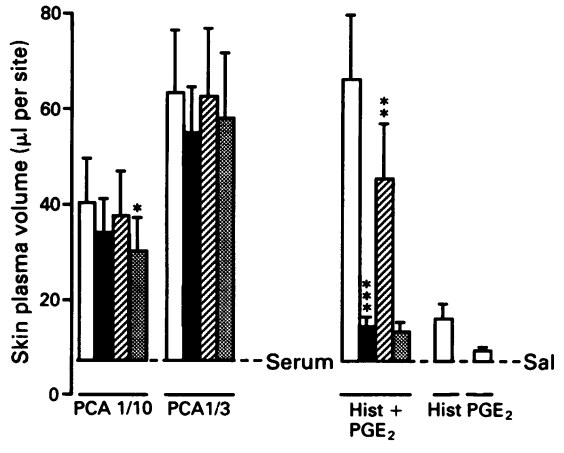

Figure 5 Effect of local administration of antihistamines on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Antigen (1 μg BGG) was injected into sensitized skin sites (1:10 and 1:3 dilutions of antiserum) either alone (open columns) or mixed with mepyramine (3×10^{-9} mol, solid columns), cimetidine (3×10^{-7} mol, hatched columns) or a combination of both drugs (stippled columns). Histamine (10^{-8} mol) was mixed with prostaglandin E_2 (PGE_2, 3×10^{-10} mol) and the antagonists before injection. The dashed lines represent the control values obtained after i.d. injection of BGG into sites pretreated with a 1:3 dilution of non-immune serum (control for PCA) or after i.d. injection of saline (control for histamine + PGE_2). Values shown are from 6 rabbits. $^*P<0.05$, $^{**}P<0.02$, $^{***}P<0.01$.

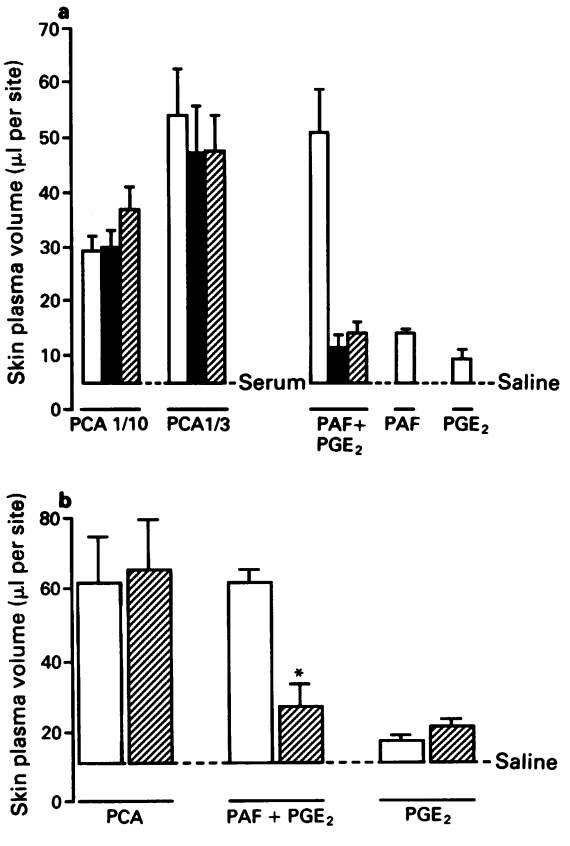

Figure 6 Effect of (a) local or (b) intravenous administration of PAF antagonists on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. (a) Antigen (1 μg BGG) was injected into sensitized skin sites (1:10 and 1:3 dilutions of antiserum) either alone (open columns) or mixed with WEB 2086 (10^{-7} mol, solid columns) or L-659,989 (5×10^{-8} mol, hatched columns). PAF (10^{-9} mol) was mixed with prostaglandin E_2 (PGE_2 3×10^{-10} mol) and the antagonists before injection. (b) Skin sites were sensitized with a 1:10 dilution of antiserum. Rabbits were treated with dimethylformamide as controls (0.1 ml kg^{-1} i.v., open columns) or with L-659,989 (5 mg kg^{-1} i.v., hatched columns). After 5 min, antigen was injected intravenously (BGG, 5 mg kg^{-1}). PAF (10^{-9} mol) was mixed with PGE_2 (3×10^{-10} mol) prior to injection. The dashed lines represent the background values obtained after i.d. injection of BGG into sites pretreated with a 1:3 dilution of non-immune serum or after i.d. injection of saline. Values shown are from 4 rabbits in (a) and from 4 pairs of rabbits in (b). $^*P<0.005$.

+ PGE_2 by 60 ± 5% ($n = 5$ rabbits), while the PCA reaction (1:10 dilution of sensitizing antibody) was unaffected: leakage in the PCA was 38 ± 5 μl and in the presence of 48740 RP was 41 ± 2 μl.

In a further series of experiments we investigated the effect of i.v. administration of L-659,989 (5 mg kg^{-1}) on oedema formation in the PCA reaction. The results are shown in Figure 6b. Oedema formation induced by PAF + PGE_2 was inhibited by 88 ± 8% ($P<0.005$), $n = 4$ pairs of rabbits. However, oedema formation in the PCA reaction was unaffected by the PAF antagonist. Weaker PCA responses (1:30 and 1:100 dilution of the sensitizing antibody were also unaffected by i.v. L-659,989 (data not shown). Thus, PAF does not appear to contribute to oedema formation in the PCA reaction.

Effect of trasylol and a bradykinin antagonist on the PCA reaction

The possibility of kinin formation in skin was investigated by use of trasylol (a kallikrein inhibitor) and a bradykinin antagonist, B-3824 (Vavrek & Stewart, 1985). Local administration of trasylol (1.5×10^{-9} mol) inhibited oedema induced by kallikrein (500 ng) + PGE_2 (3×10^{-10} mol) by 98 ± 2% (n = 3 rabbits). In the same animals, at a 1:10 dilution of sensitizing antibody, leakage in the PCA was 35 ± 1 μl and in the presence of trasylol was 38 ± 6 μl; at a 1:3 dilution of antibody the values were 65 ± 13 μl and 72 ± 19 μl, respectively (μl per skin site subtracted for values in the absence of antigen). Preliminary studies showed that plasma leakage in the PCA was also unaffected by local administration of SBTI (1 mg), PMSF (6×10^{-8} mol) or EDTA (10^{-6} mol).

In two rabbits, local administration of the antagonist (10^{-8} mol) reduced oedema formation induced by a mixture of bradykinin (10^{-10} mol) and PGE_2 (3×10^{-10} mol) by 94% and 88% respectively. Oedema formation in the PCA was not inhibited by B-3824. In fact, responses in both animals showed some increase, e.g. in one animal, oedema at 1:10 and 1:3 dilution of the antibody was 39 ± 6 μl and 82 ± 10 μl in antigen-injected sites, respectively, compared with 63 ± 9 μl ($P<0.05$) and 107 ± 5 μl ($P<0.05$) in sites injected with antigen plus B-3824. We attributed the increase to partial agonist activity of B-3824.

Effect of a 5-lipoxygenase inhibitor on oedema formation in the PCA reaction

The effect of REV-5901, a selective 5-lipoxygenase inhibitor with little anti-oxidant properties, is shown in Figure 7. REV-5901, at a dose (10^{-7} mol) which has previously been shown to inhibit 5-lipoxygenase in rabbit skin (Aked *et al.*, 1986), had no significant inhibitory effect on oedema formation in the PCA reaction. The 5-lipoxygenase inhibitory activity of the drug was evaluated in the same experiments by injecting it with a high dose of arachidonic acid (10^{-7} mol), since the leakage response to the latter is dependent on leukotriene B_4 (LTB_4) generation in rabbit skin (Aked & Foster, 1987). Oedema induced by arachidonate was inhibited by 49 ± 7% ($P<0.02$), demonstrating that 5-lipoxygenase activity in skin was reduced in these experiments.

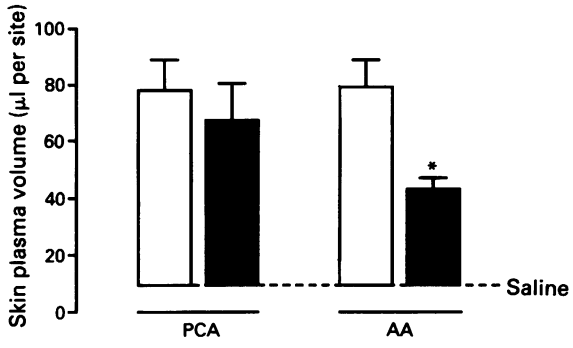

Figure 7 Effect of local administration of REV 5901 on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Plasma leakage was induced by injection of antigen (BGG, 1μg) into PCA sites (1:3 dilution of the sensitizing antibody) and i.d. injection of arachidonic acid (AA, 3×10^{-7} mol). The co-injection of REV 5901 (10^{-7} mol) is indicated by the solid columns. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 6 rabbits.
*$P < 0.02$.

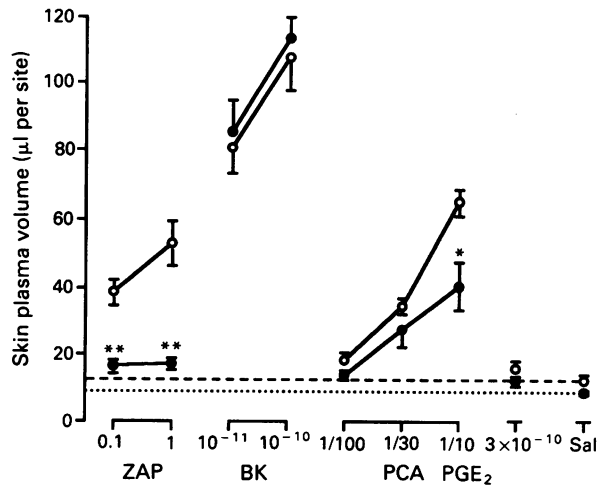

Figure 8 Effect of neutrophil depletion on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Responses were obtained in control rabbits (open symbols) and in rabbits treated 3 days previously with nitrogen mustard (1.75 mg kg^{-1}, i.v., closed symbols). C5a (ZAP, undiluted and diluted 1:10) and bradykinin (BK, 10^{-11} and 10^{-10} mol) were mixed with prostaglandin E_2 (PGE_2, 3×10^{-10} mol) prior to injection. PCA reactions were with 1:100, 1:30 and 1:10 dilutions of the sensitizing antibody given intradermally 48–72 h before the intravenous injection of antigen (BGG, 5 mg kg^{-1}). All rabbits were pretreated with mepyramine (3 mg kg^{-1}, i.v.). The dashed lines represent the background value obtained after i.d. injection of saline. Values shown are from 6 pairs of rabbits.
*$P < 0.05$; **$P < 0.001$.

Effect of anti-C5a antibodies on the PCA reaction

We have previously used anti-C5a antibodies to inhibit selectively C5a-induced inflammation in rabbit skin (Jose & Williams, 1987; Hellewell *et al.*, 1988; Collins *et al.*, 1991). Therefore, we tested the effects of these antibodies on the PCA reaction. Anti-C5a IgG, but not IgG prepared from non-immune serum, abolished oedema formation induced by C5a des Arg (ZAP) mixed with PGE_2; leakage was reduced from 35 ± 4 μl to 2 ± 2 μl (μl per skin site subtracted for values obtained after i.d. injection of PGE_2, mean ± s.e.mean for 6 replicate injections). In contrast, the inflammatory response induced by FMLP (5×10^{-11} mol) + PGE_2 was unaffected by anti-C5a (control, 53 ± 7 μl; + anti-C5a, 64 ± 4 μl) as was the PCA reaction (control, 48 ± 8 μl; + anti-C5a, 49 ± 6 μl). Thus, endogenous C5a does not appear to be involved in the PCA. Similar data were found in a second rabbit.

Role of circulating neutrophils

We have previously used nitrogen mustard and colchicine to distinguish between mediators which induce oedema formation via a neutrophil-endothelial interaction or via a direct effect on the microvascular endothelium (Wedmore & Williams, 1981; Hellewell *et al.*, 1989). Figures 8 and 9 show the effects of these anti-neutrophil treatments on oedema formation in the PCA reaction. For comparison, C5a (ZAP) and FMLP were used as standard neutrophil-dependent mediators and bradykinin as a standard neutrophil-independent mediator. In these experiments, the small contribution of the histamine was blocked by the intravenous injection of mepyramine (3 mg kg^{-1}).

Figure 8 shows the results from experiments conducted in control and nitrogen mustard-treated (neutropenic) rabbits. The circulating neutrophil counts at the time of antigen challenge were $2.1 \pm 0.3 \times 10^{-6}$ ml^{-1} and $2.5 \pm 0.6 \times 10^{-4}$ ml^{-1} respectively. Oedema formation induced by ZAP + PGE_2 was virtually abolished in neutropenic rabbits: responses obtained with undiluted and 1:10 dilutions of ZAP were inhibited by $85 \pm 3\%$ and $81 \pm 6\%$ respectively. In contrast, the responses to bradykinin + PGE_2 were unaffected by neutrophil depletion. In the PCA reaction, leakage responses at 1:100 and 1:30 dilutions were not significantly affected in neutropenic rabbits, however there was a significant inhibition ($43 \pm 10\%$, $P < 0.05$) at 1:10 dilution of the sensitizing antibody (Figure 8).

The effects of colchicine on the PCA reaction are shown in Figure 9. Figure 9a shows that i.v. administration of saline had no significant effect on oedema responses to FMLP, bradykinin or the PCA (i.e. responses over 35–65 min were the same as those measured over 0–65 min). In contrast, Figure 9b shows that systemic treatment with colchicine produced a dramatic decrease in FMLP-induced oedema formation without significantly affecting that induced by bradykinin. Responses to 5×10^{-11} and 5×10^{-12} mol FMLP were inhibited by $84 \pm 5\%$ ($P < 0.002$) and $82 \pm 6\%$ ($P < 0.002$), while responses to 10^{-10} and 10^{-11} mol bradykinin were not significantly reduced. Colchicine inhibited oedema formation in the PCA reaction: the inhibition at 1:10 dilution of the sensitizing antibody ($45 \pm 8\%$, $P < 0.05$) was similar to that seen with nitrogen mustard. At 1:3 dilution of antibody, the colchicine-dependent component was more marked; $54 \pm 9\%$ inhibition, $P < 0.02$.

Taken together, the results in Figures 8 and 9 suggest that there is a neutrophil-dependent component to oedema formation in the PCA reaction although this appears to be proportional to the magnitude of the reaction.

Generation of permeability-increasing activity in passive peritoneal anaphylaxis (PPA)

In an attempt to isolate and thus characterize inflammatory mediators in anaphylactic reactions, we carried out experiments in the peritoneal cavity. Exudate was obtained from the cavity and mixed with PGE_2 before injection in the skin of other rabbits. Figure 10a shows the time course of generation, following antigen challenge, of permeability-increasing activity in peritoneal anaphylactic exudate. Maximal activity was obtained 1–2 h after antigen challenge and had virtually disappeared at 4 h. A small amount of activity was also detected in the control exudate collected 30 min after antigen challenge of non-sensitized animals.

Figure 10b shows the results of further experiments in which exudate fluids, collected 90 min after antigen challenge in sensitized and control rabbits, were tested for inflammatory activity in the skin assay. Anaphylactic exudate

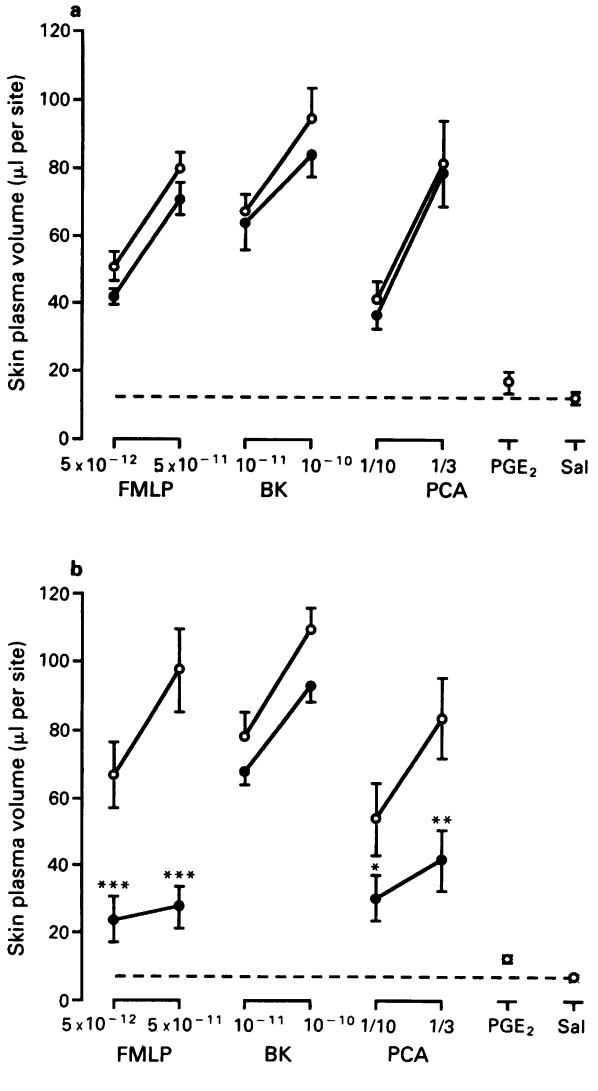

Figure 9 Effect of colchicine on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Reactions were initiated by the local injection of antigen (BGG, 1 μg) 48–72 h after the intradermal injection of sensitizing antibody. For comparison, oedema responses were induced by i.d. injection of FMLP + prostaglandin E_2 (PGE_2, 3×10^{-10} mol) and bradykinin (BK) + PGE_2. All rabbits were pretreated with mepyramine (3 mg kg^{-1} i.v.). Panel (a) shows responses before (○) and after (●) i.v. saline (1 ml kg^{-1}) and panel (b) shows responses before (○) and after (●) i.v. colchicine (1 mg kg^{-1}). The dashed lines represent the background value obtained after i.d. injection of saline. Values shown are from 6 pairs of rabbits.
*$P<0.05$, **$P<0.02$, ***$P<0.002$.

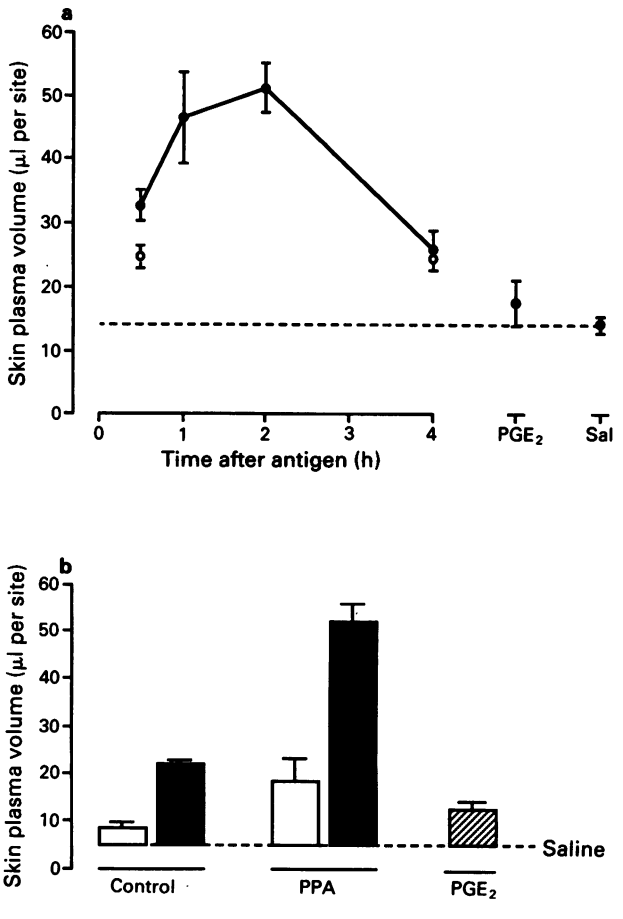

Figure 10 Generation of permeability-increasing activity in a passive peritoneal anaphylactic (PPA) reaction. Kinetics of the generation are shown in (a). Peritoneal exudate was removed at the intervals shown after intravenous antigen challenge (BGG, 5 mg kg^{-1}), mixed with prostaglandin E_2 (PGE_2 3×10^{-10} mol), injected i.d. into a naive rabbit and oedema formation assessed after 30 min (●). The control (○) represents exudate from an animal that received non-immune serum i.p. (see Methods for details). The effect of PGE_2 on oedema formation induced by control and PPA 90 min exudates is shown in (b). Exudate, removed 90 min after antigen challenge to control and sensitized animals was injected alone (open columns), or mixed with PGE_2 (3×10^{-10} mol) (solid columns). The dashed lines represent the background values obtained after i.d. injection of saline. Values shown in (a) are from 6 replicate injections per time point and those shown in (b) are from 5 control exudates and 7 PPA exudates.

showed inflammatory activity which was potentiated by the addition of PGE_2. The control exudates displayed some inflammatory activity in the skin but this was much weaker than the response to anaphylactic exudates.

The generation of inflammatory activity in the peritoneal anaphylactic exudates allows the possibility of characterizing and purifying inflammatory mediators of anaphylactic reactions in the rabbit.

Discussion

In this study we investigated which inflammatory mediators were responsible for the local oedema formation observed in the rabbit PCA reaction. In rabbit skin it has been established that oedema formation is dependent on a synergism between mediators which increase local blood flow and those which increase vascular permeability (Williams & Morley, 1973; Williams & Peck, 1977). Blocking the generation or action of either type of mediator results in a decrease in local oedema formation.

Experiments with indomethacin established that a vasodilator prostaglandin was generated in the PCA reaction although, since the reaction was not abrogated by this treatment, it is likely that other vasodilators are also generated. Possibilities include neuropeptides and we are currently investigating this. Vasodilator prostaglandins are known to be generated in other cutaneous reactions in the rabbit including those in response to zymosan (Wedmore & Williams, 1981). polycations (Needham *et al.*, 1988) and in the reversed passive Arthus reaction (Williams *et al.*, 1986).

Based on the weak inhibitory actions of a range of potent and selective pharmacological agents it appears that none of the established mediators is involved in increasing vascular permeability. The small inhibitory effect of antihistamines is consistent with earlier studies of the rabbit PCA reaction

(Lindqvist, 1968; Zvaifler & Robinson, 1969; Zvaifler et al., 1971). This is in contrast to PCA reactions in the monkey where a combination of H_1 and H_2 antihistamines completely inhibit the wheal observed after antigen challenge (Hutchcroft et al., 1979) and in man in which a combination of antihistamines reduced by approximately 50% the wheal response after antigen (Smith et al., 1980). However, there is evidence that these observed effects are due not only to histamine antagonism but to an additional mast cell stabilizing effect (Dorsch et al., 1982). Histamine has also been detected in blister fluid of skin sites challenged with antigen in sensitized individuals (Pienkowski et al., 1988) and, in the rabbit PCA, the histamine content of sensitized skin sites was reduced substantially after antigen challenge (Zvaifler et al., 1971). 5-Hydroxytryptamine (5-HT) up to 10^{-7} mol/site, has no permeability-increasing activity in rabbit skin (Hellewell, unpublished) and was therefore discounted as a mediator in the PCA; this is in contrast to its potent permeability-increasing effects in rodents. In addition, earlier studies of Zvaifler et al., (1971) found no inhibitory action of methysergide, alone or in combination with an H_1 antihistamine, on blueing in the rabbit PCA.

PAF has been proposed as an important mediator of IgE-mediated systemic anaphylaxis in the rabbit (Halonen et al., 1980). However, a recent study using the PAF antagonist WEB 2086 concluded that PAF plays a relatively minor role in respiratory, circulatory and haematological alterations that occur in anaphylaxis, although there was a significant reduction in total pulmonary resistance (Lohman & Halonen, 1990). These observations indicate that other mediators are likely to be involved in mediating the anaphylactic alterations. In man, the PAF antagonist BN-52063 has been shown to reduce significantly wheal volume in the cutaneous late phase response (at 8 h) in response to local antigen challenge in sensitized individuals (Roberts et al., 1988). However, although the immediate response was reduced by 50%, this was not a significant effect (Roberts et al., 1988). In our studies, administration of three PAF antagonists locally and one intravenously had no significant effects on local oedema formation in the PCA suggesting that endogenous PAF was unlikely to be involved in increasing vascular permeability.

There was no evidence for the generation of 5-lipoxygenase products since REV-5901 was without effect. As LTC_4 and LTD_4 are weak inducers of permeability changes when injected intradermally in rabbit skin (Ueno et al., 1981), this suggests that LTB_4, which does increase permeability (Wedmore & Williams, 1981), was not involved in the PCA reaction. A bradykinin antagonist, B-3824, and a kallikrein inhibitor, trasylol, were also without effect.

The contribution of circulating neutrophils to local oedema formation was supported by two different methods; neutrophil depletion using nitrogen mustard and interference of neutrophil function with colchicine. Both methods have been used to demonstrate neutrophil-dependent oedema formation (Wedmore & Williams, 1981; Hellewell & Williams, 1989) and Zvaifler et al. (1971) found that nitrogen mustard treatment caused very effective inhibition of bluing in the rabbit PCA reaction when combined with mepyramine. In the present study, mepyramine was used to inhibit the small contribution of histamine to oedema formation. Thus, it appeared that there was a neutrophil-dependent mediator in the rabbit PCA reaction that induced plasma exudation and that the larger the PCA reaction in terms of oedema formation, then the greater dependence on circulating neutrophils. Possibilities included C5a and C5a des Arg (Wedmore & Williams, 1981), tumour necrosis factor-α (TNF-α) (Rampart et al., 1989a), interleukin-1 (IL-1) (Buckley et al., 1991) and IL-8 (Rampart et al., 1989b). PAF (Hellewell, 1990) and LTB_4 (Wedmore & Williams, 1981) were already discounted (see above).

The likelihood that C5a was involved was small since the IgE antibody would not be expected to be complement fixing. Henson & Cochrane (1969) and Stux & Ovary (1976) both reported an anaphylactic IgG antibody in the rabbit which induced a PCA reaction that was dependent on an intact complement system. However, we could not find evidence for the existence of this antibody in our experiments. It was possible that cell-derived proteases, released in the PCA reaction, could cleave C5 in tissue fluid leading to the generation of C5a (Orr et al., 1979). Nevertheless, the contribution of C5a was mitigated by the observation that antibodies to C5a, which abrogated local oedema and neutrophil responses to i.d. ZAP and had no effect on the responses to FMLP, were without effect on oedema formation in the PCA reaction. In other studies, the same antibody effectively reduced plasma leakage and neutrophil accumulation in the reversed passive Arthus reaction in rabbit skin (Hellewell et al., 1988).

Human mast cells are a source of preformed tumour necrosis factor-α (TNF-α) (Gordon & Galli, 1990) which can be released rapidly. As TNF-α induces neutrophil accumulation and neutrophil-dependent plasma exudation in rabbit skin (Rampart et al., 1989a), its role in the PCA reaction deserves investigation. Both IL-1 and IL-8 induce neutrophil-dependent plasma leakage in rabbit skin (Buckley et al., 1991; Rampart et al., 1989b) however, there is no evidence that either cytokine is stored in cells; rather, their release depends on gene induction and protein synthesis, which takes 1–2 h to occur (Arai et al., 1990; Oppenheim et al., 1991). Nevertheless, it would still be valuable to assess their role using reagents such as blocking antibodies.

Earlier studies of the rabbit PCA focussed on the effects of antihistamines which produced varying degrees of inhibition, albeit small. Zvaifler et al. (1971) studied additional compounds which also had little effect. These included diethylcarbamazine (now known to be a non-specific 5-lipoxygenase inhibitor), disodium chromoglycate (to inhibit mast cell degranulation) and cobra venom factor (to reduce the blood complement titre). Kravis & Zvaifler (1974b) found that infusions of aminophylline, isoprenaline and propranolol were also without effect but did find reductions in local oedema formation (as assessed by diameter of bluing) after systemic treatment with PGE_1 and histamine. However, these latter treatments were associated with profound hypotension, an effect which is known to attenuate cutaneous oedema responses (Rampart & Williams, 1986). Thus, these earlier studies provide few clues as to the permeability-inducing agents that are generated in the rabbit PCA.

It is not known which cell type in skin was sensitized by the IgE antibody. Bauer et al. (1972) identified mast cells in rabbit skin and showed that these disappeared following antigen challenge in PCA sites. Furthermore, anti-IgE antibodies induce oedema formation in rabbit skin (Hellewell & Williams, 1989b) although it is not known if this is a consequence of cross-linking IgE already fixed on mast cells. In other species, including man, it is assumed that IgE-mediated cutaneous anaphylactic responses are the result of IgE-dependent mast cell degranulation (Dolovich et al., 1973; Solley et al., 1976; Dor et al., 1983). In addition, late phase responses (LPR) characterized by subcutaneous oedema and erythema (Dolovich et al., 1973; Solley et al., 1976) are also found in some individuals. We found no evidence for a late leakage response in the PCA; in fact the response was almost complete by 60 min. Moreover, we did not detect a late (up to 6 h) increase in local blood flow as assessed by a lack of potentiation of oedema when bradykinin was injected into PCA sites after a delay (data not shown).

In an attempt to isolate oedema-inducing activity in tissue fluid, we generated IgE-mediated anaphylactic reactions in the peritoneal cavity. Following antigen challenge there was an increase in permeability-increasing activity above the control exudate, as measured using the skin as an *in vivo* bioassay. This was detected within 30 min of antigen challenge and peaked after 90–120 min. Preliminary experiments confirmed that the activity was not reduced in the presence of antihistamines and was unstable to freezing. Currently, we

are attempting to isolate the activity. Studies in the guinea-pig have shown that histamine, PAF and a 5-lipoxygenase product are involved in local oedema formation in the PCA reaction (Weg *et al.*, 1991) and in allergic human subjects, skin responses to local antigen injection have been shown to involve histamine (Smith *et al.*, 1980) with evidence for the generation of PGD_2 (Pienkowski *et al.*, 1988), LTC_4 and LTD_4 (Talbot *et al.*, 1985) and interleukin-1 (Bochner *et al.*, 1990).

In conclusion, we have characterized local oedema formation in the IgE-dependent rabbit PCA reaction and found evidence for the involvement of a vasodilator prostaglandin and unknown mediators of increased vascular permeability. Current studies are attempting to identify this activity or activities which may have relevance for IgE-dependent human allergic diseases.

We thank the National Asthma Campaign and Pfizer Central Research, U.S.A. for support.

References

AKED, D.M. & FOSTER, S.J. (1987). Leukotriene B_4 and prostaglandin E_2 mediate the inflammatory response of rabbit skin to intradermal arachidonic acid. *Br. J. Pharmacol.*, **92**, 545–552.

AKED, D., FOSTER, S.J., HOWARTH, A., MCCORMICK, M.E. & POTTS, H.C. (1986). The inflammatory response of rabbit skin to topical arachidonic acid and its pharmacological modulation. *Br. J. Pharmacol.*, **89**, 431–438.

ARAI, K., LEE, F., MIYAJIMA, A., MIYATAKE, S., ARAI, N. & YOKOTA, T. (1990). Cytokines: coordinators of immune and inflammatory responses. *Annu. Rev. Biochem.*, **59**, 783–836.

BAUER, H., ZVAIFLER, N.J. & ROBINSON, J.O. (1972). IgE immunoglobulin in the rabbit. The histopathology of the homologous passive cutaneous anaphylaxis reaction. *Lab. Invest.*, **26**, 448–458.

BEHRENS, B.L., CLARK, R.A.F., PRESLEY, D.M., GRAVES, J.P., FELDSIEN, D.C. & LARSEN, G.L. (1987). Comparison of the evolving histopathology of early and late cutaneous and asthmatic responses in rabbits after a single antigen challenge. *Lab. Invest.*, **56**, 101–113.

BOCHNER, B.S., CHARLESWORTH, E.N., LICHENSTEIN, L.M., DERSE, C.P., GILLIS, S., DINARELLO, C.A. & SCHLEIMER, R.P. (1990). Interluekin-1 is released at sites of human cutaneous allergic reactions. *J. Allergy Clin. Immunol.*, **86**, 830–839.

BUCKLEY, T.L., BRAIN, S.D., COLLINS, P.D. & WILLIAMS, T.J. (1991). Inflammatory oedema induced by interactions between IL-1 and the neuropeptide calcitonin gene-related peptide. *J. Immunol.*, **146**, 3424–3430.

BURKE, J.F. & MILES, A.A. (1958). The sequence of vascular events in early infective inflammation. *J. Pathol. Bacteriol.*, **76**, 1–19.

CASALS-STENZEL, J., MUACEVIC, G. & WEBER, K-H. (1987). Pharmacological actions of WEB 2086, a new specific antagonist of platelet activating factor. *J. Pharmacol. Exp. Ther.*, **241**, 974–981.

COLLINS, P.D., JOSE, P.J. & WILLIAMS T.J. (1991). The sequential generation of neutrophil chemoattractant proteins in acute inflammation in the rabbit in vivo. Relationship between C5a and proteins with the characteristics of IL-8/neutrophil-activating protein 1. *J. Immunol.*, **146**, 677–684.

DOLOVICH, J., HARGREAVE, F.E., CHALMERS, R., SHIER, K.J. & GAULDIE, J. (1973). Late cutaneous allergic responses in isolated IgE-dependent reactions. *J. Allergy Clin. Immunol.*, **52**, 38–46.

DOR, P.J., VERVOLET, D., SAPENE, M., ANDRAC, L., BONERANDL, J.J. & CHARPIN, J. (1983). Induction of late cutaneous reaction by kallikrein injection: comparison with allergic-like response to compound 48/80. *J. Allergy Clin. Immunol.*, **71**, 363–370.

DORSCH, W., REIMANN, H.J. & NEUHAUSER, J. (1982). Histamine$_1$-histamine$_2$ antagonism: effect of combined clemastine and cimetidine pretreatment on allergen and histamine-induced reactions of the guinea pig lung in vivo and in vitro. *Agents Actions*, **12**, 113–118.

GORDON, J.R. & GALLI, S.J. (1990). Mast cells as a course of both performed and immunologically inducible TNF-alpha/cachectin. *Nature*, **346**, 274–276.

HALONEN, M., PALMER, J.D., LOHMAN, I.C., MCMANUS, L.M. & PINCKARD, R.N. (1980). Respiratory and circulatory alterations induced by acetyl glyceryl ether phosphorylcholine, a mediator of IgE anaphylaxis in the rabbit. *Am. Rev. Resp. Dis.*, **128**, 915–924.

HASLETT, C., JOSE, P.J., GICLAS, P.C., WILLIAMS, T.J. & HENSON, P.M. (1989). Cessation of neutrophil influx in C5a-induced acute experimental arthritis is associated with loss of chemoattractant activity from the joint space. *J. Immunol.*, **142**, 3510–3517.

HELLEWELL, P.G. (1990). The contribution of PAF to immune-complex mediated inflammation. In *Platelet Activating Factor in Endotoxin and Immune Diseases*. ed. Handley, D.A., Saunders, R.N., Houlihan, W.J. & Tomesch, J.C. pp. 367–386. New York: Marcel Dekker.

HELLEWELL, P.G., JOSE, P.J. & WILLIAMS, T.J. (1988). Effect of anti-C5a antibodies on oedema formation and PMN leukocyte accumulation in allergic inflammation in the rabbit. *Br. J. Pharmacol.*, **95**, 531P.

HELLEWELL, P.G. & WILLIAMS, T.J. (1986). A specific antagonist of platelet-activating factor suppresses oedema formation in an Arthus reaction but not oedema induced by leukocyte chemoattractants in rabbit skin. *J. Immunol.*, **137**, 302–307.

HELLEWELL, P.G. & WILLIAMS, T.J. (1989a). Antagonism of Paf-induced oedema formation in rabbit skin: a comparison of different antagonists. *Br. J. Pharmacol.*, **97**, 171–180.

HELLEWELL, P.G. & WILLIAMS, T.J. (1989b). An anti-inflammatory steroid inhibits tissue sensitization by IgE *in vivo*, *Br. J. Pharmacol.*, **96**, 5–7.

HELLEWELL, P.G., YARWOOD, H. & WILLIAMS, T.J. (1989). Characteristics of oedema formation induced by FMLP in rabbit skin. *Br. J. Pharmacol.*, **97**, 181–189.

HENSON, P.M. & COCHRANE, C.G. (1969). Immunological induction of increased vascular permeability. I. A rabbit passive cutaneous anaphylactic reaction requiring complement, platelets and neutrophils. *J. Exp. Med.*, **129**,153–165.

HUNTER, W.M. & GREENWOOD, F.C. (1962). Preparation of iodine^{-131} labelled human growth hormone of high specific activity. *Nature*, **194**, 495–496.

HUTCHCROFT, B.J., MOORE, E.G. & ORANGE, R.P. (1979). The effects of H_1 and H_2 receptor antagonism on the response of monkey skin to intradermal histamine, reverse-type anaphylaxis, and passive cutaneous anaphylaxis. *J. Allergy Appl. Immunol.*, **63**, 376–382.

ISHIZAKA, K., ISHIZAKA, T. & HORNBROOK, M.N. (1970). A unique rabbit immunoglobulin having homocytotropic antibody activity. *Immunochemistry*, **7**, 515–528.

JOSE, P.J., FORREST, M.J. & WILLIAMS, T.J. (1983). Detection of the complement fragment C5a in inflammatory exudates from the rabbit peritoneal cavity using radioimmunassay. *J. Exp. Med.*, **158**, 2177–2182.

JOSE, P.J. & WILLIAMS, T.J. (1987). Suppression of acute inflammatory responses by anti-C5a antibodies. *Complement*, **4**, 175.

KRAVIS, T.C. & ZVAIFLER, N.J. (1974a). Characterization of a rabbit homocytotropic antibody responsible for passive cutaneous anaphylactic reactions with a short (4-hour) latent period. *Int. Arch. Allergy Appl. Immunol.*, **46**, 60–71.

KRAVIS, T.C. & ZVAIFLER, N.J. (1974b). Alteration of rabbit PCA reaction by drugs known to influence intracellular cyclic AMP. *J. Immunol.*, **113**, 244–250.

LOHMAN, I.C. & HALONEN, M. (1990). Effects of the PAF antagonist WEB 2086 on PAF-induced physiologic alterations and on IgE anaphylaxis in the rabbit. *Am. Rev. Resp. Dis.*, **142**, 390–397.

LINDQVIST, K.J. (1968). A unique class of rabbit immunoglobulins eliciting passive cutaneous anaphylaxis in homologous skin. *Immunochemistry*, **5**, 525–542.

NEEDHAM, L., HELLEWELL, P.G., WILLIAMS, T.J. & GORDON, J.L. (1988). Endothelial functional responses and increased vascular permeability induced by polycations. *Lab. Invest.*, **59**, 538–548.

OPPENHEIM, J.J., ZACHARIAE, C.O.C., MUKAIDA, N. & MATSUSHIMA K. (1991). Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu. Rev. Immunol.*, **9**, 617–648.

ORR, F.W., VARANI, J., KREUTZER, D.L., SENIOR, R.M. & WARD, P.A. (1979). Digestion of the fifth component of complement by leukocyte enzymes. Sequential generation of chemotactic activities for leukocytes and for tumor cells. *Am. J. Pathol.*, **94**, 75–83.

PIENKOWSKI, M.M., ADKINSON, N.F., Jr., PLAUT, M., NORMAN, P.S. & LICHTENSTEIN, L.M. (1988). Prostaglandin D_2 and histamine during the immediate and latephase components of alergic cutaneous responses. *J. Allergy. Clin. Immunol.*, **82**, 95–100.

PONPIPOM, M.M., HWANG, S-B., DOEBBER, T.W., ACTON, J.J., ALBERTS, A.W., BIFTU, T., BROOKER, D.R., BUGIANESI, R.L., CHABALA, J.C., GAMBLE, N.L., GRAHAM, D.W., LAM, M-H. & WU, M.S. (1988). (±)-Trans-2-(3-methoxy-5-methyl sulfonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl) tetrahydrofuran (L-659,989), a novel, potent PAF receptor antagonist. *Biochem. Biophys. Res. Commun.*, **150**, 1213–1220.

RAMPART, M. & WILLIAMS, T.J. (1986). Polymorphonuclear leukocyte-dependent plasma leakage in the rabbit skin is enhanced or inhibited by prostacyclin, depending on the route of administration. *Am. J. Pathol.*, **124**, 66–73.

RAMPART, M., DE SMET, W., FIERS, W. & HERMAN A.G. (1989a). Inflammatory properties of recombinant tumor necrosis factor in rabbit skin. *J. Exp. Med.*, **169**, 2227–2232.

RAMPART, M., VAN DAMME, J., ZONNEKYN, L. & HERMAN A.G. (1989b). Granulocyte chemotactic peptide/interleukin-8 induces plasma leakage and neutrophil accumulation in rabbit skin. *Am. J. Pathol.*, **135**, 1–5.

REVOLTELLA, R. & OVARY, Z. (1969). Preferential production of rabbit reaginic antibodies. *Int. Arch. Allergy*, **36**, 282–289.

ROBERTS, N.M., PAGE., C.P., CHUNG, K.F. & BARNES, P.J. (1988). Effect of a PAF antagonist, BN52063, on antigen-induced, acute, and late-onset cutaneous responses in atopic subjects. *J. Allergy Clin. Immunol.*, **82**, 236–241.

SEDIVY, P., CAILLARD, C.G., CARREUTTE, A., DEREGNAUCOURT, J. & MONDOT, S. (1985). 48740 RP: selective anti-PAF agent. In *Advances in Inflammation Research* ed. Russo-Marie, F., Mencia-Huerta, J.M. & Chignard, M. pp. 171–173. New York: Raven Press.

SMITH, J.A., MANSFIELD, L.E., DE SHAZO, R.D. & NELSON, H. (1980). An evaluation of the pharmacologic inhibition of the immediate and late cutaneous reaction to allergen. *J. Allergy. Clin. Immunol.*, **65**, 118–121.

SOLLEY, G.O., GLEICH, G.J., JORDON, R.E. & SCHROETER, A.L. (1976). The late phase of the immediate wheal and flare reaction. *J. Clin. Invest.*, **58**, 408–420.

STEINBUCH, M. & AUDRAN, R. (1969). The isolation of IgG from mammalian sea with the aid of caprylic acid. *Arch. Biochem. Biophys.*, **134**, 279–284.

STETSON, C.A. (1951). Similarities in the mechanisms determining the Arthus and Schwartzman phenomena. *J. Exp. Med.*, **94**, 347–358.

STUX, S.V. & OVARY, Z. (1976). Heterogeneity of rabbit homologous skin sensitizing antibodies: IgE and a new subclass IgGa. *Int. Arch. Allergy Appl. Immunol.*, **51**, 338–358.

TALBOT, S.F., ATKINS, P.C., GOETZL, E.J. & ZWEIMAN, B. (1985). Accumulation of leukotriene C_4 and histamine in human allergic skin reactions. *J. Clin. Invest.*, **76**, 650–656.

UENO, A., TANAKA, K., KATORI, M., HAYASHI, M. & ARAI, Y. (1981). Species differences in increased vascular permeability by synthetic leukotrienes. *Prostaglandins*, **21**, 637–648.

VAVREK, J.R. & STEWART, J.M. (1985). Competitive antagonists of bradykinin. *Peptides*, **6**, 161–164.

WARREN, J.S., MANDEL, D.M., JOHNSON, K.J. & WARD, P.A. (1989). Evidence for the role of platelet activating factor in immune complex vasculitis in the rat. *J. Clin. Invest.*, **83**, 669–678.

WATANABE, N. & OVARY, Z. (1977). Antigen and antibody detection by in vivo methods; a reevaluation of passive cutaneous anaphylactic reactions. *J. Immunol. Meth.*, **14**, 381–390.

WEDMORE, C.V. & WILLIAMS, T.J. (1981). Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature*, **289**, 646–650.

WEG, V., WATSON, M.L., CORDEIRO, R.S.B. & WILLIAMS, T.J. (1991). Histamine, leukotriene D_4 and platelet activating factor in guinea pig passive cutaneous anaphylaxis. *Eur. J. Pharmacol.*, **204**, 157–163.

WILLIAMS, T.J. (1979). Prostaglandin E_2, prostaglandin I_2 and the vascular changes in inflammation. *Br. J. Pharmacol.*, **65**, 517–524.

WILLIAMS, T.J. & JOSE, P.J. (1981). Mediation of increased vascular permeability after complement activation: histamine-independent action of rabbit C5a. *J. Exp. Med.*, **153**, 136–153.

WILLIAMS, T.J. & MORLEY, J. (1973). Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature*, **246**, 215–217.

WILLIAMS, T.J. & PECK, M.J. (1977). Role of prostglandin-mediated vasodilatation in inflammation. *Nature*, **270**, 530–532.

WILLIAMS, T.J., HELLEWELL, P.G. & JOSE, P.J. (1986). Inflammatory mechanisms in the Arthus reaction. *Agents Actions*, **19**, 66–72.

ZVAIFLER, N.J., BAUER, H. & ROBINSON, J.O. (1971). IgE immunoglobulin in the rabbit. In *Biochemistry of the Acute Allergic Reactions. Second International Symposium.* ed. Austen, K.F. & Becker, E.L. pp. 33–44. Oxford: Blackwell Scientific Publications.

ZVAIFLER, N.J. & BECKER, E.L. (1966). Rabbit anaphylactic antibody. *J. Exp. Med.*, **123**, 935–950.

ZVAIFLER, N.J. & ROBINSON, J.O. (1969). Rabbit homocytotropic antibody. A unique rabbit immunoglobulin analogous to human IgE. *J. Exp. Med.*, **130**, 907–929.

(Received June 15, 1992
Revised August 13, 1992
Accepted August 17, 1992)

Br. J. Pharmacol. (1992), **107**, 1173–1180

Pharmacological profile of GR117289 *in vitro*: a novel, potent and specific non-peptide angiotensin AT_1 receptor antagonist

[2]M.J. Robertson, *J.C. Barnes, [1]G.M. Drew, K.L. Clark, *F.H. Marshall, A. Michel, †D. Middlemiss, †B.C. Ross, †D. Scopes & †M.D. Dowle

Departments of Cardiovascular and Respiratory Pharmacology, *Neuropharmacology, and †Medicinal Chemistry, Glaxo Group Research Ltd., Ware, Herts., SG12 0DP

1 This paper describes the effects of GR117289 (1-[[3-bromo-2-[2-(1H-tetrazol-5-yl)phenyl]-5-benzofuranyl]methyl]-2-butyl-4-chloro-1H-imidazole-5-carboxylic acid) at angiotensin receptors and binding sites in rabbit aorta, rat liver and bovine cerebellum preparations *in vitro*.

2 In rabbit isolated aortic strips, GR117289 (0.3, 1 and 3 nM) caused a concentration-related, insurmountable suppression of the concentration-response curve to angiotensin II (AII). When the contact time was increased, a greater degree of antagonism of AII was observed, suggesting that GR117289 is slow to reach equilibrium. A pK_B of 9.8 ± 0.1 was calculated for GR117289 after 3 h incubation. GR117289 (1 μM) did not affect contractile responses to phenylephrine or 5-hydroxytryptamine (5-HT) in the rabbit aorta.

3 GR117289 (1 nM) alone caused a marked suppression and a slight rightward displacement of the AII concentration-response curve. Co-incubation with the competitive, surmountable AT_1 receptor antagonist, losartan (10 nM, 100 nM and 1 μM), resulted in a concentration-related upward and rightward displacement of the concentration-response curve to subsequently administered AII. In separate experiments in which preparations were pre-incubated with GR117289 (1 nM), subsequent addition of losartan (1 μM) for 2, 15 or 45 min caused a further, but similar, rightward displacement of the concentration-response curve to subsequently administered AII with a *time-dependent increase* in the maximum response.

4 Suppression of AII-induced contractile responses, caused by superfusion with GR117289 (0.3, 1 or 3 nM) was not reversed by continuously washing the tissues for 3 h; in fact, the potency of GR117289 was slightly enhanced after this period.

5 In rat liver membranes, GR117289 was a potent competitor with [^{3}H]-AII for AT_1 binding sites ($pK_i = 8.7 \pm 0.1$) but in bovine cerebellum membranes, it was a very weak competitor for AT_2 binding sites ($pK_i < 6$). Pre-incubation of rat liver membranes with GR117289 had little effect on its affinity ($pK_i = 9.1 \pm 0.21$), but increasing the concentration of bovine serum albumen in the assay buffer from 0.001% to 0.1% w/v decreased affinity ($pK_i = 7.5 \pm 0.1$).

6 In saturation binding experiments in rat liver membranes, GR117289 (12 nM) increased the K_d of [^{3}H]-AII from 0.28 ± 0.06 nM to 0.37 ± 0.02 nM, and decreased B_{max} from 10.0 ± 0.1 to 5.6 ± 0.3 fmol mg^{-1} tissue. In other experiments, GR117289 (1 μM) did not alter the rate of dissociation of [^{3}H]-AII from AT_1 binding sites, following addition of excess unlabelled AII.

7 In rabbit aorta vascular smooth muscle membranes, GR117289 competed with [^{125}I]-Sar^{1}Ile^8 AII for binding to AT_1 binding sites. In the presence of 0.1% w/v bovine serum albumen, a pIC_{50} of 7.6 ± 0.1 was calculated. Under the same conditions, but with rat liver membranes, a pIC_{50} of 7.8 ± 0.1 was determined.

8 Taken together, these results show that GR117289 is a potent, specific, selective and insurmountable antagonist at angiotensin AT_1 receptors. Its profile in the rabbit aorta is consistent with the proposal that GR117289 is a slowly reversible (pseudo-irreversible) antagonist at these receptors.

Keywords: GR117289; angiotensin II (AII); AT_1 and AT_2 receptors; rabbit aorta; rat liver; bovine cerebellum; insurmountable antagonist

Introduction

The renin-angiotensin-aldosterone system plays a pivotal role in cardiovascular homeostasis, and the therapeutic success of angiotensin converting enzyme (ACE) inhibitors, such as captopril and enalapril, in the treatment of hypertension and heart failure has confirmed the involvement of this system in these disease states. Despite their clinical success, the use of ACE inhibitors is not without problems, dry cough and a propensity to induce functional renal failure being amongst the most common (Gavras & Gavras, 1988).

An alternative way of intervening in the renin-angiotensin-aldostererone axis, that might avoid the side effects of ACE inhibitors, is to prevent the action of the main effector of this system, angiotensin II (AII), at its sites of action. Until recently, angiotensin receptor antagonists have been peptidic in nature, and many have retained significant agonist activity. This, coupled with their short duration of action when administered systemically, and poor oral bioavailability, has prevented these compounds from establishing a therapeutic role. However, in 1988, Timmermans and associates reported that a series of 1-benzylimidazole-5-acetate derivatives exhibited significant affinity for angiotensin receptors in isolated tissues, including rat adrenal cortical microsomes and rabbit isolated aorta (Chiu *et al.*, 1988; 1989). The best known example of these compounds is losartan (DuP753;

[1] Author for correspondence.
[2] Present address: Department of Pharmacology, Fisons, p.l.c., Research and Development Laboratories, Pharmaceuticals Division, Bakewell Road, Loughborough, Leicestershire LE11 0RH.

Chiu et al., 1990). However, comparison of the profiles of action of compounds such as losartan, with that shown by imidazopyridines such as PD123177 (see Wong et al., 1990a), has revealed the existence of (at least) two distinct populations of angiotensin binding sites. These two binding sites are generally assumed to be synonymous with different angiotensin receptors, and it has been proposed that these be termed AT_1 and AT_2 receptors (Bumpus et al., 1991). Losartan and PD123177 are archetypal AT_1 and AT_2 receptor antagonists, respectively. The AT_1 receptors appear to mediate most, if not all, of the established effects of AII (e.g. vasoconstriction, steroidogenesis, dipsogenesis) and the role, if any, of AT_2 receptors remains unclear (e.g. see Zarahn et al., 1992).

Recently, Middlemiss et al. (1991) reported on a series of bromobenzofurans, a novel class of potent, non-peptide, angiotensin receptor antagonists. These compounds inhibit AII-induced contraction of rabbit isolated aortic strips and reduce blood pressure in renal artery-ligated, hypertensive rats. Among these compounds, GR117289 (1-[[3-bromo-2-[2-(1H-tetrazol-5-yl)phenyl]-5-benzofuranyl]methyl]-2-butyl-4-chloro-1H-imidazole-5-carboxylic acid) (Figure 1) exhibited potent AII antagonist activity *in vitro*, and exerted marked and prolonged antihypertensive activity after oral administration *in vivo*. This paper presents a detailed account of the pharmacological profile of GR117289 *in vitro*. Preliminary accounts have been presented to the British Pharmacological Society (Marshall et al., 1991; Robertson et al., 1991).

Methods

Functional studies in rabbit aorta

A detailed account of the rabbit isolated aortic strip preparation, and the experimental protocol used have been given previously (Robertson et al., 1992). Briefly, helical strips (1.5–2 cm) of thoracic aorta from male, New Zealand White rabbits were suspended in glass tissue chambers under a resting tension of 0.5 g in a physiological salt solution at 37°C, containing indomethacin (30 μM) and ascorbic acid (100 μM), and gassed with 95% O_2/5% CO_2.

In each experiment, four strips of aorta from a single rabbit were used simultaneously. In one series of experiments, the tissues were suspended under a resting tension of 0.5 g in a superfusion apparatus (see Coleman & Nials, 1989) in which the preparations were constantly superfused with physiological salt solution at 37°C.

Experimental protocols

In 4 separate, matched preparations, three consecutive concentration-contractile response curves to AII (0.1 nM–100 nM) were constructed until reproducible, by the cumulative addition of AII to the bathing fluid. The last of these was termed the 'pretest' curve. Subsequently, three tissues were exposed to one of three concentrations of GR117289 (0.3, 1 or 3 nM), for periods ranging from 45 min to 4 h. A fourth tissue was exposed to vehicle alone and served as a time-matched control. After the antagonist (or vehicle) incubation period, a final ('test') cumulative AII concentration-response curve was constructed. In a separate series of experiments, losartan (10, 100 or 1000 nM) was co-incubated for varying periods with GR117289 (1 nM) before the test AII curve. In some experiments, 5-hydroxytryptamine (5-HT, 30 nM–10 μM) or phenylephrine (10 nM–30 μM) was used in place of AII throughout.

In experiments using superfusion, cumulative AII concentration-response curves were constructed by sequentially increasing the concentration of AII in the superfusate. The flow rate over each tissue was 2 ml min^{-1}. AII was introduced into the superfusate in solution at a rate of 0.02 ml min^{-1}. GR117289 (0.3, 1 or 3 nM), or vehicle, was superfused for 45 min over the tissues (again, the flow rate of solution containing GR117289 was 0.02 ml min^{-1}). A final AII concentration-response curve (test) was then constructed, either immediately following the GR117289 (or vehicle) contact period, or after a subsequent 3 h period during which time the tissues were superfused (washed) with physiological salt solution alone.

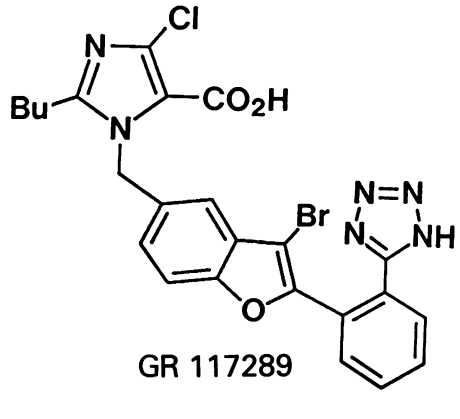

Figure 1 Chemical structure of GR117289.

Expression of results: determination of antagonist potency

For any individual preparation, the contractile response to each concentration of AII (or 5-HT, or phenylephrine) in the test curve was expressed as a percentage of the maximum contractile response to the agonist in the pretest curve. Values are shown as mean ± s.e.mean. In some cases, the concentration of agonist required to elicit a half maximum response (EC_{50}) was calculated; geometric mean (95% confidence intervals) EC_{50} values were calculated from these data.

Incubation with GR117289 reduced the maximal AII contractile response (insurmountable antagonism). For this reason, conventional Schild analysis could not be used to obtain a measure of potency of GR117289. Instead, an apparent pK_B for GR117289 was derived by use of a double-reciprocal regression plot (Kenakin, 1984). A graph of 1/A vs 1/A' was plotted, where A and A' were the equieffective concentrations of AII in the absence or presence of GR117289, respectively. The gradient (G) of this plot was then used in the Gaddum equation to obtain an estimate of pK_B: thus, $pK_B = -\log([B]/G-1)$, where B is the antagonist concentration.

Radioligand binding studies

Livers were obtained from Lister hooded rats (300–400 g) which were killed by cervical dislocation, and aortic tissue was obtained from male New Zealand White rabbits. Bovine cerebellum was obtained from Glaxo Institute for Molecular Biology, Geneva. The tissue was homogenized in 10–20 volumes (w/v) of ice cold homogenizing buffer (50 mM Tris, 5 mM EDTA, pH 7.4 at 4°C) in a Polytron P10 homogenizer. The homogenate was centrifuged at 48,000 *g* for 12 min at 4°C and the supernatant discarded. The pellet was resuspended in homogenizing buffer using the Polytron P10 and then centrifuged as before. The final pellet was resuspended in 50 mM Tris buffer (pH 7.4 at 25°C) at a tissue concentration of 400 mg ml^{-1} and stored at −70°C until required.

[^{3}H]-angiotensin II binding to rat liver and bovine cerebellum membranes

Binding assays were performed by incubating the membranes (5 mg tissue/tube) in 500 μl of assay buffer (Tris 50 mM,

NaCl 100 mM, $MgCl_2$ 10 mM, EDTA 1 mM, BSA 0.001% w/v and bacitracin 1 mM) with approximately 0.3 nM [^{3}H]-AII with or without competing compounds. Assay tubes were incubated at room temperature for 90 min, after which time, bound and free radioactivity were separated by rapid filtration through Whatman GF/B glass fibre filters, which were pretreated with 0.1% polyethyleneimine (PEI), using a Brandel cell harvester. The filters were washed with ice cold wash buffer (100 mM NaCl, 5 mM $MgCl_2$) and trapped radioactivity was determined by liquid scintillation counting using a Packard 2200Ca scintillation counter. Specific binding was defined as that displaceable by 1 μM AII. In saturation experiments, rat liver membranes were incubated with increasing concentrations of [^{3}H]-AII as described. Bound radioactivity, in the presence and absence of 1 μM AII for each concentration of [^{3}H]-AII, was determined by filtration as described above. To determine the effects of GR117289 on saturation binding, membranes were incubated with 12 nM GR117289 for 30 min prior to the addition of the [^{3}H]-AII. In dissociation experiments, [^{3}H]-AII (0.3 nM) was incubated to steady state with rat liver membranes (45 min at room temperature) in magnesium-free assay buffer. AII (1 μM), with or without GR117289 (final concentration, 1 μM), was then added and the amount bound after various time intervals between 0 and 240 min was determined by filtration as previously described. Membranes were incubated to steady state in the presence (total) or absence (nonspecific) of 1 μM AII and the dissociation rates of specific binding (total minus nonspecific binding) were determined.

[^{125}I]-Sar^1-Ile^8-angiotensin II binding

Membranes (0.1 mg tissue/tube for rat liver and 10 mg tissue/tube for rabbit aorta) were incubated in 150 μl of assay buffer (Tris 50 mM, NaCl 100 nM, $MgCl_2$ 10 mM, EDTA 1 mM, BSA 0.1% w/v and bacitracin 1 mM) with approximately 0.05 nM [^{125}I]-Sar^1-Ile^8-angiotensin II ([^{125}I]-Sarile) with or without competing compounds. Assay tubes were incubated for 90 min at room temperature after which time, bound and free radioactivity was separated as described above. Trapped radioactivity was measured with an LKB 1282 Compugamma counter. Specific binding was defined as that displaceable by 3 μM AII.

Data analysis

Competition binding data were analysed by use of iterative curve fitting techniques (Michel & Whiting, 1984). Data from [^{125}I]-Sarile binding experiments are presented as the negative logarithm of the IC_{50} (pIC_{50}). In the case of [^{3}H]-AII experiments, the IC_{50} values were corrected for the presence of the radioligand using the Cheng-Prusoff approximation (Cheng & Prusoff, 1973) and are presented as pK_i. Saturation experiments are presented in a Scatchard plot. K_d and B_{max} values were calculated by use of LIGAND (Munson & Rodbard, 1980). In dissociation experiments, dissociation rates were calculated using ENZFITTER (Biosoft, Cambridge).

Drugs used

Angiotensin II (human sequence) was obtained from Nova biochem., U.K. Ltd., Bacitracin, bovine serum albumin (BSA), 5-hydroxytryptamine (creatinine sulphate complex), phenylephrine hydrochloride and indomethacin were obtained from Sigma. [^{3}H]-angiotensin II ([^{3}H]-AII) and [^{125}I]-Sar^{1}Ile^8 angiotensin II ([^{125}I]-Sarile) were obtained from NEN, Du Pont (specific activities of 73.4 and 2200 Ci mMol^{-1}). GR117289 and losartan (potassium salt) were synthesized in the Chemistry Division, Glaxo Group Research Ltd.

Angiotensin II was dissolved in distilled water and stored frozen as aliquots (1 mM) which were thawed on the day of experimentation and diluted with distilled water. Phenylephrine and 5-HT were dissolved and diluted with distilled water. Indomethacin was dissolved in NaOH (2 M) and added directly to the physiological salt solution to give a final concentration of 30 μM. Bacitracin and BSA were dissolved directly in Tris buffer.

All other chemicals and constituents for the physiological salt solution were of Analar grade and obtained from BDH Ltd., England.

Results

The effect of GR117289 on angiotensin II-induced contraction in rabbit aortic strips

Cumulative addition of AII (0.3 nM–0.3 μM) caused concentration-related contractions of rabbit isolated aortic strips. After a 45 min incubation period, GR117289 (0.3, 1 or 3 nM) produced a concentration-related, insurmountable antagonism of AII (Figure 2a). GR117289 (3 nM) caused approximately 65% suppression of the maximum response to AII. Despite this profound, concentration-related suppression of the maximum response to AII, GR117289 did not significantly change the EC_{50} of AII. The geometric mean EC_{50} (95% confidence limits) values in the absence and presence of 0.3, 1 or 3 nM GR117289 were 5.4 (3.4–8.7), 5.3 (3.2–8.3), 5.85 (4.7–7.1), and 6.13 (5.5–6.8)nM, respectively.

For comparison, Figure 2b shows the effect of the sur-

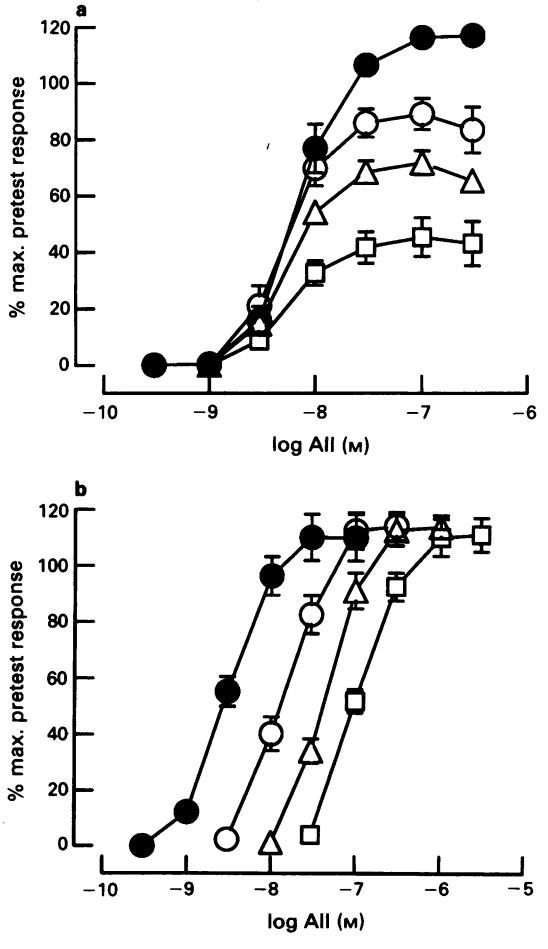

Figure 2 The contractile effect of angiotensin II (AII) in the rabbit isolated aorta, in the presence of (a) GR117289 (○ 0.3, △ 1 or □ 3 nM) or (b) losartan (○ 30, △ 100 or □ 1000 nM) or vehicle for losartan or GR117289 (●) previously incubated for 45 min ($n = 4$ for each). Results are shown as mean response (± s.e.mean, vertical bars) in the test curve, expressed as a % of the maximum response to AII in the pretest curve. Data for losartan are from Robertson *et al.* (1992) and republished with permission.

mountable, competitive angiotensin receptor antagonist, losartan (30, 100 and 300 nM) on AII-induced contractions in the rabbit aorta: losartan did not affect the maximum response to AII. As found previously (Robertson *et al.*, 1992), the pA_2 of losartan was calculated to be 8.2 ± 0.1 (slope = 1.1 ± 0.1) by Schild analysis.

Like AII, both 5-HT (30 nM–10 μM) and phenylephrine (10 nM–30 μM) caused concentration-related contractions in the rabbit aorta. GR117289 (1 μM) had no significant effect on the contractile response to either agonist (data not illustrated).

AII concentration-response curves, before and after a 3 h incubation with the vehicle for GR117289 (pretest and test curves) were highly reproducible. Compared with their respective time-matched vehicle controls, a 3 h incubation with GR117289 (0.3 nM) caused a more profound reduction (77% suppression, Figure 3a) of the maximum response to AII than had been observed after only 45 min (25% suppression, Figure 2a) or 2 h incubation (36% suppression, data not shown). In tissues incubated with vehicle for 4 h, AII responses became more variable (Figure 3b). However, GR117289 (0.3 nM), caused little further reduction (81% suppression, Figure 3b) of the maximum response to AII under these conditions, compared with that seen after 3 h incubation. Thus, GR117289 seemed to have reached equilibrium after approximately 3 h incubation. Using the method of Kenakin (1984), the pK_B of GR117289 was derived from 7 separate experiments in which GR117289 (0.3 nM) was incubated with aortic tissue for 3 h: a pK_B of 9.8 ± 0.1 was calculated. The geometric mean EC_{50} (95% confidence interval) of AII was significantly greater ($P < 0.005$, Student's unpaired t test) in the presence of GR117289 (8.2 (5.1–13.2) nM), than in its absence (2.7 (1.5–4.9) nM), indicating a small rightward displacement of the AII concentration-response curve.

The effect of losartan on GR117289-induced antagonism of angiotensin II

In tissues incubated with GR117289 (1 nM) for 2 h 45 min, the AII concentration-response curve was markedly suppressed and displaced to the right, compared with time-matched, vehicle-treated controls. In preparations which were co-incubated with losartan (10 nM, 100 nM or 1 μM) for the final 45 min of the GR117289 incubation period, the subsequent AII concentration-response curves were displaced upwards (with 10 nM, 100 nM or 1 μM losartan) and to the right (with 100 nM or 1 μM losartan), in a concentration-related manner (Figure 4).

In a related series of experiments, GR117289 (1 nM) incubated with rabbit aorta for 2 h, again caused a marked suppression and rightward displacement of the AII concentration-response curve, although this was not as marked as that seen when the incubation time was 2 h 45 min (compare Figure 5 with Figure 4). In the same series of experiments, GR117289 (1 nM) was incubated for 2 h and then losartan (1 μM) was also added for either 2, 15 or 45 min, before construction of the test AII curve. Co-incubation of losartan with GR117289 caused a larger rightward displacement of the AII concentration-response curve

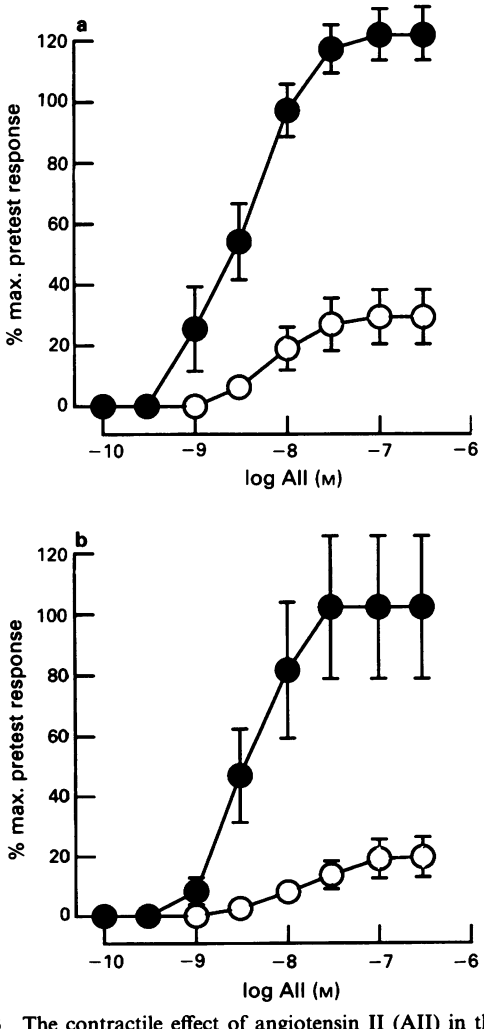

Figure 3 The contractile effect of angiotensin II (AII) in the rabbit isolated aorta, in the presence of GR117289 (○ 0.3 nM) or vehicle for GR117289 (●) incubated for either (a), 3 h or (b), 4 h ($n = 4$ for each).

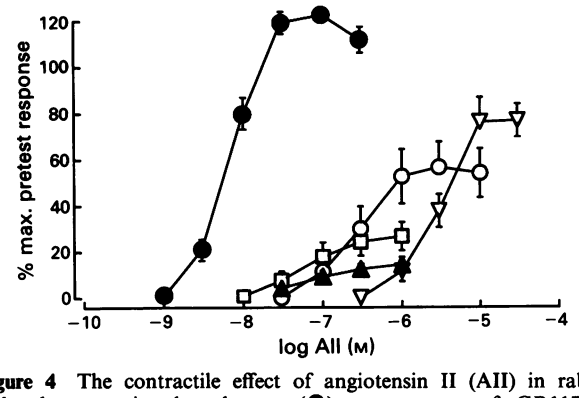

Figure 4 The contractile effect of angiotensin II (AII) in rabbit isolated aorta, in the absence (●) or presence of GR117289 (▲ 1 nM, 2 h 45 min incubation) alone, or losartan (□ 10 nM, ○ 100 nM, or ▽ 1 μM) co-incubated with GR117289 for the final 45 min of the 2 h 45 min incubation period ($n = 4$).

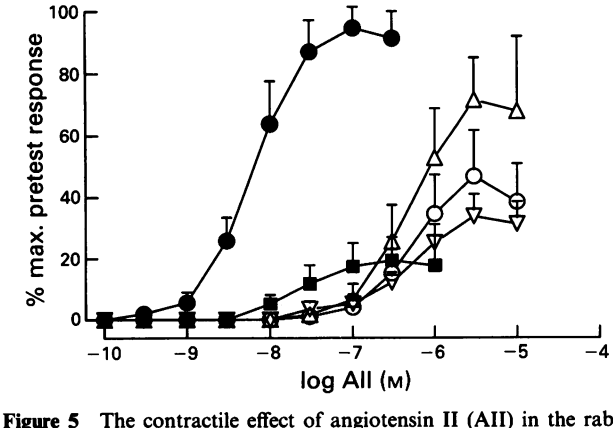

Figure 5 The contractile effect of angiotensin II (AII) in the rabbit isolated aorta, in the absence (●) or presence of GR117289 (■ 1 nM, 2 h incubation) alone, or co-incubated with losartan (1 μM) for a further 2 min (▽), 15 min (○) or 45 min (△) ($n = 5$).

than incubation with GR117289 alone. The concentrations of AII required to produce 50% of the maximum response in the test curve, in preparations co-incubated with losartan and GR117289 were similar, regardless of the duration of the losartan incubation (geometric mean EC_{50} values (95% confidence limits) after 2, 15 and 45 min losartan co-incubation were 731 (224–2381), 621 (260–1483) and 625 (209–1875)nM, respectively). In contrast, co-incubation with losartan caused a time-related increase of the maximum response to AII compared with that observed in preparations treated with GR117289 alone (Figure 5).

The effect of continuous washing on GR117289 antagonism of angiotensin II in superfused rabbit aortic strips

In superfused rabbit aortic strips, increasing concentrations of AII (1–30 nM) caused concentration-related increases in the contractile tension. The sensitivity of the preparations to AII was similar to that seen in the experiments carried out in a fixed volume of bathing fluid. As before, GR117289 (0.3, 1 or 3 nM) perfused over the tissues for 45 min, caused a concentration-related, insurmountable suppression of the contractile response to AII. The highest concentration of GR117289 used (3 nM), reduced the maximum response to AII by approximately 92%. When separate tissues from the same rabbits were treated in the same way with GR117289, but then superfused with drug-free physiological salt solution alone for another 3 h, subsequent AII responses were not restored; rather they were reduced further (see Figure 6b).

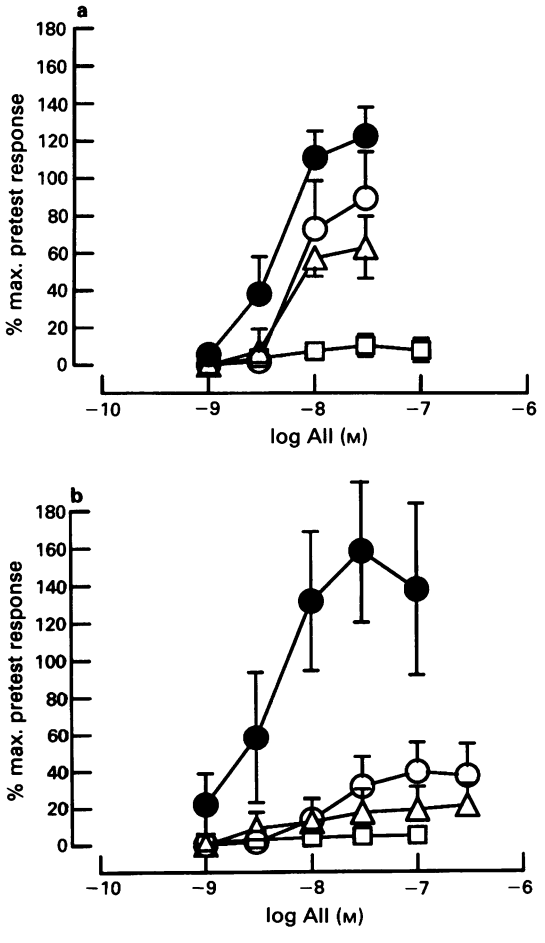

Figure 6 The contractile effect of angiotensin II (AII) in rabbit superfused isolated aorta, (a) in the absence of GR117289 (●) or after GR117289 (○ 0.3, △ 1 or □ 3 nM) was superfused for 45 min or, (b) in the absence of GR117289 (●) or after GR117289 (○ 0.3, △ 1 or □ 3 nM) were superfused for 45 min and followed by drug-free physiological salt solution (prolonged washing) for 3 h, respectively ($n = 3$).

Characteristics of GR117289 binding in rat liver and bovine cerebellum

In competition binding studies using [^{3}H]-AII at angiotensin AT_1 receptors in rat liver membranes, GR117289 was potent with a pK_i of 8.7 ± 0.1 ($n = 12$) and a Hill coefficient (nH) close to unity (1.11). In contrast, in bovine cerebellum, GR117289 had negligible affinity for angiotensin AT_2 sites ($pK_i < 6$, $n = 2$).

The AT_1 receptor has been shown to exist in high and low affinity states depending on its G-protein coupling. The competition studies described above were conducted in the presence of Mg^{2+}, which would result in the majority of the receptors being in the high affinity state with respect to agonist binding. In order to determine whether GR117289 could discriminate between high and low affinity states of the AT_1 receptor, competition studies were carried out in the presence and absence of Mg^{2+}. Under these conditions, the pIC_{50} values were found to be 8.5 ± 0.1 ($n = 3$, nH = 0.99) and 8.7 ± 0.2 ($n = 3$, nH = 1.31), respectively.

In order to determine whether GR117289 attained equilibrium during the 90 min incubation period of the [^{3}H]-AII binding assay, the liver membranes were preincubated with the antagonist for 1 h before addition of the radioligand. A 1 h preincubation resulted in a small, but not significant, increase in the pK_i from 8.7 ± 0.3 ($n = 3$, nH = 1.06) to 9.1 ± 0.21 ($n = 3$, nH = 1.4).

To establish the nature of the antagonism exerted by GR117289, its effect on the saturation curve of [^{3}H]-AII binding was determined. The results for GR117289 (12 nM) are shown in Figure 7 in the form of a Scatchard analysis. GR117289 (12 nM) decreased the B_{max} from 10.0 ± 0.1 fmol mg^{-1} tissue to 5.6 ± 0.3 fmol mg^{-1} tissue and increased the K_d of [^{3}H]-AII-binding from 0.28 ± 0.06 nM to 0.37 ± 0.02 nM ($n = 3$).

In order to investigate further the nature of the antagonism, the effect of GR117289 (1.0 μM) on the dissociation of [^{3}H]-AII, caused by addition of an excess of unlabelled AII, was measured. There was no significant difference between the dissociation rate in the absence ($t_{\frac{1}{2}} = 6.5 \pm 1.1$ min) or presence ($t_{\frac{1}{2}} = 7.4 \pm 1.9$ min) of GR117289 (1.0 μM, $n = 3$).

The affinity of GR117289 in binding assays was found to be markedly dependent on the concentration of BSA in the assay buffer. Increasing the BSA from 0.001% to 0.1% w/v resulted in a large rightward displacement of the inhibition curve which described the competition of [^{3}H]-AII with GR117289. In the presence of 0.1% w/v BSA, the pK_i of GR117289 in the rat liver decreased to 7.5 ± 0.1 (nH = 1.11, $n = 5$).

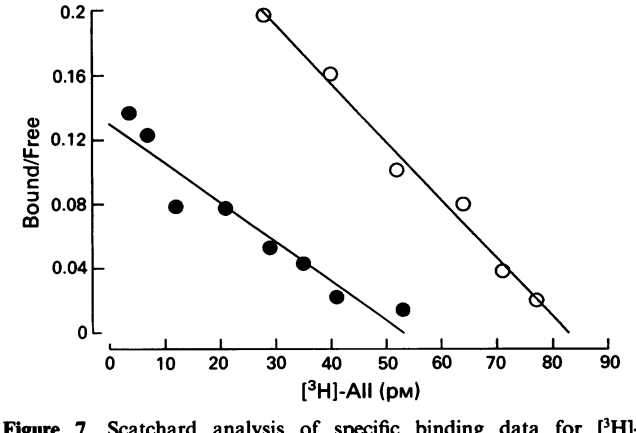

Figure 7 Scatchard analysis of specific binding data for [^{3}H]-angiotensin II ([^{3}H]-AII) in the absence (○) or presence (●) of GR117289 (12 nM). Data are taken from a representative experiment.

Characteristics of GR117289 binding in rabbit aorta

In an attempt to correlate findings in functional and radioligand binding sites, the affinity of GR117289 for angiotensin binding sites in rabbit aorta smooth muscle membranes was determined. Because of the low density of receptors in this tissue, a higher specific activity ligand, [^{125}I]-Sarile was used. In addition, to increase specific binding, the assay buffer contained 0.1% w/v BSA. Under these conditions, GR117289 competed with [^{125}I]-Sarile for binding to angiotensin receptors and yielded a mean pIC_{50} of 7.6 ± 0.1 (nH = 1.13, $n = 3$). Under the same conditions, but with rat liver membranes, the pIC_{50} for GR117289 was 7.8 ± 0.1 (nH = 1.08, $n = 5$).

Discussion

GR117289 is a potent antagonist against AII in rabbit aortic strips, and competes with [^{3}H]-AII for binding sites in rat liver and with [^{3}H]-AII or [^{125}I]-Sarile on rabbit aorta vascular smooth muscle membranes. These preparations were chosen because it is well established that the angiotensin receptors present belong to the AT_1 subtype (Wong *et al.*, 1990a; de Gasparo *et al.*, 1990; Chang & Lotti, 1991) and AT_1 receptors are the only subtype that, thus far, are known to mediate the major physiological effects of AII. In contrast, GR117289 had negligible affinity for AT_2 sites in bovine cerebellum, thus establishing its high selectivity for AT_1 receptors.

We have previously confirmed functionally that losartan is a potent, competitive, surmountable antagonist ($pA_2 = 8.2$) of AII in the rabbit aorta, using a tissue contact time of 45 min (Robertson *et al.*, 1992). However, the most striking difference between GR117289 and losartan in this preparation, is the way in which the two compounds displace the concentration-response curves to AII. Losartan caused a concentration-dependent, parallel, rightward displacement, without significantly affecting the maximum contractile response to AII. In contrast, GR117289 caused a concentration-related, insurmountable suppression of the AII concentration-response-curve. This profile of action could be explained by a number of different mechanisms: GR117289 may be (1) a 'non-specific' antagonist, (2) an allosteric antagonist, (3) an irreversible antagonist, or (4) a slowly reversible (pseudo-irreversible) antagonist, of AII.

Is GR117289 a 'non-specific' antagonist?

In this context, the term 'non-specific' is used to describe an antagonist which blocks, at some point, the chain of events leading to the production of a response by AII. Alternatively, the compound may antagonize at a receptor unrelated to angiotensin.

Even at the relatively high concentration of 1 μM, GR117289 did not affect contractile responses induced by 5-HT or phenylephrine in the rabbit aorta. This indicates not only that GR117289 has a low affinity ($pK_B < 6$) for both 5-HT_2 receptors and α_1-adrenoceptors in this tissue, but also that GR117289 does not interfere with the signal-transduction process that mediates the contraction to AII since AII (Griendling *et al.*, 1989), 5-HT (Roth *et al.*, 1986) and phenylephrine (Homcy & Graham, 1985), all induce smooth muscle contraction by receptor mediated hydrolysis of phosphatidylinositol leading to increased $[Ca^{2+}]_i$. Thus, these data suggest that the site of action of GR117289 is the AT_1 receptor macromolecule or a closely related (e.g. allosteric) site.

Further evaluation in other functional and radioligand binding studies has shown that, at $\geqslant 1$ μM, GR117289 has no detectable affinity for a wide range of receptors/binding sites/enzymes including adrenoceptors (α_2-, β_1-, β_2-), adenosine (A_1, A_2), bradykinin, dopamine (D_1, D_2), 5-hydroxytryptamine (5-HT_1, 5-HT_3), γ-aminobutyric acid ($GABA_A$, $GABA_B$), glycine, muscarinic (M_1, M_2), opioid (μ-, δ-, κ-), neuropeptide Y and neurotensin receptors, ACE, renin or adenylate cyclase (unpublished observations).

Is GR117289 an allosteric antagonist?

An allosteric antagonist is one that binds to a site on the receptor macromolecule or cell membrane, close to, but not at, the site at which the agonist binds. Nevertheless, occupation of the allosteric site by the antagonist impairs the ability of the agonist-receptor complex to generate a response. Such a phenomenon has been proposed by Kaumann & Frenken (1985) to explain the insurmountable suppression, by methysergide, of vasoconstrictor responses to 5-HT in calf coronary arteries. The functional experiments described in this report cannot exclude the possibility that GR117289 behaves as an allosteric antagonist of AII. However, in radioligand binding studies (see below), GR117289 was shown not to affect the rate of dissociation of [^{3}H]-AII from AT_1 receptors in rat liver membranes, as measured in isotope dilution experiments. Therefore, provided that GR117289 binds to AT_1 receptors in the rabbit aorta and rat liver membranes in a comparable manner, an allosteric mechanism of action in the aorta is unlikely.

Is GR117289 an irreversible antagonist?

Insurmountable antagonism can also occur when an antagonist forms an irreversible covalent bond with the receptor, such that the receptor number is effectively reduced to the point where a full agonist response cannot be achieved. However, it is unlikely that GR117289 binds irreversibly to AT_1 receptors. Evidence for this view comes from two main observations. Firstly, although the extent of the blocking action of GR117289 is progressive over 3 h of incubation, increasing the incubation time to 4 h did not appear to result in any greater degree of antagonism. Thus, GR117289 seemed eventually to reach equilibrium with AT_1 receptors in the rabbit aorta. Secondly, the degree of suppression, induced by GR117289, of the maximal response to AII, was reduced by co-incubation with losartan. Increasing concentrations of losartan displaced the concentration-response curve to AII, in the presence of GR117289, upward and to the right (Figure 4). If GR117289 had bound covalently to the AT_1 receptor, then co-incubation with a competitive antagonist would not have been expected to have any effect on the AII maximum.

Is GR117289 a slowly reversible (pseudo-irreversible) antagonist?

A further explanation of insurmountable antagonism is that slow dissociation of the antagonist from the receptor occurs. In this situation, the agonist cannot reach equilibrium with the antagonist/receptor complex under the time constraints of the experiments (see Craig *et al.*, 1990). In other words, the antagonist, once bound to the receptor, may dissociate from it so slowly as to appear irreversibly bound (pseudo-irreversible antagonism). Thus, a simple explanation for the findings of the experiments described above in which GR117289 and losartan were co-incubated, is that GR117289 dissociates slowly from the AT_1 receptors but, as it does, vacated receptors become occupied by losartan. Subsequently administered AII then competes for receptors occupied by losartan, as well as those still occupied by GR117289. As the concentration of losartan is increased, proportionally more receptors are occupied by losartan than by GR117289. Because AII can surmount the antagonism exerted by losartan, the maximal response to AII increases in relation to the proportion of receptors occupied by losartan rather than by GR117289. If GR117289 had bound irreversibly to the AT_1 receptors, co-incubation with losartan would merely have

produced further rightward displacement of the already suppressed AII curves. Similar to the present data, Wienen *et al.* (1990) and Wong & Timmermans (1991), demonstrated that the suppression of the AII curve produced by Sar^1Ile^8-AII and EXP3892, respectively, could also be reversed by losartan in rabbit aorta. More recent data (Entzeroth *et al.*, 1991) have confirmed and expanded these observations.

For this explanation of the interaction between GR117289 and losartan to be valid, it is necessary that losartan reaches equilibrium with the AT_1 receptor more rapidly than GR117289. In the present study, no experiments were conducted to determine whether losartan had reached equilibrium within the 45 min incubation period. However, other published evidence suggests that equilibrium is achieved very quickly. Using incubation times of less than 45 min, other groups have reported pA_2 values for losartan against AII in the rabbit aorta, similar to the value (8.2) obtained by Robertson *et al.* (1992) (e.g. Rhaleb *et al.*, 1991, 10 min incubation time, pA_2 8.27; Wong *et al.*, 1990b, 15 min incubation time, pA_2 8.48). It would therefore appear that, unlike GR117289, losartan reaches equilibrium with the AT_1 receptor rapidly, and certainly within 45 min. Further evidence in support of this concept is provided by the results shown in Figure 5, in which a single concentration of losartan was co-incubated for varying periods of time after pretreatment with GR117289. In these experiments, similar rightward displacements of the AII curve were seen after 2 min or 45 min incubation with losartan, again suggesting a rapid equilibration of losartan with the receptors. Figure 5 also shows that the increase in the maximum response to AII, in the presence of both antagonists, was progressive as the duration of losartan incubation was increased. This may be a measure of the slow rate of dissociation of GR117289 from the receptor.

The results obtained from the radioligand binding studies broadly support those from functional studies. In rat liver homogenates, the pK_i estimate for GR117289 was 8.7 ± 0.1. This is lower than was obtained in the rabbit aorta ($pK_B = 9.8$) and the Hill coefficient suggested that GR117289 and [^{3}H]-AII competed reversibly for the binding sites. However, it should be remembered that the competing agents were administered simultaneously in the binding studies, whereas the aorta was exposed to GR117289 for 3 h before challenge with AII. Pre-incubation of liver membranes with GR117289 for 1 h before addition of [^{3}H]-AII had little effect on the affinity estimate for GR117289. It was impractical to use longer pre-incubation times to determine whether the affinity of GR117289 would increase further.

The failure of GR117289 to increase the rate of dissociation of [^{3}H]-AII from liver membranes, following isotope dilution with excess, unlabelled AII argues against the binding of GR117289 to an allosteric site on the AT_1 receptor macromolecule. However, examination of the data shown in Figure 7 shows that the nature of the binding of GR117289 to liver membranes is characteristic of neither a simple, competitive antagonist, nor of a wholly irreversible antagonist. GR117289 both reduced the B_{max} and increased the K_d of [^{3}H]-AII binding. These findings are, therefore, consistent with the view that GR117289 may be a slowly reversible antagonist at AT_1 receptors.

Direct evidence for occupation of AT_1 receptors on rabbit aorta by GR117289, was obtained from binding studies carried out on smooth muscle membranes from this tissue. However, the low density of receptors present in these preparations meant that the experimental conditions were different from those previously used with rat liver membranes. In particular, the BSA concentration was 0.1% w/v in these experiments. This almost certainly accounts for the low pIC_{50} value (7.6 ± 0.1) obtained for GR117289 in this preparation, a view supported by the finding of a similarly low pIC_{50} (7.8 ± 0.1) in rat liver membranes used under identical conditions. Chiu *et al.* (1991) have previously reported that BSA inhibits the binding of several di-acid analogues of losartan. Thus, it is highly likely that GR117289 occupies a common binding site (i.e. receptor) in the two tissues, and that the measurements of potency obtained under these experimental conditions are underestimates of its true affinity.

The accuracy of the estimate of the dissociation constant made for GR117289, after 3 h incubation in the rabbit aorta, is debatable. It assumes that GR117289 simply occupies the same receptor site as AII and that their interaction reflects this phenomenon. However, the potency estimate made from functional studies is approximately 5 times higher than that determined from binding studies. This may be attributable to differences in experimental protocols (e.g. temperature, pretreatment time, whole tissue or membranes, the presence or absence of BSA). Although the results obtained in experiments in which rabbit aortic strips were continuously superfused with drug-free physiological salt solution for 3 h, after 45 min exposure to GR117289, are consistent with its being a very slowly dissociating antagonist, other factors may contribute to the long-lasting inhibition of responses to subsequently administered AII. In particular, GR117289 is a highly lipophilic agent (cLog P = 7.5). Thus, it is conceivable that the profile of activity of GR117289 is attributable, at least in part, to retention or, perhaps, even concentration within the membrane lipid. Apart from making it difficult to remove GR117289 by washing, this property might influence the estimation of its affinity for the angiotensin receptor in the rabbit aorta. For example, GR117289 might modulate the interaction between AII and its active site, not by changing its affinity (see above), but by reducing its efficacy as a result of altering the balance between receptor internalisation and expression, as has been suggested for some peptide antagonists of AII (Liu *et al.*, 1992). Thus, a smaller stimulus would be generated for any given agonist concentration. This would be reflected by a suppression, with little displacement, of the AII concentration-response curve. For this reason, the pK_B of 9.8 for GR117289 in this preparation should be regarded as no more than an approximation of its true affinity.

Taken as a whole, the data suggest that the most likely explanation for the insurmountable antagonism of AII by GR117289 in the rabbit aorta is attributable to its slow association with, and dissociation from, the AT_1 receptor (pseudo-irreversible antagonism). However, other explanations of the data are possible. For example, de Chaffoy de Courcelles *et al.* (1986) have described a receptor-transducer coupling model to explain insurmountable antagonism at 5-HT_2 receptor sites. In this model, an insurmountable antagonist produces its effect by binding to the receptor in such a way as to induce a conformational change in the receptor, which subsequently results in a decrease in the efficiency of stimulus-response coupling. Wong & Timmermans (1991) have tentatively applied this model to explain the insurmountable antagonism produced by EXP3892 (2'propyl-4'trifluoromethyl-5'-carboxylic acid derivative of losartan) at angiotensin AT_1 receptors in the rabbit aorta. In this model, competitive, surmountable antagonists can take the place of insurmountable antagonists, and this would explain the interaction we have observed between losartan and GR117289. However, it is not possible to distinguish between this type of interaction and pseudo-irreversible antagonism.

There are several precedents in the angiotensin literature which suggest that insurmountable antagonism is not unique to GR117289. For example, in rabbit aortic strips, suppression of the AII curve has been also reported for sarcosine substituted peptide angiotensin receptor antagonists such as Sar^1-$Cys(Me)^8$-AII (70% suppression; Freer *et al.*, 1980), Sar^1Ile^8-AII (40–90% suppression; Wienen *et al.*, 1990) or Sar^1-Ala^8-AII (saralasin, 50% suppression; Freer *et al.*, 1980; Chiu *et al.*, 1990). Similar findings have been made with non-peptide antagonists such as EXP3174 (5' carboxylic acid metabolite of losartan, 40% suppression; Wong *et al.*, 1990c)

and EXP3892, (40% suppression; Wong & Timmermans, 1991).

In conclusion, the present studies suggest that GR117289 is a potent, non-peptide, specific, selective and insurmountable, angiotensin AT_1 receptor antagonist. The mechanism of the insurmountable antagonism is debatable, but the data are consistent with GR117289 being a slowly reversible (pseudo-irreversible) antagonist.

The authors wish to thank Jason Brown, Amanda Dixon, Danen Cunoosamy and Donna Boxall for expert technical assistance and Beverley Gummer for typing the manuscript.

References

BUMPUS, F.M., CATT, K.J., CHIU, A.T., DE GASPARO, M., GOODFRIEND, T., HUSAIN, A., PEACH, M.J., TAYLOR, D.G. & TIMMERMANS, P.B.M.W.M (1991). Nomenclature for angiotensin receptors. A report of the nomenclature committee of the council for high blood pressure research. *Hypertension*, **17**, 720–721.

CHANG, R.S.L. & LOTTI, V.J. (1991). Angiotensin receptor subtypes in rat, rabbit and monkey tissues. *Life Sci.*, **49**, 1485–1490.

CHENG, Y.-C. & PRUSOFF, W.H. (1973). The relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

CHIU, A.T., CARINI, D.J., DUNCIA, J.V., LEUNG, K.H., MCCALL, D.E., PRICE, W.A., WONG, P.C., SMITH, R.D., WEXLER, R.R. & TIMMERMANS, P.B.M.W.M. (1991). DuP532: a second generation of nonpeptide angiotensin II receptor antagonists. *Biochem. Biophys. Res. Commun.*, **177**(1), 209–211.

CHIU, A.T., CARINI, D.J., JOHNSON, A.L., MCCALL, D.E., PRICE, W.A., THOOLEN, M.J.M.C., WONG, P.C., TABER, R.I. & TIMMERMANS, P.B.M.W.M. (1988). Non-peptide angiotensin II receptor antagonists. II. Pharmacology of S-8308. *Eur. J. Pharmacol.*, **157**, 13–21.

CHIU, A.T., DUNCIA, J.V., MCCALL, D.E., WONG, P.C., PRICE, W.A., THOOLEN, M.J.M.C., CARINI, D.J., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1989). Nonpeptide angiotensin II receptor antagonist III. Structure-function studies. *J. Pharmacol. Exp. Ther.*, **250**, 867–874.

CHIU, A.T., MCCALL, D.E., PRICE, W.A., WONG, P.C., CARINI, D.J., DUNCIA, J.V., WEXLER, R.R., YOO, S.E., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1990). Nonpeptide angiotensin II receptor antagonists. VII. Cellular and biochemical pharmacology of DuP753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.*, **252**, 711–718.

COLEMAN, R.A. & NIALS, A.T. (1989). Novel and versatile superfusion system. Its use in the evaluation of some spasmogenic and spasmolytic agents using guinea-pig isolated tracheal smooth muscle. *J. Pharmacol. Meth.*, **21**, 71–86.

CRAIG, D.A., ORNSTEIN, A.G. & CLARKE, D.E. (1990). XI. An analysis of unsurmountable antagonism to 5-hydroxytryptamine in the isolated perfused rat kidney: evidence for pseudoirreversible inhibition. In *Cardiovascular Pharmacology of 5-Hydroxytryptamine*. pp. 143–155. ed., Saxena, P.R., Wallis, D.J. & Bevan, P. Dordrecht, Netherlands: Kluwer Academic Publishers.

DE CHAFFOY DE COURCELLES, D., LEYSEN, J.E., ROEVENS, P. & VAN BELLE, H. (1986). The serotonin-S_2 receptor-transducer coupling model to explain insurmountable antagonist effects. *Drug Dev. Res.*, **8**, 173–178.

DE GASPARO, M., WHITEBREAD, S., MELE, M., MOTANI, A.S., WHITCOMBE, P.J., RAMJOUE, H.-P. & KAMBER, B. (1990). Biochemical characterisation of two angiotensin II receptor subtypes in the rat. *J. Cardiovasc. Pharmacol.*, **16**(suppl 4), S31–S35.

ENTZEROTH, M., MAUZ, A.B.M., VAN MEEL, J.C.A. & WIENEN, W. (1991). Different receptor interactions of peptide and nonpeptide angiotensin II antagonists revealed by biochemical and pharmacological studies. 238 (Abs) in *8th Camerino symposium: Trends in Receptor Research*, September 1991.

FREER, R.J., SUTHERLAND, J.C. & DAY, A.R. (1980). Synthesis and pharmacology of a non-competitive antagonist of angiotensin-induced contractions of vascular smooth muscle. *Circ. Res.*, **46**, 720–725.

GAVRAS, H. & GAVRAS, I. (1988). Angiotensin converting enzyme inhibitors. Properties and side effects. *Hypertension*, **11**(suppl II), II-37–II-41.

GRIENDLING, K.K., TSUDA, T., BERK, B.C. & ALEXANDER, R.W. (1989). Angiotensin II stimulation of vascular smooth muscle. *J. Cardiovasc. Pharmacol.*, **14**(6), S27–S33.

HOMCY, C.J. & GRAHAM, R.M. (1985). Molecular characterisation of adrenergic receptors. *Circ. Res.*, **56**, 635–650.

KAUMANN, A.J. & FRENKEN, M. (1985). A paradox: the 5-HT2-receptor antagonist restores the 5-HT-induced contraction depressed by methysergide in large coronary arteries of calf. Allosteric regulation of 5-HT2-receptors. *Naunyn-Schmiederbergs Arch. Pharmacol.*, **328**, 295–300.

KENAKIN, T.P. (1984). Drugs and drug responses in isolated tissues. *Pharmacol. Rev.*, **36**, 165–222.

LIU, Y.J., SHANKLEY, N.P., WELSH, N.J. & BLACK, J.W. (1992). Pharmacological analysis of angiotensin-II receptor ligand interactions in the rabbit isolated aorta assay. *Br. J. Pharmacol.*, **106**, 233–241.

MARSHALL, F.H., BARNES, J.C., BROWN, J.D., MICHEL, A.D. & TYERS, M.B. (1991). The interaction of GR117289 with the angiotensin AT_1 and AT_2 binding sites. *Br. J. Pharmacol.*, **104**, 425P.

MICHEL, A.D. & WHITING, R.L. (1984). Analysis of ligand binding data using a microcomputer. *Br. J. Pharmacol.*, **83**, 406P.

MIDDLEMISS, D., DREW, G.M., ROSS, B.C., ROBERTSON, M.J., SCOPES, D.I.C., DOWLE, M.D., AKERS, J.S., CARDWELL, K.L., CLARK, K.L., COOTE, S., ELDRED, C.D., HAMBLETT, J., HILDITCH, A., HIRST, G.C., JACK, T., MONTANA, J., PANCHAL, T.A., PATON, J.M.S., SHAH, P., STUART, G. & TRAVERS, A. (1991). Bromobenzofurans: a new class of potent, non-peptide antagonists of angiotensin II. *Bioorg. Med. Chem. Lett.*, **1**, 771–776.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: a versatile computerised approach for the characterisation of ligand binding systems. *Anal. Biochem.*, **107**, 220–239.

RHALEB, N., ROUISSI, N., NANTEL, F., D'ORLEANS-JUSTE, P. & REGOLI, D. (1991). DuP753 is a specific antagonist for the angiotensin receptor. *Hypertension*, **17**, 480–484.

ROBERTSON, M.J., CUNOOSAMY, M.P. & CLARK, K.L. (1992). Effects of peptidase inhibition on angiotensin receptor agonist and antagonist potency in rabbit isolated thoracic aorta. *Br. J. Pharmacol.*, **106**, 166–172.

ROBERTSON, M.J., MIDDLEMISS, D., ROSS, B.C., DREW, G.M., SCOPES, D.I.C. & DOWLE, M.D. (1991). GR117289: a novel potent and specific non-peptide angiotensin receptor antagonist. *Br. J. Pharmacol.*, **104**, 300P.

ROTH, B.L., NAKAKI, T., CHUANG, D. & COSTA, E. (1986). 5-Hydroxytryptamine$_2$ receptors coupled to phospholipase C in rat aorta: modulation of phosphoinositide turnover by phorbol ester. *J. Pharmacol. Exp. Ther.*, **238**, 480–485.

WIENEN, W., DIEDEREN, W.W., MAUZ, A.B.M. & VAN MEEL, J.C.A. (1990). Reversal of the non-competitive behaviour of the angiotensin II-antagonist Sar^{1}Ile^8-AII by a nonpeptide AII-antagonist in vitro and in vivo. *Eur. J. Pharmacol.*, **183**, 1553.

WONG, P.C., HART, S.D., ZASPEL, A.M., CHIU, A.T., ARDECKY, R.J., SMITH, R.D. & TIMMERMANS, P.B.M.W.M. (1990a). Functional studies of nonpeptide angiotensin II receptor subtype-specific ligands: DuP753 (AII-1) and PD123177 (AII-2). *J. Pharmacol Exp. Ther.*, **255**, 584–592.

WONG, P.C., PRICE, W.A., CHIU, A.T., CARINI, D.J., DUNCIA, J.V., JOHNSON, A.L., WEXLER, R.R. & TIMMERMANS, P.B.M.W.M. (1990b). Nonpeptide angiotensin II receptor antagonists: studies with EXP9270 and DuP753. *Hypertension*, **15**, 823–834.

WONG, P.C., PRICE, W.A., CHIU, A.T., DUNCIA, J.V., CARINI, D.J., WEXLER, R.R., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1990c). Nonpeptide angiotensin II receptor antagonists. XI. Pharmacology of EXP3174: an active metabolite of DuP753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.*, **255**, 211–217.

WONG, P.C. & TIMMERMANS, P.B.M.W.M. (1991). Nonpeptide angiotensin II receptor antagonists: insurmountable angiotensin II antagonism of EXP3892 is reversed by the surmountable antagonist DuP753. *J. Pharmacol Exp. Ther.*, **258**, 49–57.

ZARAHN, E.D., YE, X., ADES, A.M., REAGAN, L.P. & FLUHARTY, S.J. (1992). Angiotensin-induced cyclic GMP production is mediated by multiple receptor subtypes and nitric oxide in N1E-115 neuroblastoma cells. *J. Neurochem.*, **58**, 1960–1963.

(Received July 9, 1992
Accepted August 17, 1992)

Br. J. Pharmacol. (1992), **107**, 1181–1191

Involvement of nitric oxide in the regional haemodynamic effects of perindoprilat and captopril in hypovolaemic Brattleboro rats

[1]Sheila M. Gardiner & Terence Bennett

Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH

1 Male, homozygous Brattleboro (i.e. vasopressin-deficient) rats were chronically instrumented with pulsed Doppler flow probes and intravascular catheters, and were studied 5 h after a subcutaneous injection of an hyperoncotic solution of polyethylene glycol to render them hypovolaemic, and hence dependent on the renin-angiotensin system for maintenance of haemodynamic status. Pilot experiments showed that, in this model, primed infusion of perindoprilat (0.05 mg kg^{-1} bolus, 0.05 mg kg^{-1} h^{-1} infusion) or captopril (0.2 mg kg^{-1} bolus, 0.2 mg kg^{-1} h^{-1} infusion) just abolished the pressor effect of angiotensin I (120 pmol), and had similar initial hypotensive and renal hyperaemic vasodilator effects.

2 Perindoprilat had more sustained hypotensive, and mesenteric and hindquarters vasodilator effects than captopril in the presence of saline. In the presence of N^G-nitro-L-arginine methyl ester (L-NAME 3 mg kg^{-1} h^{-1}), the renal vasodilator effects of perindoprilat were unchanged, whereas the other haemodynamic effects of perindoprilat and captopril were reduced. Hence, in the presence of L-NAME, all haemodynamic effects of perindoprilat were greater than those of captopril.

3 The renal hyperaemic vasodilator effects of acetylcholine were abolished by L-NAME and by perindoprilat, and were markedly reduced by captopril. However, since perindoprilat and captopril caused such marked renal hyperaemic vasodilatation themselves, it is feasible this change in baseline status contributed to their effects. It is unlikely this could be a full explanation of the results, because the haemodynamic effects of lemakalim were unchanged under any experimental conditions.

4 Bradykinin alone, or in the presence of saline, caused mesenteric hyperaemic vasodilatation whereas, in the presence of perindoprilat or captopril, bradykinin caused marked renal and mesenteric vasoconstrictions. However, in the additional presence of L-NAME, the mesenteric vasoconstriction was reduced, yet the hypotensive effect of bradykinin was augmented. One possible explanation of these observations is that, in the presence of L-NAME and either perindoprilat or captopril, bradykinin caused marked coronary vasoconstriction, leading to a reduction in cardiac output.

5 Neither perindoprilat nor captopril impaired the pressor, or renal, mesenteric, or hindquarters vasoconstrictor effects of L-NAME. Indeed, in their presence, the effects of L-NAME were generally enhanced, consistent with perindoprilat and captopril causing activation of nitric oxide-dependent mechanisms that were subsequently inhibited by L-NAME.

Keywords: Perindoprilat; captopril; nitric oxide; haemodynamic

Introduction

There is increasing evidence from *in vitro* studies (Kerth & Vanhoutte, 1991; Goldschmidt & Tallarida, 1991; Mombouli *et al.*, 1991; Wiemer *et al.*, 1991; Clozel, 1991; Mombouli & Vanhoutte, 1991; Illiano *et al.*, 1991; Henrion *et al.*, 1991) that various angiotensin-converting enzyme (ACE) inhibitors influence endothelial cell function. In one instance the interaction was seen with captopril, but not with enalaprilat (Goldschmidt & Tallarida, 1991), indicating that sulphydryl groups might be responsible. However, in the other studies cited above, non-sulphydryl-containing ACE inhibitors were found to exert endothelial-mediated effects, so the question is unresolved.

At the time the present study was planned there were no data regarding putative interactions between ACE inhibitors and endothelial-mediated processes *in vivo*, so one of our aims was to provide such data. However, while the experiments described here were in progress, Cachofeiro *et al.* (1992) published findings relating to the ability of the nitric oxide synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA), to attenuate the hypotensive effects of captopril or ramiprilat in spontaneously hypertensive rats. Unfortunately, Cachofeiro *et al.* (1992) carried out experiments on acutely prepared animals and provided no regional haemodynamic data.

In previous studies we had found that Brattleboro (i.e. vasopressin-deficient) rats rendered hypovolaemic by water deprivation or by subcutaneous (s.c.) injection of an hyperoncotic solution of polyethylene glycol, became exquisitely sensitive to the hypotensive and vasodilator effects of ACE inhibitors such as captopril, enalaprilat and lisinopril (Gardiner & Bennett, 1985; 1986; Gardiner *et al.*, 1988; 1989; Tomlinson *et al.*, 1990; Muller *et al.*, 1990). Therefore, we considered this model might be one in which putative interactions between ACE inhibitors and endothelial function would be particularly marked. Our major aims were, by performing experiments in conscious, chronically-instrumented Brattleboro rats rendered hypovolaemic by s.c. injection of polyethylene glycol, to determine whether or not the actions of perindoprilat and captopril were influenced by the nitric oxide (NO) synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) and to assess the influence of perindoprilat and captopril on haemodynamic responses to acetylcholine, the K^+ channel opener, lemakalim (BRL

[1] Author for correspondence.

38227), and bradykinin (i.e. vasodilators with differing degrees of 'endothelial dependence').

Methods

Male, homozygous (350–450 g) Brattleboro rats were anaesthetized (sodium methohexitone, 60 mg kg^{-1} i.p., supplemented as required) and, through a midline laparotomy, had miniaturised, pulsed Doppler probes (Haywood *et al.*, 1981) implanted around the left renal and superior mesenteric arteries and the distal abdominal aorta (to monitor hindquarters flow).

Following surgery, animals were given ampicillin (7 mg kg^{-1}, i.m. Penbritin, Beecham) and returned to individual home cages with free access to tap water and food (Biosure, GLP grade diet 41B (M)). At least 7 days later, animals were briefly anaesthetized (sodium methohexitone 40 mg kg^{-1}, i.p.) and had implanted an intra-arterial catheter in the distal abdominal aorta (via the ventral caudal artery) for blood pressure and heart rate recording, 3 catheters in the right jugular vein for drug or peptide administration, and a single s.c. catheter; they were then allowed to recover for at least 48 h before experiments were begun. The total group of 32 fully-instrumented animals was randomized into 4 subgroups of 8 (Groups 1, 2, 3 and 4).

At 07 h 00 min on the day of the experiment, animals in all groups received (through the previously implanted s.c. catheter) an injection of 5 ml of an hyperoncotic solution of polyethylene glycol (PEG; carbowax 20 M, 30% in isotonic saline) warmed to body temperature (Gardiner *et al.*, 1989). Subsequently, animals were not allowed access to drinking water, in order to achieve isosmotic hypovolaemia (Gardiner & Bennett, 1986; Gardiner *et al.*, 1989). The experimental protocol began 5 h after the injection of PEG (Gardiner *et al.*, 1989). Continuous recordings (on a Gould ES 1000 system) were made of mean and phasic arterial blood pressures, instantaneous heart rate and mean and phasic Doppler shift signals from renal, mesenteric and hindquarters probes. The latter were monitored to ensure the signals were of an acceptable quality (signal: noise $>$ 20:1). Vascular conductance changes were calculated from mean Doppler shift signals and mean arterial blood pressure (Gardiner *et al.*, 1990a,b,c).

Pilot experiments

From these experiments ($n = 10$, in total) it was found that captopril at a dose of 0.2 mg kg^{-1} bolus, 0.2 mg kg^{-1} h^{-1} infusion and perindoprilat at a dose of 0.05 mg kg^{-1} bolus, 0.05 mg kg^{-1} h^{-1} infusion just caused complete inhibition of the haemodynamic effects of angiotensin I (120 pmol), in rats treated 5 h previously with PEG. Furthermore, at these doses the initial hypotensive and renal haemodynamic effects of the ACE inhibitors were similar, and hence these doses were chosen for the full experiments.

We planned, originally, to give randomized, 3 min infusions of acetylcholine (55 nmol kg^{-1} min^{-1}) (Gardiner *et al.*, 1991a) lemakalim (35 nmol kg^{-1} min^{-1}) (Gardiner *et al.*, 1991b) and bradykinin (36 nmol kg^{-1} min^{-1}) (Gardiner *et al.*, 1992a). However, it became apparent during further pilot experiments that the dose of lemakalim caused hypotensive and tachycardic effects that were too persistent to allow a systematic protocol to be run; in addition, in the presence of the ACE inhibitors, bradykinin infusion caused irreversible cardiovascular deterioration. Eventually we determined that 3 min infusions of acetylcholine (55 nmol kg^{-1} min^{-1}) and lemakalim (8.8 nmol kg^{-1} min^{-1}) and a bolus injection of bradykinin (2.4 nmol kg^{-1}), always given in that order, evinced the most reproducible responses, so this was the protocol used in the full experiments.

Full experiments

Animals were randomized into 4 groups with similar body weights (Group 1 = 421 ± 8 g (mean ± s.e.mean); Group 2 = 403 ± 9 g; Group 3 = 418 ± 4 g; Group 4 = 412 ± 9 g). All groups were initially challenged with 3 min infusions of acetylcholine (55 nmol kg^{-1} min^{-1}) and lemakalim (8.8 nmol kg^{-1} min^{-1}) and a bolus injection of bradykinin (2.4 nmol kg^{-1}). Thereafter, animals in Groups 1 and 2 received continuous i.v. infusion of saline (0.3 ml h^{-1}) and beginning 30 min later, were re-challenged with acetylcholine, lemakalim and bradykinin (i.e. in the same order as before). Sixty min after the onset of saline infusion, animals in Group 1 were given a primed infusion of perindoprilat (0.05 mg kg^{-1} bolus, 0.05 mg kg^{-1} h^{-1} infusion) and, beginning 30 min later, were re-challenged with acetylcholine, lemakalim and bradykinin. Two h after the onset of saline infusion (i.e. 1 h after onset of perindoprilat infusion) these animals received an L-NAME infusion (3 mg kg^{-1} h^{-1}, 0.3 ml h^{-1}) for 30 min. This dose of L-NAME was chosen on the basis of previous experiments (Gardiner *et al.*, 1991a) showing that a lower dose did not abolish vasodilator responses to acetylcholine.

Animals in Group 2 were treated identically to those in Group 1 except that they received a primed infusion of captopril (0.2 mg kg^{-1} bolus, 0.2 mg kg^{-1} h^{-1} infusion) rather than perindoprilat.

Animals in Groups 3 and 4 were treated as those in Groups 1 and 2, respectively, except that they received a continuous L-NAME infusion instead of saline infusion initially (i.e. from 30 to 180 min) and saline infusion instead of L-NAME infusion at the end of the protocol (i.e. from 150 to 180 min).

Animals in Groups 1 and 2 received acetylcholine, lemakalim and bradykinin through one catheter, and saline and the ACE inhibitor, separately, through the other two catheters. L-NAME was given for the last 30 min of the experiment through the catheter which had been used to deliver the vasodilator challenges.

Identical procedures were followed for animals in Groups 3 and 4, except that L-NAME was given through a separate, unused catheter, and saline was given at the end through the catheter which had been used to deliver the vasodilator challenges.

Data analysis

All raw data were recorded on a Gould ES 1000 system in the form of hard copy of the analogue signals. Following an experiment, measurements (by hand) were made of mean arterial blood pressure, instantaneous heart rate and mean renal, mesenteric and hindquarters Doppler shift signals. These variables were averaged (by eye) over epochs of 20 s starting immediately before any intervention and, depending on the profile of response, at appropriate time points thereafter. In the case of acetylcholine and lemakalim, measurements were made for the 20 s epochs straddling the 1, 2 and 3 min time points during infusion. For bradykinin, measurements were made at the peaks or nadirs of the mesenteric and hindquarters flow changes. Following administration of perindoprilat or captopril, the values for the 20 s epochs at 5, 10 and 30 min were recorded, while for L-NAME and saline those at 30 min were noted.

For ease of presentation, group data have been rendered into the form of means ± s.e.mean, both for individual time points and for areas under or over curves (AUC and AOC, respectively). All calculations (means and s.e.mean, AUC or AOC, % changes etc) were made with a Fortran programme running on a mainframe (Vax) computer.

Data were analysed by non-parametric tests, i.e. Wilcoxon's test, Kruskal-Wallis test and Friedman's test (Theodorsson-Norheim, 1987), as appropriate. A P value < 0.05 was taken as significant.

Drugs, peptides and chemicals

Perindoprilat was supplied by Servier R & D; captopril was obtained from the Squibb Institute (U.S.A.), and lemakalim (BRL 38227) from SmithKline Beecham (UK). Acetylcholine chloride and L-NAME were obtained from Sigma (UK) and bradykinin from Bachem (UK). Polyethylene glycol (Carbowax 20 M) was obtained from BDH (UK). Perindoprilat and captopril were dissolved in isotonic saline and buffered to pH 7.4–7.6 with Na_2CO_3 (0.5%). Acetylcholine, lemakalim, L-NAME, PEG and bradykinin were dissolved in isotonic saline. In the case of bradykinin the saline contained 1% bovine serum albumin (Sigma, UK).

Results

Resting cardiovascular variables in all 4 experimental groups at the beginning of each protocol are shown in Table 1. There were no significant differences between the groups.

Effects of acetylcholine (ACh)

In all 4 groups, the first infusion of ACh caused hypotension, tachycardia, marked renal vasodilatation, slight hindquarters vasodilatation and a variable mesenteric vasoconstriction (Figures 1 and 2, Table 2). A similar picture was seen in the presence of saline, although there was a tendency for the hypotensive effect of ACh to be less (Figure 1, Table 2, Groups 1 and 2). However, during combined infusions of saline and perindoprilat there was no significant renal vasodilator response to ACh, whereas in the presence of saline and captopril, there was still a significant renal vasodilator response to ACh, albeit significantly smaller than in the presence of saline alone (Figure 1, Table 2, Groups 1 and 2). There was an increase in mesenteric vascular conductance in response to ACh in the presence of saline and either perindoprilat or captopril, which was significantly different from the mesenteric vasoconstriction seen in the presence of saline alone (Figure 1, Table 2, Groups 1 and 2).

In the presence of L-NAME, with or without captopril or perindoprilat, the renal vasodilator response to ACh was markedly attenuated, although other changes were not significantly affected (Figure 2, Table 2, Groups 3 and 4). However, in the presence of L-NAME and either ACE inhibitor, the mesenteric vascular response to ACh was significantly different from that seen in the presence of saline and either ACE inhibitor (Figures 1 and 2, Table 2).

Effects of lemakalim

In all 4 groups, the first infusion of lemakalim caused slight hypotension and a tachycardia accompanied by marked mesenteric vasodilatation, and modest and variable renal and hindquarters vasodilatations (Figures 1 and 2, Table 3).

Similar effects of lemakalim were seen in the presence of saline, or of L-NAME, and in the additional presence of perindoprilat or captopril (Figures 1 and 2, Table 3). There were no inter-group differences in the responses to lemakalim at any stage of the experimental protocols (Figures 1 and 2, Table 3).

Effects of bradykinin (BK)

In all 4 groups, the initial bolus injection of BK caused tachycardia and a tendency towards hypotension, associated with an early mesenteric vasodilatation followed by hindquarters vasodilatation; there was slight and variable renal vasodilatation (Figures 1 and 2, Table 4). A similar picture was seen in the presence of saline (Figure 1, Table 4, Groups 1 and 2). During combined infusions of saline and either captopril or perindoprilat, BK caused hypotension, marked bradycardia, renal and mesenteric vasoconstriction and hindquarters vasodilatation (Figure 1, Table 4, Groups 1 and 2). All these changes were significantly different from those seen in the presence of saline alone. There was no difference between the responses seen in the presence of captopril and those seen in the presence of perindoprilat.

The effects of BK in the presence of L-NAME differed from those in the presence of saline in respect of mean arterial blood pressure (which tended to rise, rather than fall) and renal vascular conductance (which tended to fall, rather than rise, Figures 1 and 2, Table 4).

During combined infusions of L-NAME and perindoprilat, or L-NAME and captopril, BK caused marked hypotension and bradycardia, and renal vasoconstriction and hindquarters vasodilatation (Figure 2, Table 4, Groups 3 and 4). However, there was mesenteric vasoconstriction in response to BK in the presence of L-NAME and perindoprilat, and this was significantly different from the response seen in the presence of L-NAME and captopril (Figure 2, Table 4). Moreover, the hypotensive response to BK in the presence of L-NAME and perindoprilat was significantly greater than the hypotensive response in the presence of saline and perindoprilat, consistent with the mesenteric vasoconstrictor effect of BK being greater in the latter condition (Figures 1 and 2, Table 2). The lack of mesenteric vasoconstrictor response to BK in the presence of L-NAME and captopril was associated with a tendency towards an enhanced hypotensive response, but this did not reach significance (Figures 1 and 2, Table 4), possibly because the hindquarters vasodilator effect of BK was significantly less than in the presence of saline and captopril (Table 4).

Effects of perindoprilat or captopril

Although the pilot experiments, and the results from the full experiments, indicated that the doses of perindoprilat and captopril were matched for their ability to inhibit the haemodynamic effects of angiotensin I, and for their initial

Table 1 Resting cardiovascular variables in the 4 separate experimental groups

	Group 1	*Group 2*	*Group 3*	*Group 4*
Heart rate (beats min^{-1})	328 ± 11	305 ± 10	293 ± 19	308 ± 10
Mean BP (mmHg)	114 ± 2	109 ± 2	108 ± 3	111 ± 2
Doppler shift (kHz)				
Renal	6.8 ± 1.0	7.2 ± 0.9	6.7 ± 0.8	6.3 ± 0.7
Mesenteric	5.5 ± 0.5	5.3 ± 0.5	5.3 ± 0.5	5.5 ± 0.8
Hindquarters	2.9 ± 0.3	2.6 ± 0.3	2.7 ± 0.4	2.7 ± 0.3
Vascular conductance ([kHz $mmHg^{-1}$]10^3)				
Renal	59 ± 8	66 ± 7	61 ± 5	56 ± 6
Mesenteric	48 ± 5	48 ± 4	50 ± 5	50 ± 8
Hindquarters	26 ± 3	24 ± 2	25 ± 3	24 ± 3

Values are mean ± s.e.mean, $n = 8$ (in all groups)

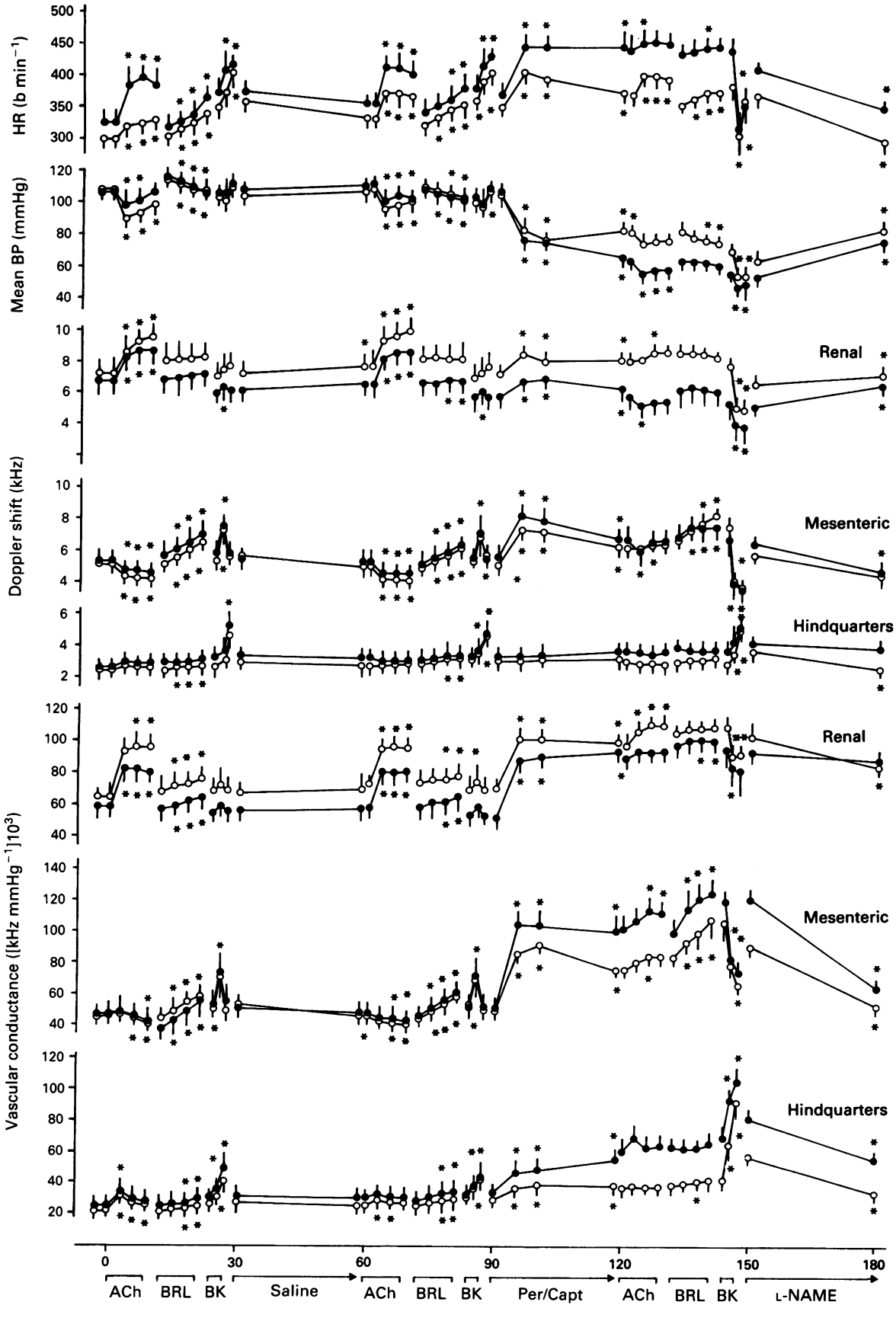

Figure 1 Cardiovascular responses to 3 min infusions of acetylcholine (ACh) or lemakalim (BRL) or bolus injection of bradykinin (BK) before and during infusion of saline, and perindoprilat (Per, ●, Group 1) or captopril (Capt, ○, Group 2) in separate groups ($n = 8$ in each) of conscious Brattleboro rats. At the end of the experiment, both groups were given a 30 min infusion of N^G-nitro-L-arginine methyl ester (L-NAME). BP = blood pressure; HR = heart rate. Values are mean and vertical bars are s.e.mean. $^*P < 0.05$ for change relative to the corresponding pre-intervention resting value. Statistics for AUC or AOC are given in the tables.

Table 2 Cardiovascular changes (AUC or AOC, arbitrary units) in response to 3 min infusions of acetylcholine under basal conditions, during infusion of saline (Groups 1 and 2) or N^G-nitro-L-arginine methyl ester (L-NAME, Groups 3 and 4), and during infusion of saline plus perindoprilat (Group 1), saline plus captopril (Group 2), L-NAME plus perindoprilat (Group 3) and L-NAME plus captopril (Group 4)

	Group 1	*Group 2*	*Group 3*	*Group 4*
ΔHeart rate	166 ± 24*	54 ± 11*[a]	83 ± 15*	105 ± 20*
ΔMean BP	−35 ± 12*	−48 ± 8*	−31 ± 6*	−50 ± 7*
ΔRenal conductance	60 ± 11*	73 ± 4*	64 ± 8*	59 ± 7*
ΔMesenteric conductance	−9 ± 3*	−9 ± 3*	−7 ± 2	−6 ± 2
ΔHindquarters conductance	14 ± 7*	16 ± 5*	14 ± 4*	20 ± 4*
	Saline		*L-NAME*	
ΔHeart rate	145 ± 17*	100 ± 14*	50 ± 10*	37 ± 14
ΔMean BP	−27 ± 6*	−28 ± 5*	−20 ± 7*	−20 ± 7*
ΔRenal conductance	57 ± 7*	63 ± 3*	8 ± 2[c]	9 ± 4*[d]
ΔMesenteric conductance	−9 ± 2*	−13 ± 4*	−6 ± 3	−9 ± 3*
ΔHindquarters conductance	6 ± 4	8 ± 2*	13 ± 4*	8 ± 2*
	Saline		*L-NAME*	
	Perindoprilat	*Captopril*	*Perindoprilat*	*Captopril*
ΔHeart rate	33 ± 7*	72 ± 13*	35 ± 13	38 ± 13
ΔMean BP	−21 ± 4*	−17 ± 6	−24 ± 9*	−28 ± 10*
ΔRenal conductance	12 ± 3	29 ± 5*[a]	11 ± 4	12 ± 3
ΔMesenteric conductance	20 ± 5*	19 ± 6*	−30 ± 8[c]	−15 ± 4*[d]
ΔHindquarters conductance	18 ± 8	8 ± 3	14 ± 4*	13 ± 4*

Values are mean ± s.e.mean, $n = 8$ (all groups).
*$P < 0.05$ for change, [a]$P < 0.05$ Group 2 vs Group 1; [c]$P < 0.05$ Group 3 vs Group 1; [d]$P < 0.05$ Group 4 vs Group 2

Table 3 Cardiovascular changes (AUC or AOC, arbitrary units) in response to 3 min infusions of lemakalim under basal conditions, during infusion of saline (Groups 1 and 2) or N^G-nitro-L-arginine methyl ester (L-NAME, Groups 3 and 4), and during infusion of saline plus perindoprilat (Group 1), saline plus captopril (Group 2), L-NAME plus perindoprilat (Group 3) and L-NAME plus captopril (Group 4)

	Group 1	*Group 2*	*Group 3*	*Group 4*
ΔHeart rate	69 ± 11*	57 ± 11*	33 ± 10	58 ± 11*
ΔMean BP	−16 ± 2*	−15 ± 2*	−11 ± 2*	−11 ± 2*
ΔRenal conductance	13 ± 3*	12 ± 3*	8 ± 3*	12 ± 2*
ΔMesenteric conductance	24 ± 4*	22 ± 3*	24 ± 3*	26 ± 5*
ΔHindquarters conductance	5 ± 1*	9 ± 1*	8 ± 2	4 ± 1
	Saline		*L-NAME*	
ΔHeart rate	51 ± 9*	61 ± 12*	20 ± 6 *	45 ± 18*
ΔMean BP	−17 ± 3*	−12 ± 2*	−18 ± 5*	−14 ± 2*
ΔRenal conductance	12 ± 2*	8 ± 1*	11 ± 3*	15 ± 3*
ΔMesenteric conductance	22 ± 3*	20 ± 3*	17 ± 3*	17 ± 3*
ΔHindquarters conductance	8 ± 2*	8 ± 2*	4 ± 1	5 ± 1
	Saline		*L-NAME*	
	Perindoprilat	*Captopril*	*Perindoprilat*	*Captopril*
ΔHeart rate	23 ± 7*	41 ± 8*	49 ± 9*	40 ± 8*
ΔMean BP	−6 ± 2	−11 ± 2*	−5 ± 2	−9 ± 4
ΔRenal conductance	10 ± 2*	7 ± 2	10 ± 4*	10 ± 4*
ΔMesenteric conductance	27 ± 4*	37 ± 6*	21 ± 4*	32 ± 8*
ΔHindquarters conductance	4 ± 2	7 ± 2	7 ± 3	5 ± 1

Values are mean ± s.e.mean, $n = 8$ (all groups).
*$P < 0.05$ for change

hypotensive and renal haemodynamic actions (Figure 1), differences between the effects of the two ACE inhibitors appeared during the 30 min following their administration. Thus, in the presence of saline, perindoprilat had significantly greater hypotensive, tachycardic and mesenteric and hindquarters vasodilator effects than captopril (Table 5).

In the presence of L-NAME, the renal vasodilator effect of perindoprilat was unchanged but the hypotension, tachycardia and mesenteric and hindquarters vasodilatation were all significantly smaller than in the absence of L-NAME (Table 5, Groups 1 and 3). In contrast, all three vascular beds showed significantly smaller vasodilatations in response to captopril during L-NAME infusion, compared to the responses seen in the absence of L-NAME (Table 5, Groups 2 and 4). Thus, the hypotensive and renal, mesenteric and hindquarters vasodilator effects of perindoprilat were all significantly greater than those of captopril during L-NAME infusion (Figure 2, Table 5, Groups 3 and 4).

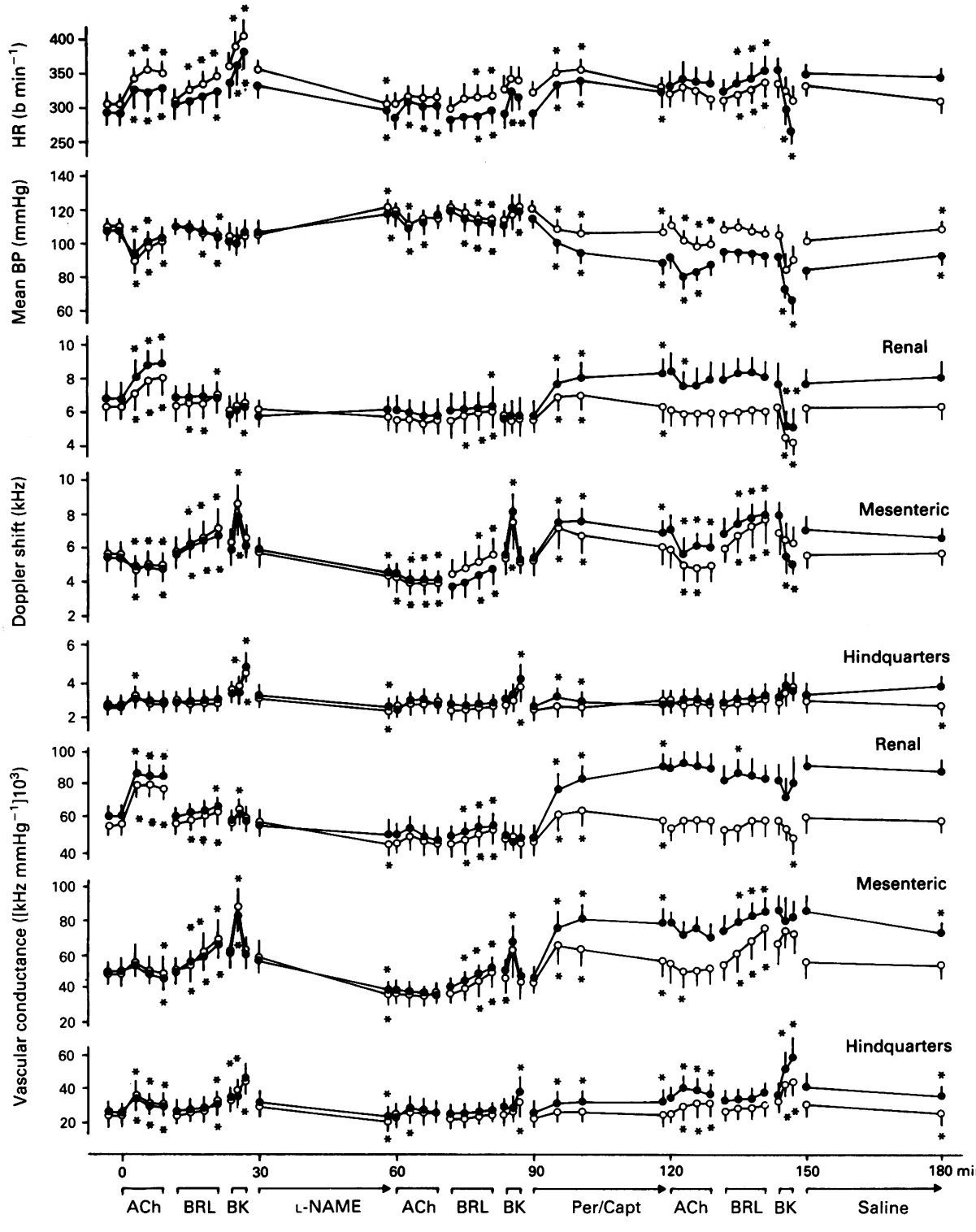

Figure 2 Cardiovascular responses to 3 min infusions of acetylcholine (ACh) or lemakalim (BRL) or bolus injection of bradykinin (BK) before and during infusion of N^G-nitro-L-arginine methyl ester (L-NAME) and perindoprilat (Per, ●, Group 3) or captopril (Capt, ○, Group 4) in separate groups ($n = 8$ in each) of conscious Brattleboro rats. At the end of the experiment, both groups were given a 30 min infusion of saline. BP = blood pressure; HR = heart rate.
Values are mean and vertical bars are s.e.mean. $^*P < 0.05$ for change relative to the corresponding pre-intervention resting value. Statistics for AUC or AOC are given in the tables.

Effects of L-NAME

L-NAME, alone, increased mean arterial blood pressure in association with bradycardia, and constrictions in renal, mesenteric and hindquarters vascular beds (Figure 2, Table 6).

In the presence of saline and perindoprilat or captopril the pattern of response to L-NAME was similar to that seen with L-NAME alone, but, with the exception of the renal vasoconstrictor response to L-NAME, all variables showed significantly greater changes in the presence of ACE inhibitors (Figures 1 and 2, Table 6).

Table 4 Cardiovascular changes (AUC or AOC, arbitrary units) in response to bolus injections of bradykinin under basal conditions, during infusion of saline (Groups 1 and 2) or N^G-nitro-L-arginine methyl ester (L-NAME, Groups 3 and 4), and during infusion of saline plus perindoprilat (Group 1), saline plus captopril (Group 2), L-NAME plus perindoprilat (Group 3) and L-NAME plus captopril (Group 4)

	Group 1	*Group 2*	*Group 3*	*Group 4*
ΔHeart rate	56 ± 6*	57 ± 15*	55 ± 14*	54 ± 5*
ΔMean BP	−15 ± 6	−13 ± 6	−9 ± 5	−11 ± 4
ΔRenal conductance	10 ± 3	8 ± 3	5 ± 2	10 ± 3*
ΔMesenteric conductance	26 ± 7*	23 ± 4*	28 ± 8*	34 ± 9*
ΔHindquarters conductance	15 ± 4*	12 ± 2*	10 ± 2*	12 ± 4*
	Saline		*L-NAME*	
ΔHeart rate	59 ± 6*	58 ± 9*	48 ± 12*	37 ± 10*
ΔMean BP	−12 ± 4	−10 ± 4	21 ± 8*[c]	14 ± 4[d]
ΔRenal conductance	8 ± 3	6 ± 1*	−13 ± 6[c]	−6 ± 2[d]
ΔMesenteric conductance	24 ± 6*	21 ± 3*	22 ± 8*	23 ± 6*
ΔHindquarters conductance	13 ± 3*	12 ± 3*	9 ± 4*	8 ± 3*
	Saline		*L-NAME*	
	Perindoprilat	*Captopril*	*Perindoprilat*	*Captopril*
ΔHeart rate	−229 ± 44*	−153 ± 39*	−138 ± 19*	−75 ± 26
ΔMean BP	−16 ± 3*	−31 ± 7*	−44 ± 6*[c]	−45 ± 12*
ΔRenal conductance	−24 ± 7*	−38 ± 9*	−32 ± 9*	−18 ± 4*
ΔMesenteric conductance	−77 ± 20*	−71 ± 13*	−24 ± 7[c]	16 ± 5[bd]
ΔHindquarters conductance	43 ± 8*	48 ± 8*	30 ± 7*	19 ± 2*[d]

Values are mean ± s.e.mean, $n = 8$ (all groups).
*$P < 0.05$ for change, [b]$P < 0.05$ Group 4 vs Group 3; [c]$P < 0.05$ Group 3 vs Group 1; [d]$P < 0.05$ Group 4 vs Group 2

Table 5 Cardiovascular changes (AUC or AOC, arbitrary units) over a 30 min period following administration of perindoprilat in the presence of saline (Group 1) or N^G-nitro-L-arginine methyl ester (L-NAME, Group 3), or captopril in the presence of saline (Group 2) or L-NAME (Group 4)

	Group 1 (Saline + perindoprilat)	*Group 2 (Saline + captopril)*	*Group 3 (L-NAME + perindoprilat)*	*Group 4 (L-NAME + captopril)*
ΔHeart rate	2089 ± 256*	1185 ± 269*[a]	1223 ± 269*[c]	652 ± 165*
ΔMean BP	−1022 ± 78*	−714 ± 67*[a]	−597 ± 59*[c]	−373 ± 64*[bd]
ΔRenal conductance	1081 ± 75*	859 ± 67*	997 ± 97*	435 ± 73*[bd]
ΔMesenteric conductance	1375 ± 145*	989 ± 65*[a]	871 ± 117*[c]	493 ± 106*[bd]
ΔHindquarters conductance	508 ± 64*	239 ± 48*[a]	212 ± 39*[c]	83 ± 17*[bd]

Values are mean ± s.e.mean, $n = 8$ (all groups).
*$P < 0.05$ for change; [a]$P < 0.05$ Group 2 vs Group 1; [b]$P < 0.05$ Group 4 vs Group 3; [c]$P < 0.05$ Group 3 vs Group 1; [d]$P < 0.05$ Group 4 vs Group 2

Table 6 Cardiovascular changes (AUC or AOC, arbitrary units) over a 30 min period following administration of N^G-nitro-L-arginine methyl ester (L-NAME) in the presence of saline and perindoprilat (Group 1), saline and captopril (Group 2) or alone (Groups 3 and 4)

	Group 1 ($n = 8$)	*Group 2 ($n = 8$)*	*Group 3 and Group 4 ($n = 16$)*
ΔHeart rate	−1706 ± 318*	−2100 ± 252*	−1284 ± 151*[b]
ΔMean BP	351 ± 51*	302 ± 63*	193 ± 23*[ab]
ΔRenal conductance	−291 ± 135*	−546 ± 99*	−222 ± 50*[b]
ΔMesenteric conductance	−1683 ± 296*	−1123 ± 303*	−535 ± 82*[ab]
ΔHindquarters conductance	−780 ± 151*	−779 ± 197*	−279 ± 33*[ab]

*$P < 0.05$ for change; [a]$P < 0.05$ Groups 3 and 4 vs Group 1; [b]$P < 0.05$ Group 3 and 4 vs Group 2

Discussion

The experimental design allowed us to study (1) the regional haemodynamic effects of perindoprilat and captopril and the influence of L-NAME thereon, (2) the effects of the ACE inhibitors, in the absence or presence of L-NAME, on the haemodynamic responses to vasodilators with differing degrees of 'endothelial dependence', and, incidentally, (3) the

effects of L-NAME in the absence and presence of ACE inhibition. The following discussion is divided into the corresponding sections.

Effects of perindoprilat or captopril

Although, during infusion of saline, both perindoprilat and captopril caused marked hypotension, tachycardia and increases in renal and mesenteric blood flow, and renal, mesenteric and hindquarters vascular conductances, all effects (except the renal vasodilatation) were greater with perindoprilat than with captopril. Hence, any interaction between captopril and NO-mediated events (Goldschmidt & Tallarida, 1991) did not confer any enhanced vasodilator ability on captopril. Indeed, it appeared that the vasodilator effects of perindoprilat were better maintained than those of captopril and this was particularly true in the presence of L-NAME (Figure 2). Under these conditions, all the haemodynamic effects of captopril were significantly less than those of perindoprilat and, proportionately, were more reduced than were those of perindoprilat, relative to the respective responses in the presence of saline. In fact, the renal vasodilator effect of perindoprilat was not significantly affected by L-NAME, although the mesenteric and hindquarters vasodilatations were. Thus, it appears that the renal vasodilator effects of perindoprilat are independent of NO, although NO may contribute to its mesenteric and hindquarters vasodilator effects, but to a lesser extent than with captopril.

The lack of an effect of L-NAME on the renal hyperaemic vasodilator action of perindoprilat is particularly striking, since it would be expected that the increase in the renal blood flow, itself, might have stimulated NO release (Hutcheson & Griffith, 1991), and Haji-ali & Zimmerman (1992) have reported that the renal hyperaemic vasodilator effects of the non-sulphydryl ACE inhibitor, lisinopril, are inhibited by N^G-nitro-L-arginine. Whatever the explanation of our results, they indicate that perindoprilat could be capable of promoting renal blood flow in the presence of impaired endothelial function, when the renal haemodynamic effects of other ACE inhibitors might be compromised.

Recently, Cachofeiro *et al.* (1992) reported that the NO synthesis inhibitor, N^G-monomethyl-L-arginine (L-NMMA), attenuated hypotensive responses to captopril, ramiprilat or the nonpeptide AT_1-receptor antagonist, losartan, in spontaneously hypertensive rats. They suggested this was not a non-specific effect, since hypotensive responses to sodium nitroprusside were not changed. However, Cachofeiro *et al.* (1992) found that the responses to sodium nitroprusside were enhanced by L-NMMA in normotensive rats, and thus the lack of change in the hypertensive animals could have represented an abnormality of the sensitization that usually occurs to nitrovasodilators following NO synthesis inhibition (Moncada *et al.*, 1991; Gardiner *et al.*, 1991a). Nevertheless, our present results, showing a diminished hypotensive response to perindoprilat or captopril in the presence of L-NAME, corroborates the finding of Cachofeiro *et al.* (1992), and extends it by demonstrating that different haemodynamic effects underlie this event in the case of the perindoprilat and captopril.

It is feasible that the haemodynamic effects of ACE inhibitors are contributed to by inhibition of degradation of endogenous BK (e.g. Wiemer *et al.*, 1991; Cachofeiro *et al.*, 1992). However, in water-deprived, Brattleboro rats, captopril is devoid of any haemodynamic effects if it is administered in the presence of losartan (Batin *et al.*, 1991a,b), indicating that ACE inhibition has no additional consequences in this circumstance. Moreover, in the present work, the complex profile of effects of exogenous BK indicates that accumulation of endogenous BK following ACE inhibition could not, alone, explain the haemodynamic effects of perindoprilat or captopril.

Effects of vasodilators

As reported elsewhere (Gardiner *et al.*, 1990c; 1991a,b; 1992a,b) we observed that ACh caused renal hyperaemia, whereas lemakalim elicited mesenteric hyperaemia and BK caused an initial mesenteric, followed by hindquarters, hyperaemia. In those vascular beds in which flow increases did not occur, any change in vascular conductance which was associated with a maintenance, or relative maintenance of flow, could have been autoregulatory. Clearly, in those instances where flow fell in association with a reduction in vascular conductance there was an active vasoconstriction that may have been direct and/or indirect (reflex or otherwise) in origin.

Acetylcholine: Although there was an indication of desensitization to ACh with repeated infusions (cf. responses to ACh alone compared to responses to ACh in the presence of saline, Table 2), L-NAME caused clear-cut, and almost total, inhibition of the renal haemodynamic effects of ACh (Figure 2, Table 2). This effect was much more dramatic than we have previously seen with acute L-NAME treatment in Long Evans rats (Gardiner *et al.*, 1990c; 1991a), although in those instances the animals were normovolaemic. However, from previous experiments on Brattleboro rats with isosmotic hypovolaemia induced by s.c. injection of PEG (Gardiner *et al.*, 1989), or hyperosmotic hypovolaemia induced by water deprivation (Gardiner *et al.*, 1988), it appears that the renal circulation is relatively well preserved and in the present work the renal vasodilator effects of ACh were not substantially different from those seen in Long Evans or Brattleboro rats under normovolaemic conditions (Gardiner *et al.*, 1991a; 1992b). Thus, the susceptibility to L-NAME of the renal haemodynamic effects of ACh in the present experiments is not likely to have been due to factors such as elevated renal vasomotor tone or impaired renal perfusion, but was probably accounted for by the infusion of a higher dose of L-NAME than in previous studies (Gardiner *et al.*, 1991a).

In the presence of saline and perindoprilat, the renal hyperaemic vasodilator effect of ACh was abolished (as in the presence of L-NAME). At first sight, it thus appears that perindoprilat has a potent inhibitory effect on NO-mediated renal haemodynamic changes. However, it should be noted that, in the experimental model employed, perindoprilat itself caused marked hypotension and hyperaemic renal vasodilatation (Figure 1, Table 5). Hence, prior to ACh infusion, systemic and renal haemodynamics were markedly different from baseline, and the lack of response to ACh could have been due to the renal haemodynamic variables being at maximal levels. However, this is not likely to be a complete explanation, since there was a renal vasodilator response to ACh in the presence of saline and captopril and this was not seen in the presence of L-NAME and captopril (Figure 2, Table 2). Thus, these findings indicate the renal vasodilator effects of ACh are NO-dependent, and they are relatively less diminished in the presence of captopril than of perindoprilat.

There are few data available relating to the effects of perindoprilat on endothelium-dependent vasorelaxations in response to ACh, and what data there are show regional heterogeneity. For example, Kerth & Vanhoutte (1991) reported that, in endothelium-intact ring preparations of the left anterior descending coronary artery of the dog, precontracted with prostaglandin $F_{2\alpha}$ and pretreated with indomethacin, the concentration-dependent relaxations evoked by ACh, BK or thrombin were enhanced by perindoprilat. However, perindoprilat was without effect on the ACh or thrombin-induced, endothelium-dependent, relaxation of rings of canine femoral arteries. Moreover, perindoprilat was devoid of any direct effect on vascular smooth muscle and did not stimulate the release of endothelium-derived relaxing factor(s). The fact that Kerth & Vanhoutte (1991) did not observe any inhibitory effects of perindoprilat on endothelium-dependent vasorelaxation might indicate that

the effects reported here were indirect rather than direct.

As indicated above, the hindquarters vasodilator effect of ACh could have been autoregulatory, consistent with its being unchanged under any experimental condition. In contrast, ACh caused a variable mesenteric vasoconstriction (possibly reflex in origin), both in the presence of saline and of L-NAME; thus, it appears that the effects of ACh in the mesenteric vasculature under these condiitons were not modulated by NO. However, when ACh was given in the presence of saline, and either of the ACE inhibitors, there was mesenteric vasodilatation. While we cannot dismiss the possibility that this vasodilatation was autoregulatory (since there was no increase in flow), it is notable that it did not occur in the presence of L-NAME and hence it is likely that NO contributed to the effect. If this were the case, then it appears that any NO-mediated vasodilator effects of ACh in the mesenteric vascular bed are not inhibited by perindoprilat in the same way as the NO-mediated effects of ACh in the kidney appear to be, at least in PEG-treated, Brattleboro rats.

Lemakalim: In our earlier experiments (Gardiner *et al.*, 1991b) we had considered the use of lemakalim as an internal reference to control for the haemodynamic effects of L-NAME itself, acknowledging problems resulting from the development of supersensitivity to nitrovasodilators following inhibition of NO synthase (Moncada *et al.*, 1991; Gardiner *et al.*, 1991a). However, we also pointed out difficulties of interpretation of responses to 'enothelium-independent' vasodilators *in vivo* (Gardiner *et al.*, 1991b). Indeed, one could argue that the haemodynamic effects of any vasoactive substance *in vivo* cannot be endothelium-independent, since, even if its primary action was not on endothelial cells, any changes in haemodynamics it caused would influence endothelial function through changes in shear forces and pulsatility (Hutcheson & Griffith, 1991). That being said, the present results indicate the absolute responses to lemakalim were not affected under any experimental condition, in spite of marked changes in baseline haemodynamics at various stages of the protocols. Unfortunately, lemakalim does not cause renal hyperaemia, and hence its effects do not provide a particularly useful comparator for those of ACh.

Bradykinin: Although BK is generally to be considered an 'endothelium-dependent' vasodilator, it has complex effects *in vivo* involving direct and indirect vasodilator and vasoconstrictor actions (Gardiner *et al.*, 1990c; 1992a; Fasciolo *et al.*, 1990; Cowan & Cohen, 1992). Initially, we had intended to administer BK by 3 min infusion but in pilot experiments we found that this intervention, in the presence of perindoprilat or captopril, caused irreversible cardiovascular deterioration. Therefore, we decided to administer BK by bolus injection at a lower dose than we have used previously (Gardiner *et al.*, 1990c; 1992a), since the PEG-treated Brattleboro rats were particularly susceptible to its hypotensive effects in the presence of ACE inhibitors. The tendency towards hypotension, and the tachycardic and mesenteric and hindquarters vasodilator effects we saw with this low dose of BK were generally similar to those observed with higher doses of BK previously (Gardiner *et al.*, 1990c; 1992a). However, the modest hindquarters vasodilator effect of BK was unaffected by L-NAME. Whilst it is feasible that this effect of BK may differ between Brattleboro and Long Evans rats (since, in the latter, L-NAME inhibits the hindquarters vasodilator effect of BK, Gardiner *et al.*, 1990c), it is also possible that any NO-mediated effects of BK in the hindquarters of PEG-treated Brattleboro rats were offset by activation of sympathetic efferent tone supported by the renin-angiotensin system (Gardiner & Bennett, 1986; Gardiner *et al.*, 1989). Furthermore, we cannot preclude the possibility that a higher dose of BK would have exerted some hindquarters vasodilator effect involving NO (see below).

A relative lack of effect of L-NAME on the mesenteric vasodilator action of BK is consistent with our previous findings (Gardiner *et al.*, 1990c; 1992a), and indicates that NO-independent mechanisms may be involved in this phenomenon (e.g. Cowan & Cohen, 1992).

In the presence of saline and perindoprilat, or saline and captopril, BK caused significant hypotension and renal and mesenteric vasoconstriction, accompanied by augmented hindquarters vasodilatation. While the latter, and the hypotensive effects of BK under these conditions, are entirely consistent with enhancement of the effects of BK, due to inhibition of its degradation by the ACE inhibitors, the explanation of the renal and mesenteric vasoconstrictions is less straightforward, particularly since these effects were associated with such clear reductions in flow. It is feasible these responses were an amalgam of the vasoconstrictor effects of BK (Fasciolo *et al.*, 1990) together with indirect actions and reflex vasoconstriction in response to the hypotension. However, additional factors must have been involved since, in the presence of L-NAME and perindoprilat, or L-NAME and captopril, the hypotensive effect of BK was much greater than in the absence of L-NAME, yet the mesenteric vasoconstriction was less (perindoprilat) or absent (captopril). At first sight this is paradoxical, since there is evidence for involvement of NO in the hypotensive and other vasodilator effects of BK (Gardiner *et al.*, 1990c; 1992a); indeed, consistent with this, the hindquarters vasodilator effect of BK was less in the presence of L-NAME and captopril than in the presence of captopril and saline (Table 4). One possibility is that, in the presence of L-NAME and perindoprilat, or L-NAME and captopril, there was a marked coronary vasoconstrictor effect of BK and this resulted in a fall in cardiac output which amplified the hypotension. It is clear there was an unusual interaction between BK and the heart in the presence of the ACE inhibitors, because profound bradycardia, rather than the usual tachycardia, was seen. However, the bradycardia itself was not responsible for the augmented hypotensive response to BK in the presence of L-NAME and the ACE inhibitors, because a similar bradycardic effect was seen in the absence of L-NAME (Table 4).

Consistent with the influence of captopril on the effects of ACh in the renal vascular bed, it appeared that the ability of captopril to enhance the hindquarters vasodilator action of BK was dependent on a substantial L-NAME-sensitive component (Table 4). In contrast, the augmentation by perindoprilat of the hindquarters vasodilator effect of BK was not significantly affected by L-NAME. It does not seem likely that the difference between perindoprilat and captopril in this regard can be explained by different degrees of BK accumulation, due to differential extents of local ACE inhibition, since the hindquarters vasodilator response to BK was the same in the presence of saline and perindoprilat as in the presence of saline and captopril.

Effects of L-NAME

Similar to its effects in animals under normal conditions (Gardiner *et al.*, 1990b), L-NAME caused hypertension and bradycardia in association with renal, mesenteric and hindquarters vasoconstrictions in PEG-treated, Brattleboro rats. Interestingly, the pressor and mesenteric and hindquarters vasoconstrictor effects of L-NAME were augmented in the presence of perindoprilat or captopril, consistent with the mesenteric and hindquarters vasodilator effects of the ACE inhibitors being dependent, to an extent, on NO (see above). Furthermore, the similar renal vasoconstrictor effect of L-NAME in the presence of saline or perindoprilat, compared to the enhanced renal vasoconstrictor effect of L-NAME in the presence of captopril (Table 6), supports the proposition that the renal vasodilator effects of the latter involve NO, whereas those of perindoprilat do not. The greater effects of L-NAME in the presence of ACE inhibition indicate that the renin-angiotensin system is not involved indispensibly in the

systemic pressor or regional haemodynamic responses to L-NAME in PEG-treated Brattleboro rats, consistent with findings in normovolaemic Long Evans rats (Gardiner *et al.*, 1990c). However, as noted earlier, in the presence of the ACE inhibitors, mean arterial blood pressure was markedly reduced and there were substantial elevations in renal, mesenteric and hindquarters vascular conductances; hence, these changes in baseline status could have affected the absolute changes in cardiovascular variables evoked by L-NAME, but this does not explain why the renal vasoconstrictor effects of L-NAME were unchanged in the presence of perindoprilat. Thus, it is more likely that all the other effects of L-NAME were enhanced in the presence of the ACE inhibitors due to the latter augmenting NO-dependent mechanisms. While it is feasible that such an interaction could occur at the level of the endothelial cells, through a direct influence on release and/or inactivation of NO, the haemodynamic response to the ACE inhibitors might have enhanced NO release through changes in the physical forces acting on the endothelial cells. However, as mentioned earlier, it is not clear why such a phenomenon should not be apparent in the renal hyperaemic vasodilator effect of perindoprilat.

In conclusion, in the PEG-treated, Brattleboro rat, perindoprilat exerts more marked and sustained hypotensive, and hyperaemic vasodilator effects in mesenteric and hindquarters vascular beds than does captopril, in spite of the effects of the latter showing more dependence on NO-mediated processes. However, both ACE inhibitors appear to inhibit ACh-induced renal hyperaemic vasodilatations (perindoprilat significantly more so than captopril), but whether or not this is a direct effect, and the extent to which endogenous BK is involved in the haemodynamic actions of perindoprilat and captopril remain to be determined.

This work was supported by Institut de Recherches Internationales Servier. We are grateful to Drs Chloe Brown and Yves Joulin for their constructive comments.

References

BATIN, P., GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1991a). Differential regional haemodynamic effects of the non-peptide angiotensin II antagonist, DuP 753, in water-replete and water-deprived Brattleboro rats. *Life Sci.*, **48**, 733–739.

BATIN, P., GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1991b). Cardiac haemodynamic effects of the non-peptide, angiotensin II-receptor antagonist, DuP 753, in conscious Long Evans and Brattleboro rats. *Br. J. Pharmacol.*, **103**, 1585–1591.

CACHOFEIRO, V., SAKAKIBARA, T. & NASJLETTI, A. (1992). Kinins, nitric oxide, and the hypotensive effect of captopril and ramiprilat in hypertension. *Hypertension*, **19**, 138–145.

CLOZEL, M. (1991). Mechanism of action of angiotensin converting enzyme inhibitors on endothelial function in hypertension. *Hypertension*, **18** (suppl. II), II-37–II-42.

COWAN, C.L. & COHEN, R.A. (1992). Different mechanisms of relaxation of pig coronary artery to bradykinin and cromakalim are distinguished by potassium channel blockers. *J. Pharmacol. Exp. Ther.*, **260**, 248–253.

FASCIOLO, J.C., VARGAS, L., LAMA, M.C. & NOLLY, H. (1990). Bradykinin-induced vasoconstriction of rat mesenteric arteries precontracted with noradrenaline. *Br. J. Pharmacol.*, **101**, 344–348.

GARDINER, S.M. & BENNETT, T. (1985). Interactions between neural mechanisms, the renin-angiotensin system and vasopressin in the maintenance of blood pressure during water deprivation: studies in Long Evans and Brattleboro rats. *Clin. Sci.*, **68**, 647–657.

GARDINER, S.M. & BENNETT, T. (1986). Pressor contributions from angiotensin and vasopressin after polyethylene glycol. *Am. J. Physiol.*, **251**, R769–R774.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1988). Regional hemodynamic effects of atrial natriuretic peptide or captopril in Brattleboro rats. *Am. J. Physiol.*, **255**, R737–R743.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1989). Regional hemodynamic changes following hypovolemia in conscious rats. *Am. J. Physiol.*, **256**, R1076–R1083.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1990a). Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension*, **15**, 486–492.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990b). Regional and cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats. *Br. J. Pharmacol.*, **101**, 625–631.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990c). Regional and cardiac haemodynamic responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in conscious rats: effects of N^G-nitro-L-arginine methyl ester. *Br. J. Pharmacol.*, **101**, 632–639.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1991a). Effects of N^G-nitro-L-arginine methyl ester on vasodilator responses to acetylcholine, 5'-N-ethylcarboxamidoadenosine or salbutamol in conscious rats. *Br. J. Pharmacol.*, **103**, 1725–1732.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1991b). Effects of N^G-nitro-L-arginine methyl ester on vasodilator responses to adrenaline or BRL 38227 in conscious rats. *Br. J. Pharmacol.*, **104**, 731–737.

GARDINER, S.M., KEMP, P.A., BENNETT, T., BOSE, C., FOULKES, R. & HUGHES, B. (1992a). Involvement of β_2-adrenoceptors in the regional haemodynamic responses to bradykinin in conscious rats. *Br. J. Pharmacol.*, **105**, 839–848.

GARDINER, S.M., KEMP, P.A., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1992b). Renal vasodilator responses to acetylcholine in Brattleboro rats before, during and after chronic oral ingestion of N^G-monomethyl-L-arginine (L-NMMA). *Br. J. Pharmacol.*, **105**, 93P.

GOLDSCHMIDT, J.E. & TALLARIDA, R.J. (1991). Pharmacological evidence that captopril possesses an endothelium-mediated component of vasodilation: effect of sulfhydryl groups on endothelium-derived relaxing factor. *J. Pharmacol. Exp. Ther.*, **257**, 1136–1145.

HAJJ-ALI, A.F. & ZIMMERMAN, B.G. (1992). Nitric oxide participation in renal hemodynamic effect of angiotensin converting enzyme inhibitor lisinopril. *Eur. J. Pharmacol.*, **212**, 279–281.

HAYWOOD, J.R., SHAFFER, R., FASTENOW, C., FINK, G.D. & BRODY, M.J. (1981). Regional blood flow measurement with pulsed Doppler flowmeter in conscious rat. *Am. J. Physiol.*, **241**, H273–H278.

HENRION, D., CHILLON, J.M., CAPDEVILLE-ATKINSON, C. & ATKINSON, J. (1991). Treatment with the converting enzyme inhibitor, perindopril, protects endothelial function in a rat model of calcium overload. *Br. J. Pharmacol.*, **102**, 326P.

HUTCHESON, I.R. & GRIFFITH, T.M. (1991). Release of endothelium-derived relaxing factor is modulated both by frequency and amplitude of pulsatile flow. *Am. J. Physiol.*, **261**, H257–H262.

ILLIANO, S.C., MOMBOULI, J.V., NAGAO, T. & VANHOUTTE, P.M. (1991). Converting enzyme inhibitors potentiate hyperpolarizations and nitric oxide-independent relaxations induced by bradykinin. *FASEB J.*, **5**, A1727.

KERTH, P.A. & VANHOUTTE, P.M. (1991). Effects of perindoprilat on endothelium-dependent relaxation and contractions in isolated blood vessels. *Am. J. Hypertens.*, **4**, 226S–234S.

MOMBOULI, J.-V., NEPTHALI, M. & VANHOUTTE, P.M. (1991). Effects of the converting enzyme inhibitor cilazeprilat on endothelium-dependent responses. *Hypertension*, **18** (suppl. II), II-22–II-29.

MOMBOULI, J.-V. & VANHOUTTE, P.M. (1991). Perindoprilat amplifies endothelium-dependent relaxations to bradykinin and unmasks those to kininogen. *FASEB J.*, **5**, A401.

MONCADA, S., REES, D.D., SCHULZ, R. & PALMER, R.M.J. (1991). Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 2166–2170.

MULLER, A.F., GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1990). Regional haemodynamic effects of captopril, enalaprilat and lisinopril in conscious water-replete and water-deprived Brattleboro rats. *Clin. Sci.*, **79**, 393–401.

THEODORSSON-NORHEIM, E. (1987). Friedman and Quade tests: BASIC computer program to perform non-parametric two-way analysis of variance and multiple comparisons on ranks of several related samples. *Comput. Biol. Med.*, **17**, 85–89.

TOMLINSON, K.C., GARDINER, S.M. & BENNETT, T. (1990). Hypotensive effects of angiotensin II analogues and angiotensin converting enzyme inhibitors in water-deprived Brattleboro rats. *J. Cardiovasc. Pharmacol.*, **15**, 562–568.

WIEMER, G., SCHÖLKENS, B.A., BECKER, R.H.A. & BUSSE, R. (1991). Ramiprilat enhances endothelial autacoid formation by inhibiting breakdown of endothelium-derived bradykinin. *Hypertension*, **18**, 558–563.

(Received June 11, 1992
Revised August 10,1992
Accepted August 17,1992)

Br. J. Pharmacol. (1992), **107**, 1192–1197

Palmitoyl-DL-carnitine has calcium-dependent effects on cultured neurones from rat dorsal root ganglia

*[1]Simon R. Stapleton, Kevin P.M. Currie, Roderick H. Scott & *B. Anthony Bell

Department of Physiology and *Division of Clinical Neuroscience, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE

1 The effects of palmitoyl-DL-carnitine (0.01 to 1 mM) on whole cell voltage-activated calcium channel currents carried by calcium or barium and Ca^{2+}-activated chloride currents were studied in cultured neurones from rat dorsal root ganglia.

2 Palmitoyl-DL-carnitine applied to the extracellular environment or intracellularly via the patch solution reduced Ca^{2+} currents activated over a wide voltage range from a holding potential of −90 mV. Inhibition of high voltage activated Ca^{2+} channel currents was dependent on intracellular Ca^{2+} buffering and was reduced by increasing the EGTA concentration from 2 to 10 mM in the patch solution. Barium currents were significantly less sensitive to palmitoyl-DL-carnitine than Ca^{2+} currents.

3 The amplitude of Ca^{2+}-activated Cl^- tail currents was reduced by palmitoyl-DL-carnitine. However, the duration of these Cl^- currents was greatly prolonged by palmitoyl-DL-carnitine, suggesting slower removal of free Ca^{2+} from the cytoplasm following Ca^{2+} entry through voltage-activated channels.

4 Palmitoyl-DL-carnitine evoked Ca^{2+}-dependent inward currents which could be promoted by activation of the residual voltage-activated Ca^{2+} currents and attenuated by intracellular application of EGTA.

5 We conclude that palmitoyl-DL-carnitine reduced the efficiency of intracellular Ca^{2+} handling in cultured dorsal root ganglion neurones and resulted in enhancement of Ca^{2+}-dependent events including inactivation of voltage-activated Ca^{2+} currents. The activation of inward currents by palmitoyl-DL-carnitine may involve Ca^{2+}-induced Ca^{2+} release from intracellular stores, or direct interaction of palmitoyl-DL-carnitine with Ca^{2+} stores.

Keywords: Voltage-activated calcium currents; chloride currents; lipid metabolites; intracellular calcium

Introduction

The loss of the ability of certain cells to maintain low intracellular calcium ion concentrations ($[Ca^{2+}]_i$) while still allowing Ca^{2+} influx for the activation of essential cellular processes may underlie a mechanism of neuronal damage produced by hypoxic or ischaemic insults. Alterations in Ca^{2+} entry through voltage-activated Ca^{2+} channels is one potential mechanism whereby such deleterious changes in Ca^{2+} homeostasis may occur during hypoxia or ischaemia (Choi, 1988).

Cellular metabolites produced in excess during ischaemia (Liedtke *et al.*, 1978) may alter calcium homeostasis by interaction with mechanisms of calcium regulation within the cell or its membranes. These mechanisms include Na^+/Ca^{2+} exchange, ATP-dependent Ca^{2+} pumps, Ca^{2+} binding proteins and intracellular Ca^{2+} storage organelles (McBurney & Neering, 1987). Such metabolites include the long chain acyl carnitines, intermediaries in the transport of fatty acids into mitochondria prior to β-oxidation. During ischaemia, mitochondrial β-oxidation is blocked with the subsequent accumulation of cytosolic fatty acyl carnitines including the palmitoyl derivative. As a result of myocardial ischaemia, levels of palmitoyl carnitine in the sarcolemma may rise 70 fold. This is associated with impaired inotropic function of the heart and the production of dysrhythmias (Knabb *et al.*, 1986). Palmitoyl carnitine itself has been demonstrated to inhibit the Na^+/K^+-ATPase and reduce [^{3}H]-ouabain binding in canine ventricular muscle (Adams *et al.*, 1979). The Ca^{2+}-ATPase and Ca^{2+} binding to sarcolemmal membranes are also inhibited by palmitoyl carnitine at 50–200 μM concentrations (Pitts *et al.*, 1978; Adams *et al.*, 1979). Further, Inoue & Pappano (1983) demonstrated the similarity of the effects of palmitoyl carnitine and those of elevated extracellular Ca^{2+} concentration in chick ventricular muscle in increasing the maximal amplitude and prolonging the Ca^{2+}-dependent action potential. Palmitoyl carnitine produced during ischaemia may act as an endogenous voltage-activated Ca^{2+} channel activator thus mediating some of the Ca^{2+} overload associated with myocardial ischaemia (Spedding & Mir, 1987; Patmore *et al.*, 1989). The effects of palmitoyl carnitine were shown not to be restricted to smooth muscle and cardiac myocyte preparations; selective interactions with brain cortical membranes have also been investigated. Palmitoyl carnitine inhibited binding of the Ca^{2+} channel ligands nitrendipine, verapamil and diltiazem to rat brain cortical membranes suggesting an interaction with the neuronal voltage-activated Ca^{2+} channels (Spedding & Mir, 1987). Although the metabolism of fatty acids is not a major energy source in the normal brain, (Bird *et al.*, 1985) abnormalities of lipid metabolism do occur during severe ischaemia (Gardiner *et al.*, 1981). Furthermore palmitoyl carnitine derived from non-neuronal ischaemic tissues may affect neuronal activity.

Inhibition of neuronal voltage-activated Ca^{2+} channels may have a role in offering some protection against the damage caused by cerebral ischaemia. We have studied the mechanism of action of palmitoyl carnitine on voltage-activated Ca^{2+} channel currents and Ca^{2+}-activated currents in cultured rat dorsal root ganglion neurones to gain an understanding of the possible modes of action of the lipid and its potential role in mediating neuronal damage. A preliminary account of this work has previously been communicated (Scott *et al.*, 1992a).

[1] Author for correspondence at: Department of Physiology, St George's Hospital Medical School, Cranmer Terrace, London, SW17 0RE

Methods

Primary cell cultures

Dorsal root ganglion (DRG) neurones were obtained from 2 day old Wistar rats and following dissociation the cells were plated on laminin-polyornithine coated cover-slips and maintained in culture for 2–3 weeks at 37°C in humidified air containing 5% CO_2. F14 culture medium (Imperial Laboratories), supplemented with 10% heat-inactivated horse serum (GIBCO), nerve growth factor (Sigma), penicillin and streptomycin (Flow Laboratories), was used.

Electrophysiology

Voltage-activated Ca^{2+} currents and Ca^{2+}-activated currents were studied by the whole-cell variant of the patch-clamp technique (Hamill *et al.*, 1981). Cells were voltage-clamped by use of an Axoclamp-2A switching amplifier, sampling at 28–35 kHz, or an Axopatch 1D, with 70–80% series resistance compensation. Low resistance (3–7 MΩ) borosilicate glass micropipettes were used in this study. Recording medium contained (in mM): choline chloride 130, KCl 3.0, $MgCl_2$ 0.6, $NaHCO_3$ 1.0, HEPES 10, glucose 4.0, tetraethylammonium (TEA) bromide 25, tetrodotoxin (TTX, Sigma) 0.0025 and $CaCl_2$ or $BaCl_2$ 2. The pH and osmolarity were adjusted to 7.4 and 320 mOsM with NaOH and sucrose respectively. Choline chloride based recording medium was used to prevent any contribution from TTX-insensitive sodium currents. Patch pipettes were filled with a solution containing (in mM): CsCl or Cs acetate 140, $MgCl_2$ 2.0, Na-ATP 2.0, HEPES 10, $CaCl_2$ 0.1 and EGTA 2 or 10 to give $[Ca^{2+}]_i$ of 8.4 or 1.6 nM respectively. The pH was adjusted with Tris to 7.2 and the osmolarity to 310 mOsM with sucrose. Stock solutions of 10 mM to 1 mM palmitoyl-DL-carnitine (PC) (Sigma) were prepared freshly each day by dissolving in recording medium or patch solution. The pH was subsequently adjusted with NaOH to 7.2 or 7.4 accordingly and to assure complete dissolution. When appropriate, dilution of the stock solution was carried out as soon as the PC was completely dissolved. Palmitoyl-DL-carnitine was applied extracellularly by low pressure ejection from a pipette (tip diameter approx. 10 μm) placed within 100 μm of the cell being recorded. Palmitoyl-DL-carnitine was applied to the intracellular environment by inclusion in the patch pipette solution.

All electrophysiological recordings were stored on a digital audio tape recorder (Biologic) and analysed with Cambridge Electronic Design computer software. Voltage-activated Ca^{2+} and Ba^{2+} currents were obtained after scaled linear subtraction of leakage and capacitance currents. All data are given as mean ± standard error of mean (s.e.mean). Statistical significance was assessed by use of a 'two-way' Student's *t* test; paired or unpaired when appropriate.

Results

Actions of palmitoyl-DL-carnitine on voltage-activated Ca^{2+} channel currents

Extracellular application of 1 mM PC inhibited voltage-activated Ca^{2+} currents (I_{Ca}) activated over a wide voltage-range; − 30 mV to + 100 mV (Figure 1). The maximum inward high voltage-activated I_{Ca} evoked by depolarizing voltage step commands from − 90 mV to 0 mV were inhibited by extracellular application of PC (1 mM); only modest recovery, usually less than 25% was observed 5 to 10 min after removal of the pressure ejection pipette containing the lipid. The mean peak amplitude of the control high voltage-activated I_{Ca} was − 1.63 ± 0.15 nA and this current inactivated to − 0.91 ± 0.13 nA ($n = 9$) by the end of a 100 ms voltage step command. After 3 to 5 min application of 1 mM

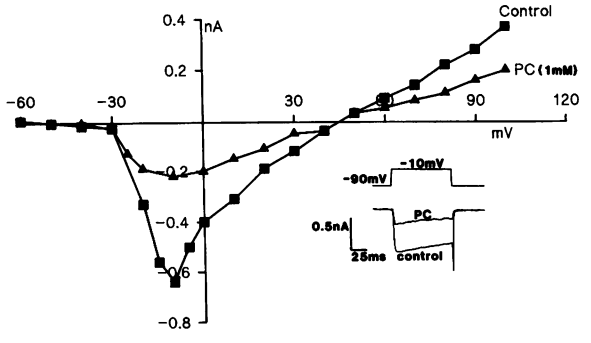

Figure 1 Current-voltage relationship showing that palmitoyl-DL-carnitine (PC, 1 mM) reduced Ca^{2+} currents activated over a wide voltage range, in a cell voltage-clamped at a holding potential of − 90 mV. Both inward Ca^{2+} currents activated at clamp potentials between − 30 mV and + 40 mV, (this cell had no low threshold T-type current) and outward Ca^{2+} channel currents activated at potentials positive to the null potential of + 45 mV were inhibited. PC (1 mM) had no effect on the null potential, (the voltage at which no net current flows through Ca^{2+} channels). The graphs show control data (■) and data generated after 3 to 5 min application of 1 mM PC (▲). The inset traces show the inhibition of the maximum inward Ca^{2+} current activated by 100 ms voltage step commands to − 10 mV.

PC, the mean peak amplitude of I_{Ca} was significantly reduced to − 1.12 ± 0.12 nA and this current inactivated to − 0.33 ± 0.15 nA ($n = 9$; $P < 0.01$ and $P < 0.001$ for inhibition of peak and end current respectively). The I_{Ca} measured at the end of the voltage step command was inhibited to a greater extent by PC (1 mM) compared with the peak I_{Ca} measured at the beginning of the voltage step. This is reflected by the increased inactivation of I_{Ca} which was 43 ± 7% under control conditions and 71 ± 10% ($n = 9$) in the presence of PC (1 mM). Outward Ca^{2+} channel currents carried by monovalent ions and activated by voltage step commands to potentials positive to the null potential were inhibited by 1 mM PC (Figure 1). Additionally the low threshold T-type I_{Ca} was also attenuated by 1 mM PC (Figure 2). Extracellular application of 100 μM PC for 5 min was less effective than 1 mM PC and inhibited the peak I_{Ca} and end I_{Ca} by 23 ± 11% and 23 ± 10% respectively. Lower concentrations of PC (1 μM and 10 μM) applied extracellularly had no significant action on I_{Ca} ($n = 2$ and 4 respectively). In 2 cases PC (10 μM and 100 μM) caused transient increases in high voltage-activated I_{Ca} in the first minute of PC application; however, this observation was not found consistently.

The inhibitory actions of PC (1 mM) applied extracellularly on high voltage-activated Ca^{2+} channel currents were dependent on intracellular Ca^{2+} buffering and divalent cation charge carrier. Increasing the concentration of the Ca^{2+} chelator, EGTA, in the patch solution from 2 mM to 10 mM reduced the free Ca^{2+} concentration from 8.4 nM to 1.6 nM and increased the Ca^{2+} buffering capacity of the solution. Palmitoyl carnitine (1 mM) was significantly less effective when applied to cells which were recorded from using a patch solution with 10 mM EGTA and with 2 mM Ca^{2+} in the extracellular environment. Similarly, changing extracellular Ca^{2+} for 2 mM Ba^{2+} also reduced the inhibitory actions of PC (1 mM) (Table 1, Figure 2).

Studies were also carried out on the effects of intracellular application of PC (10 μM and 100 μM) which was applied via the patch solution. Palmitoyl carnitine (10 μM) had no significant effect on high voltage-activated inward I_{Ca}. In contrast, 100 μM PC reduced peak and end I_{Ca} over 6 min (Table 2). However, data were gathered for only 6 min because intracellular PC (100 μM) gave rise to unstable recordings (see below). Control data showed that I_{Ca} did not significantly run down during 20 min of recording ($n = 5$).

Actions of palmitoyl-DL-carnitine on Ca^{2+}-activated Cl^- tail currents

Tail currents which were predominantly due to Ca^{2+}-activated Cl^- currents were observed as slowly decaying inward currents in a proportion of DRG neurones loaded with CsCl patch solution (Currie & Scott, 1992). In 5 cells, extracellular application of 1 mM PC in addition to reducing Ca^{2+} influx through voltage-activated Ca^{2+} channels, also reduced in all cases the amplitude of the accompanying Ca^{2+}-activated Cl^- tail currents measured 20 ms after the end of the voltage step command by 29 ± 5%. The mean amplitude of the Cl^- tail current measured 20 ms after the end of the voltage step command was reduced from − 0.98 ± 0.27 nA to − 0.69 ± 0.20 nA, (n = 5) by PC (1 mM). However in 4 out of 5 cells the decay of the Cl^- tail currents were greatly slowed by extracellular application of PC (1 mM) (Figure 3). The mean time for the Cl^- tail currents to decay by 63% of the maximum current was increased from 1390 ± 580 ms to approximately 3600 ms (n = 5) after 3 min application of PC (1 mM). The time courses of the very prolonged Cl^- tail currents were difficult to measure accurately thus limiting quantification, although they did decay completely (Scott *et al.*, 1992a).

Similar observations were made when comparing Cl^- tail currents activated in cells with 10 μM PC applied via the patch solution to the intracellular environment. Intracellular

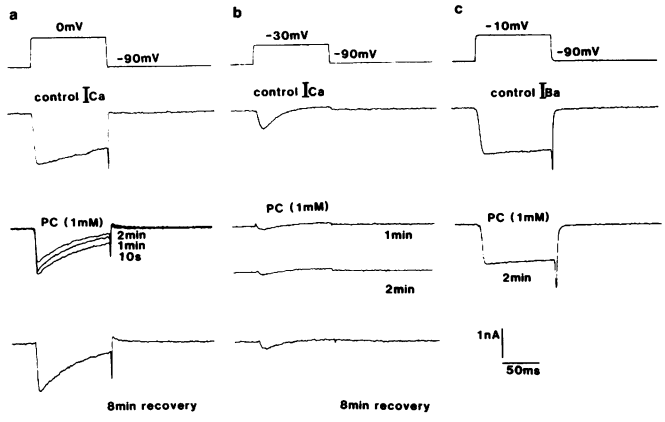

Figure 2 Palmitoyl-DL-carnitine (PC, 1 mM) inhibits voltage-activated Ca^{2+} channel currents carried by Ca^{2+} more effectively than currents carried by Ba^{2+}. (a) Traces showing inhibition of the high voltage-activated Ca^{2+} current during 10 s, 1 and 2 min application of 1 mM PC. Note the accelerated inactivation of the Ca^{2+} current in the presence of PC. (b) Traces showing inhibition of low voltage-activated T-type Ca^{2+} current by 1 and 2 min application of 1 mM PC. The predominant action is inhibition of the peak T-type Ca^{2+} current. Traces in (a) and (b) are from the same cell. Modest recovery of high voltage-activated and T-type Ca^{2+} current 8 min after removing the pipette containing PC is also illustrated. (c) Traces showing the modest inhibition of the high voltage-activated Ba^{2+} current by 2 min application of PC (1 mM). Note that PC (1 mM) produced no change in Ba^{2+} current inactivation.

Table 1 Percentage inhibition of high voltage-activated Ca^{2+} channel currents by extracellular palmitoyl-DL-carnitine (PC, 1 mM)

	2 mM$[Ca^{2+}]_o$ 2 mM[EGTA]$_i$	2 mM$[Ca^{2+}]_o$ 10 mM[EGTA]$_i$	2 mM$[Ba^{2+}]_o$ 2 mM[EGTA]$_i$
Peak I_{Ca}	34 ± 7%	14 ± 5% NS	18 ± 11% NS
End I_{Ca}	62 ± 9%	32 ± 8% †	27 ± 13% *
n	9	7	5

NS: not significant.
*P < 0.05; †P < 0.03 comparing Ba^{2+} and high EGTA data with Ca^{2+} and low EGTA data. Values are given as the mean percentages ± s.e.mean.
PC was applied for 3 to 5 min until steady state was achieved and peak and end I_{Ca} were measured at the beginning and end of 100 ms voltage step commands.

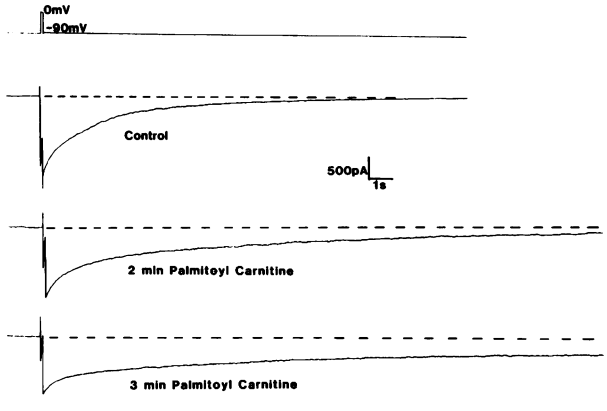

Figure 3 Palmitoyl-DL-carnitine (PC, 1 mM) prolongs Ca^{2+}-activated Cl^- tail currents. Traces show high voltage activated I_{Ca} activated by depolarizing step commands from − 90 mV to 0 mV. Ca^{2+} currents are accompanied by slowly deactivating inward Cl^- tail currents. Currents were activated under control conditions and 2 and 3 min after continuous application of 1 mM PC. Palmitoyl-DL-carnitine (1 mM) inhibited I_{Ca} and reduced the amplitude of the Ca^{2+}-activated Cl^- tail currents measured 20 ms after the end of the voltage step command. However PC greatly slowed the deactivation of the Ca^{2+}-activated Cl^- tail currents. The time course of decay of the Cl^- tail currents recorded in the presence of PC appears incomplete because the time base has been expanded to show I_{Ca}. However after more than 20 s the Ca^{2+}-activated Cl^- tail currents deactivated completely, in the presence of PC.

Table 2 Palmitoyl-DL-carnitine (PC, 100 μM) applied to the intracellular environment inhibits high voltage-activated I_{Ca} recorded from cultured DRG neurones

	10 μM PC		*100 μM PC*	
Time (min)	*Peak* I_{Ca} (nA)	*End* I_{Ca} (nA)	*Peak* I_{Ca} (nA)	*End* I_{Ca} (nA)
0.5	− 2.25 ± 0.21	− 0.97 ± 0.28	− 1.63 ± 0.43	− 0.68 ± 0.31
3.0	− 2.24 ± 0.30	− 1.34 ± 0.26	− 1.25 ± 0.32	− 0.78 ± 0.21
6.0	− 1.90 ± 0.34	− 1.24 ± 0.30	− 0.82 ± 0.25	− 0.49 ± 0.16
	NS	NS	*	NS

n = 6 for both 10 μM and 100 μM PC. Time is the duration of recording after entering the whole cell recording configuration. Peak I_{Ca} and end I_{Ca} are the mean amplitudes of I_{Ca} measured at the beginning and end of 100 ms voltage step command.
NS: not significant. *P < 0.01 comparing currents activated at 0.5 min with those activated after 6 min. Peak I_{Ca} and End I_{Ca} measured in the presence of 100 μM PC were significantly smaller than currents measured in the presence of 10 μM PC, (P < 0.03 and P < 0.05 respectively) throughout.

PC (10 μM) did not significantly affect I_{Ca} (Table 1) or the amplitude of Ca^{2+}-activated Cl^- tail currents. However the Cl^- tail currents were greatly prolonged when comparing currents activated 30 s and 3 min after entering the whole cell configuration. After 30 s recording the Cl^- tail currents decayed by 63% of the maximum current in 1500 ± 200 ms ($n = 3$); after 3 min recording the value was > 3000 ms in all cases.

Palmitoyl-DL-carnitine activates Ca^{2+}-dependent currents

Extracellular application of PC (1 mM) evoked inward currents after a delay of approximately 30 s in cells voltage clamped at − 90 mV. These PC activated currents were usually transient in nature, but repeated oscillating currents were observed even after PC was no longer being applied (Figure 4a,b,c). It is difficult to quantify these responses to 1 mM PC, however, the mean maximum transient inward current amplitude was 1280 ± 80 pA ($n = 6$). After the PC-induced currents were activated the cells usually stabilized; however, if a train of 20 voltage step commands was applied to activate the residual I_{Ca} after a delay the activity was restarted ($n = 3$) (Figure 5a). These responses were not observed in control cells ($n = 5$) and repeated activation of larger control I_{Ca} did not induce oscillating inward currents. Palmitoyl-DL-carnitine (1 mM) could also produce damage to some of the cultured DRG neurones ($n = 3$). Cell damage was identified by sustained increases in inward current (Figure 4a,b) and in some cases the current records became very noisy (Figure 4a). Additionally cell swelling was observed and developed with the sustained current. The cells being recorded from by the whole cell technique were particularly vulnerable to PC-induced damage; neighbouring cells from which recordings were not made but which were exposed to PC did not undergo physical changes such as swelling. This may be a consequence of loss of cytoplasmic

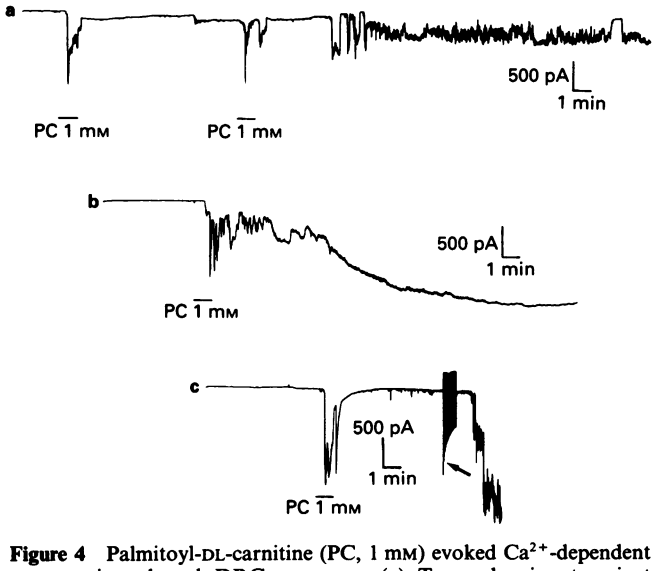

Figure 4 Palmitoyl-DL-carnitine (PC, 1 mM) evoked Ca^{2+}-dependent currents in cultured DRG neurones. (a) Trace showing transient responses to two periods of application of 1 mM PC followed by sustained noisy inward current which was associated with cell swelling. (b) Trace showing a series of inward current oscillations in response to 1 mM PC. The oscillating currents were followed by a large slowly developing sustained inward current and cell swelling. (c) PC (1 mM) activated several transient inward currents. Once the cell stabilized twenty 100 ms voltage step commands were applied at a frequency of 0.33 Hz to activate repeatedly the residual I_{Ca} (arrow) and load the cell with Ca^{2+}. Note the rapid inactivation of I_{Ca} with repeated activation. After I_{Ca} was activated at a clamp potential of 0 mV there was a short delay and then a rapid but large stepwise increase in inward current which was accompanied by cell swelling. Cells in (a), (b) and (c) were voltage-clamped at a holding potential of − 90 mV.

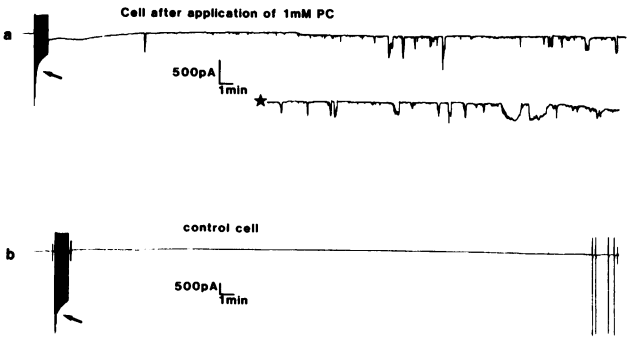

Figure 5 Repeated activation of high voltage-activated Ca^{2+} currents after application of palmitoyl-DL-carnitine (PC) triggers repeated spontaneous inward currents. (a) After 2 min application of 1 mM PC a single 1 nA transient current was observed and the cell stabilized (not illustrated). Five min after application of PC the cell was loaded with Ca^{2+} by activating I_{Ca} with twenty 100 ms voltage step commands to 0 mV at a frequency of 0.33 Hz (arrow). After a delay, transient oscillating inward currents were observed. The current record continues at (★). (b) Record from a control cell where repeated activation of the high voltage activated I_{Ca} (arrow) using the same protocol as described in (a), failed to evoke oscillating inward currents. At the end of the record, four Ca^{2+} currents and one leak current were activated to show that I_{Ca} was still present and that no run down had occurred. Both cells in (a) and (b) were voltage-calmped at a holding potential of − 90 mV. The amplification in each trace is different (note the scale bars). The high voltage-activated Ca^{2+} currents were much smaller when PC (1 mM) had been applied (a) and inactivation of I_{Ca} during the twenty voltage step commands was much more apparent compared with the control cell (b).

constituents involved in Ca^{2+} and/or cell volume regulation, with the whole cell recording technique.

The PC-activated currents were dependent on intracellular $[Ca^{2+}]_i$. Increasing the EGTA concentration in the patch solution from 2 mM to 10 mM increased intracellular Ca^{2+} buffering and greatly attenuated PC-activated currents; delaying onset, reducing occurrence and decreasing current amplitude. Of 6 cells, PC (1 mM) activated transient inward currents in only 1 cell (Figure 6a). The onset of this activity was delayed for 8 min and the maximum current amplitude observed was only 700 pA. Palmitoyl carnitine-induced repeated spontaneous inward currents could not be triggered by 20 voltage step commands to activate I_{Ca} repeatedly in cells containing patch solution with 10 mM EGTA ($n = 3$). Intracellular application via the patch solution of 100 μM PC also activated transient inward currents in 2 out of 6 cells.

The ionic nature of the repeated spontaneous inward currents activated by PC was not investigated in detail; however, these currents were still observed when Cs acetate based patch solution was used. In all 6 cells studied PC-activated currents were seen. The mean maximum transient inward current observed was 920 ± 130 pA ($n = 6$). Many of the events seen in the presence of Cs acetate patch solution decayed more slowly than the fast transient events observed when the cells were loaded with CsCl (Figure 6b). This anion effect is similar to that previously reported when caffeine was used to release Ca^{2+} from stores (Currie & Scott, 1992).

Palmitoyl-DL-carnitine-activated currents are attenuated by caffeine pretreatment

We have investigated the possibility that PC mobilizes Ca^{2+} from a caffeine-sensitive intracellular store. Dorsal root ganglion cells were pretreated with 1 mM caffeine in Ba^{2+} rather than Ca^{2+} containing recording medium for two 7.5 min periods. The cells were then washed three times with Ba^{2+} containing recording medium. Following caffeine pretreat-

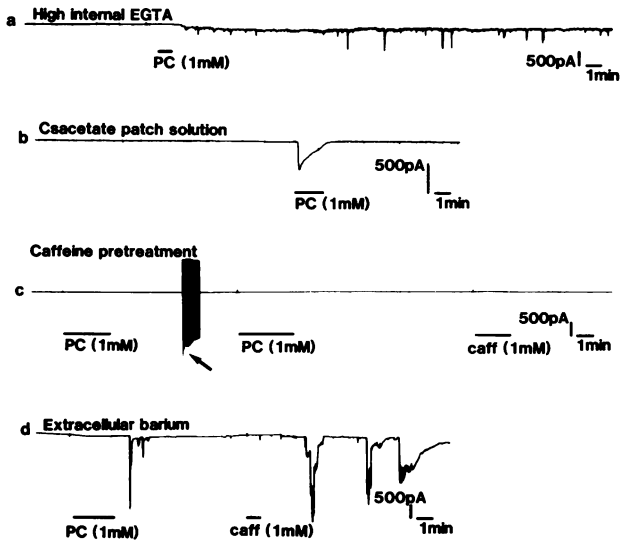

Figure 6 Ca^{2+}-dependence of palmitoyl-DL-carnitine (PC)-activated spontaneous transient inward currents recorded from DRG neurones at a holding potential of −90 mV. (a) Trace shows PC-activated currents delayed and attenuated by intracellular EGTA (10 mM), recorded from a cell with extracellular Ca^{2+} and CsCl based patch solution. (b) Inward current activated by PC (1 mM) recorded with Cs acetate based patch solution. (c) Current trace recorded from a DRG neurone pretreated with caffeine (Caff, 1 mM) in the presence of Ba^{2+} containing recording medium. Application of PC (1 mM) and caffeine (1 mM) failed to elicit spontaneous transient inward currents. Twenty 100 ms voltage step commands to 0 mV applied at a frequency of 0.33 Hz to activate barium currents (see arrow), again failed to elicit these inward transients or to alter the sensitivity of the cell to either PC or caffeine. (d) PC (1 mM) and caffeine (1 mM) activate spontaneous transient inward currents in a cell bathed in medium containing Ba^{2+} and not pretreated with caffeine. Compare with (c).

ment and in the presence of extracellular Ba^{2+} rather than Ca^{2+}, PC (1 mM) did not evoke repeated spontaneous inward currents in 5 cells. Under these conditions caffeine responses were also abolished ($n = 5$). Repeated activation of Ba^{2+} currents by 100 ms voltage step commands failed to sensitize the cells to PC or caffeine (Figure 6c).

With extracellular Ba^{2+} but without caffeine pretreatment, both PC (1 mM) and caffeine (1 mM) responses could still be elicited (Figure 6d).

Discussion

Both extracellular and intracellular application of PC reduced voltage-activated Ca^{2+} currents recorded from cultured DRG neurones. The actions of PC on high voltage-activated Ca^{2+} currents were at least in part Ca^{2+}-dependent which raises the possibility that the reductions in current observed were due to enhanced Ca^{2+}-dependent inactivation. Calcium-dependent processes play an important role in the inactivation of high voltage-activated Ca^{2+} currents (Chad & Eckert, 1986). Intracellular EGTA (Eckert & Tillotson, 1981) and substitution of extracellular Ca^{2+} by Ba^{2+} (Tillotson, 1979) reduced current inactivation. In this study, PC was less effective at reducing high voltage-activated Ca^{2+} channel currents carried by Ba^{2+} and I_{Ca} recorded from cells with 10 mM intracellular EGTA. Since the action of PC on high voltage-activated I_{Ca} was quite rapid in onset (10 s), the effect of PC was not likely to be rate-limited by the Ca^{2+}-induced inactivation process which is fast, occurring with a time constant < 10 ms (Morad *et al.*, 1988).

The role of Ca^{2+} in mediating the action of PC was supported by the finding that PC slowed deactivation of Ca^{2+}-activated Cl^- tail currents. The deactivation of Ca^{2+}-activated Cl^- tail currents is slowed by both increased Ca^{2+} entry through voltage-activated Ca^{2+} channels (Mayer, 1985; Currie & Scott, 1992) and by impairing $[Ca^{2+}]_i$ homeostatic mechanisms; for example with cyanide (Duchen, 1990) or caffeine (Scott *et al.*, 1992b). The maximal amplitude of the Ca^{2+}-activated Cl^- tail currents was reduced by PC. This was probably due to the decrease in I_{Ca}, resulting in less Ca^{2+} being available to activate the tail current.

Palmitoyl-DL-carnitine also activated inward currents which were similar to those observed following caffeine-induced Ca^{2+} release from intracellular stores (Currie & Scott, 1992). Like these caffeine-induced responses, PC-induced currents were attenuated by intracellular EGTA, suggesting that the currents were Ca^{2+}-dependent. These inward currents activated by PC were predominantly due to cation influx, however, we cannot exclude the possibility that when using CsCl based patch solution there is not also a component due to Cl^- efflux.

Several mechanisms may be associated with the PC action: (1) PC may reduce the efficiency of Ca^{2+} homeostatic mechanisms and allow a build up of free cytoplasmic Ca^{2+} with subsequent Ca^{2+}-induced Ca^{2+} release from intracellular stores. Support for this hypothesis comes from the finding that PC inhibits Ca^{2+}-ATPase and Ca^{2+} transport (Pitts *et al.*, 1978; Adams *et al.*, 1979). ATP-dependent Ca^{2+} transport plays an important role in regulation of $[Ca^{2+}]_i$ in DRG neurones (Thayer & Miller, 1990). Additionally, inhibition of Ca^{2+}-ATPase has been found to slow recovery from a Ca^{2+} load produced by voltage-activation of Ca^{2+} currents (Benham *et al.*, 1989). (2) PC may enter DRG neurones and itself trigger Ca^{2+} release from an intracellular store. Spedding (Zernig, 1991) has recently reported that acylcarnitines cause cell damage in part by activation of a ryanodine-sensitive Ca^{2+} release process associated with sarcoplasmic reticulum.

The use of Ba^{2+} as the charge carrier limits activation of Ca^{2+}-dependent processes including filling and release from caffeine-sensitive stores and activation of Ca^{2+}-dependent currents. However PC and caffeine-induced release of Ca^{2+} from intracellular stores still occurs with Ba^{2+} in the extracellular environment. Evidence from this study where caffeine pretreatment blocks PC responses suggests that in part, PC activates spontaneous transient inward currents by either direct or indirect Ca^{2+} release from a caffeine-sensitive store. L-Palmitoyl carnitine responses in single ventricular myocytes have previously been shown to be blocked by ryanodine, also suggesting a role for intracellular Ca^{2+} stores in PC responses (Mészàros & Pappano, 1990). PC has a lipophilic tail and was active whether applied intra- or extracellularly, so an intracellular site of action cannot be excluded. Although significant effects of PC were only observed with concentrations of 0.1 and 1 mM applied extracellularly, intracellular application of PC (0.1 mM) was more effective at reducing I_{Ca}. The same concentrations of PC as used in this study have previously been found to interact with Ca^{2+} channel ligand binding (Spedding & Mir, 1987).

Following the initiation of PC-induced Ca^{2+}-dependent inward currents, cell swelling and sustained increases in inward current were sometimes observed. These phenomena were not studied in detail but may reflect current generated by stretch-activated ion channels or activity of other channels associated with volume regulation. These effects may have developed as a result of disruption of cation gradients and osmotic balance after PC application or as a result of mobilization of intracellular Ca^{2+} and activation of inward currents. These effects of PC on $[Ca^{2+}]_i$ homeostasis may underlie an important mechanism of cell damage produced by ischaemic or hypoxic insults.

Not all the actions of PC can be accounted for by altered handling of intracellular free Ca^{2+}. The reduction of T-type low voltage-activated I_{Ca} probably involves a different mechanism because this current does not show marked Ca^{2+}-dependent inactivation (Bossu & Feltz, 1986; Carbone &

Lux, 1987). Furthermore, T-type I_{Ca} can be isolated from high voltage-activated I_{Ca} because it is much less sensitive to raised $[Ca^{2+}]_i$ (Carbone & Lux, 1987). T-type I_{Ca} is very sensitive to agents such as 1-octanol (Llinás, 1988) and an arginine polyamine (Scott *et al.*, 1992c) which interact with lipid components of cell membranes. The action of PC on T-type I_{Ca} may involve modification of the lipid microenvironment of the Ca^{2+} channels rather than direct interaction.

We have not investigated the actions of other long chain acyl carnitines on our preparation. However, altering the fatty acid chain length has been shown to attenuate fatty acid responses in other systems (Criddle *et al.*, 1990). Fatty acids are known to have regulatory actions on a variety of ion channels (Ordway *et al.*, 1991). This may be due to direct interaction with channels or indirect effects via second messenger formation or on the lipid microenvironment. Actions on Na^+ and Ca^{2+} currents have been described in neuroblastoma cells where fatty acids appear to attenuate these currents by a protein kinase C-mediated mechanism (Linden & Routtenberg, 1989).

At present we cannot exclude the possibility that PC has some inhibitory properties which involve direct interactions with voltage-activated Ca^{2+} channels. We have not observed any consistent increase in I_{Ca} produced by PC in this functional assay system. However the dominant action of PC to reduce I_{Ca} observed in this study may have masked neuronal Ca^{2+} channel activator properties previously reported (Spedding & Mir, 1987).

In conclusion we have observed actions of PC on DRG neurones which are consistent with reduced cellular homeostatic control of $[Ca^{2+}]_i$.

We thank The Wellcome Trust and Medical Research Council for support. We also thank Dr A. Hughes for the computer programme for calculating free Ca^{2+} levels and Ms S. Maddox for help with the preparation of the manuscript.

References

ADAMS, R.J., COHEN, D.W., GUPTE, S., WALLICK, E.T., WANG, T. & SCHWARTZ, A. (1979). In vitro effects of palmitoyl carnitine on cardiac plasma membrane Na, K-ATPase, and sarcoplasmic reticulum Ca^{2+}-ATPase and Ca^{2+} transport. *J. Biol. Chem.*, **254**, 12404–12410.

BENHAM, C.D., EVANS, M.L. & MCBAIN, C.J. (1989). Inhibition of Ca-ATPase slows recovery from voltage-gated Ca_i load in cultured neurones from rat dorsal root ganglia. *J. Physiol.*, **415**, 21P.

BIRD, M.I., MUNDAY, L.A., SAGGERSON, E.D. & CLARK, J.B. (1985). Carnitine acyl transferase activities in rat brain mitochondria. *Biochem. J.*, **226**, 323–330.

BOSSU, J-L. & FELTZ, A. (1986). Inactivation of the low-threshold transient calcium current in rat sensory neurones: evidence for a dual process. *J. Physiol.*, **376**, 341–357.

CARBONE, E. & LUX, H.D. (1987). Kinetics and selectivity of a low voltage-activated calcium current in chick and rat sensory neurones. *J. Physiol.*, **386**, 547–570.

CHAD, J.E. & ECKERT, R. (1986). An enzymatic mechanism for calcium current inactivation in dialysed Helix neurones. *J. Physiol.*, **378**, 31–51.

CHOI, D.W. (1988). Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischaemic damage. *Trends Neurosci.*, **11**, 465–469.

CRIDDLE, D.N., DEWAR, G.H., WATHEY, W.B. & WOODWARD, B. (1990). The effects of novel long chain acyl carnitine esters in the isolated perfused heart of the rat. *Br. J. Pharmacol.*, **99**, 477–480.

CURRIE, K.P.M. & SCOTT, R.H. (1992). Calcium-activated currents in cultured neurones from rat dorsal root ganglia. *Br. J. Pharmacol.*, **106**, 593–602.

DUCHEN, M.R. (1990). Effects of metabolic inhibition on the membrane properties of isolated mouse primary sensory neurones. *J. Physiol.*, **424**, 387–409.

ECKERT, R. & TILLOTSON, D.L. (1981). Calcium-mediated inactivation of the calcium conductance in caesium loaded giant neurones of *Aplysia californica*. *J. Physiol.*, **314**, 265–280.

GARDINER, M., NILSSON, B., REHNCRONA, S. & SEISJÖ, B.K. (1981). Free fatty acids in the rat brain in moderate and severe hypoxia. *J. Neurochem.*, **36**, 1500–1505.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

INOUE, D. & PAPPANO, A.J. (1983). L-Palmitylcarnitine and calcium act similarly on excitatory ionic currents in avian ventricular muscle. *Circ. Res.*, **52**, 625–634.

KNABB, M.T., SAFFITZ, J.E., CORR, P.B. & SOBEL, B.E. (1986). The dependence of electrophysiological derangements on accumulation of endogenous long-chain acyl carnitine in hypoxic neonatal rat myocytes. *Circ. Res.*, **58**, 230–240.

LIEDTKE, A.J., NELLIS, S. & NEELY, J.R. (1978). Effects of excess free fatty acids on mechanical and metabolic function in normal and ischaemic myocardium in swine. *Circ. Res.*, **43**, 652–661.

LINDEN, D.J. & ROUTTENBERG, A. (1989). Cis-fatty acids which activate protein kinase C, attenuate Na^+ and Ca^{2+} currents in mouse neuroblastoma cells. *J. Physiol.*, **419**, 95–119.

LLINÁS, R.R. (1988). The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function. *Science*, **242**, 1654–1664.

MCBURNEY, R.N. & NEERING, I.R. (1987). Neuronal calcium homeostasis. *Trends Neurosci.*, **10**, 164–169.

MAYER, M.L. (1985). A calcium-activated chloride current generates the after-depolarisation of rat sensory neurones in culture. *J. Physiol.*, **364**, 217–239.

MÉSZÁROS, J. & PAPPANO, A.J. (1990). Electrophysiological effects of L-palmitoyl carnitine in single ventricular myocytes. *Am. J. Physiol.*, **258**, H931–H938.

MORAD, M., DAVIES, N.W., KAPLAN, J.H. & LUX, H.D. (1988). Inactivation and block of calcium channels by photo-released Ca^{2+} in dorsal root ganglion neurones. *Science*, **241**, 842–844.

ORDWAY, R.W., SINGER, J.J. & WALSH, J.V. (1991). Direct regulation of ion channels by fatty acids. *Trends Neurosci.*, **14**, 96–100.

PATMORE, L., DUNCAN, G.P. & SPEDDING, M. (1989). Interaction of palmitoyl carnitine with calcium antagonists in myocytes. *Br. J. Pharmacol.*, **97**, 443–450.

PITTS, B.J.R., TATE, C.A., VAN WINKLE, W.B., WOOD, J.M. & ENTMAN, M.L. (1978). Palmitylcarnitine inhibition of the calcium pump in cardiac sarcoplasmic reticulum; a possible role in myocardial ischaemia. *Life Sci.*, **23**, 391–402.

SCOTT, R.H., STAPLETON, S.R. & CURRIE, K.P.M. (1992a). Ca^{2+}-dependent actions of palmitoyl DL-carnitine on cultured rat DRG neurones. *Br. J. Pharmacol.*, **105**, 118P.

SCOTT, R.H., CURRIE, K.P.M., SUTTON, K.G. & DOLPHIN, A.C. (1992b). Modulation of neuronal Ca^{2+}-dependent currents by neurotransmitters, G-proteins and toxins. *Biochem. Soc. Trans.*, **20**, 443–449.

SCOTT, R.H., SWEENEY, M.I., KOBRINSKY, E.M., PEARSON, H.A., TIMMS, G.H., PULLAR, I.A., WEDLEY, S. & DOLPHIN, A.C. (1992c). Actions of arginine polyamine on voltage and ligand-activated whole cell currents recorded from cultured neurones. *Br. J. Pharmacol.*, **106**, 199–207.

SPEDDING, M. & MIR, A.S. (1987). Direct activation of Ca^{2+} channels by palmitoyl carnitine, a putative endogenous ligand. *Br. J. Pharmacol.*, **92**, 457–468.

THAYER, S.A. & MILLER, R.J. (1990). Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurones in vitro. *J. Physiol.*, **425**, 85–115.

TILLOTSON, D. (1979). Inactivation of Ca conductance dependent on entry of Ca ions in molluscan neurons. *Proc. Natl. Acad Sci. U.S.A.*, **76**, 1497–1500.

ZERNIG, G. (1991). Clinical future for Ca^{2+} antagonists looks more promising. *Trends Pharmacol. Sci.*, **12**, 439–442.

(Received May 13, 1992
Revised June 30, 1992
Accepted August 19, 1992)

Br. J. Pharmacol. (1992), **107**, 1198–1204

Characteristics of the contractile response of rabbit aorta produced by cromakalim in calcium-free solution

[1]S. Duty & [2]A.H. Weston

Smooth Muscle Research Group, Department of Physiological Sciences, Stopford Building, University of Manchester, Oxford Road, Manchester, M13 9PT

1 The effect of potassium channel opening compounds has been investigated in the smooth muscle of rabbit aorta under Ca-free conditions. Examination of the characteristics of the response has been performed using cromakalim as the prototype compound.

2 In order of potency, Ro 31-6930, cromakalim, minoxidil sulphate and pinacidil each produced a contraction in rabbit aortic strips bathed in Ca-free MOPS-buffered physiological salt solution (PSS). In contrast, forskolin, glyceryl trinitrate and nifedipine each failed to increase tension under identical conditions. Cromakalim also evoked contraction of bovine trachealis muscle bathed in Ca-free PSS.

3 The contractile response to cromakalim in rabbit aortic strips was of delayed onset (15–20 min) and reached a plateau after approximately 120 min (1.8 g maximum with 1 μM cromakalim). No cromakalim-induced tension changes were observed in either 1 mM or 2.5 mM Ca-containing PSS.

4 Raising the [KCl] of the Ca-free PSS to 65.9 mM fully inhibited the cromakalim-induced contraction in rabbit aortic strips. In addition, pretreatment of aortic strips with the sulphonylurea glibenclamide antagonized the subsequent mechanical response to cromakalim.

5 In Ca-free PSS, cromakalim (1 μM) stimulated ^{42}K-efflux with a time-course corresponding to the contractile event. Glibenclamide (1 μM) inhibited this cromakalim-induced ^{42}K-efflux.

6 In sharp microelectrode studies in bovine trachealis, cromakalim (10 μM) produced a sustained membrane hyperpolarization in normal PSS. In contrast, the cromakalim-induced hyperpolarization in Ca-free PSS was not sustained. The fading of the hyperpolarization was temporally correlated with the increase in tension under these experimental conditions.

7 It is concluded that the K-channel opener-induced smooth muscle contractile response revealed in Ca-free PSS is the consequence of K-channel opening. The nature of the detailed mechanism which underlies this contractile phenomenon remains to be determined.

Keywords: Cromakalim; Ro 31-6930; minoxidil sulphate; pinacidil; rabbit aorta; bovine trachealis; K-channels; glibenclamide; ^{42}K-efflux; calcium

Introduction

Potassium (K) channel openers such as cromakalim are well-recognised as potent smooth muscle relaxants (Hamilton & Weston, 1989). These mechano-inhibitory agents exert their effects on a variety of pre-contracted smooth muscles and inhibit the development not only of spontaneous tension changes but also of a variety of agonist-induced contractions (Newgreen *et al.*, 1990; Bray *et al.*, 1991). One consequence of K-channel opening is electrical hyperpolarization which indirectly restricts Ca entry through potential-dependent plasmalemmal Ca-channels (Hamilton *et al.*, 1986). In addition, recent studies with fura-2 fluorescent microscopy have more directly shown that K-channel openers reduce both Ca entry through receptor-operated routes and intracellular Ca release in smooth muscle preparations (Yanagisawa *et al.*, 1990; Duty & Weston, 1991a; Ito *et al.*, 1991).

In complete contrast to these numerous results regarding the smooth muscle relaxant properties of the K-channel openers, the present paper describes the contractile responses to these agents which are revealed under Ca-free conditions. A preliminary account of some of these findings has already been published (Bray *et al.*, 1989; Duty & Weston, 1991b).

Methods

Tissue bath experiments

Preparation of tissues Most of the experiments were performed on strips of rabbit aorta isolated from male Half-Lop rabbits (2.5–3.0 kg; supplied by the University of Manchester Animal Unit) which were killed by stunning and exsanguination. The thoracic aorta was removed and carefully cleaned of fat and connective tissue. The aorta was cut into segments of approximately 5 mm in length. Each of these was opened along the longitudinal axis to form a flat sheet. The vascular endothelium was then removed by gentle rubbing of the intimal surface with a cotton bud moistened with physiological salt solution (PSS). A fine pin with thread attached was inserted into the mid-part of each of the longitudinally-cut edges of the segment to enable isometric tension recording. Other investigations were performed in small strips of smooth muscle (approximately 5 mm × 10 mm), which were dissected from bovine trachealis muscle obtained freshly from the Manchester Abattoir on the morning of the experiment.

Tension recordings were performed in both types of tissue. In addition, radiolabelled ion flux experiments were carried out in the aortic preparations and microelectrode experiments were performed with the trachealis preparations. In all of these studies tissues were bathed in either a Ca-containing MOPS-buffered PSS or in a Ca-free MOPS-buffered physiological salt solution containing EGTA (Ca-free PSS).

[1] Current address: Muscle Cell Function Laboratory, Department of Physiology (F13), University of Sydney, NSW 2006, Australia.
[2] Author for correspondence.

Isometric tension recording Segments of rabbit aorta denuded of endothelium were mounted for isometric tension recording in PSS at 37°C, bubbled with 100% oxygen. Tissues were equilibrated for at least 60 min under a resting tension of 2 g. Where appropriate, tissues were subsequently transferred for equilibration in Ca-free PSS, with three changes of bathing solution at 5 min intervals, before experiments commenced in the continuing presence of Ca-free PSS. In the experiments with bovine trachealis muscle, these tissues were mounted under a resting tension of 1 g but were otherwise treated as described for the rabbit aorta denuded of endothelium.

Contractile effects of K-channel openers in Ca-free PSS Segments of aorta bathed in Ca-free PSS were exposed to the following K-channel openers by use of a cumulative protocol; cromakalim (0.1–1 μM), BRL 38226 (the inactive (+)- enantiomeric component of cromakalim: 1–100 μM), minoxidil sulphate (0.1–1 μM), pinacidil (0.1–10 μM) and Ro 31-6930 (0.01–1 μM). A contact time of at least 30 min was allowed for each concentration of agonist and the resulting increase in tension was allowed to develop fully before increasing the agonist concentration. Time-matched vehicle controls were performed and the EC_{50} values were determined for each agent following correction for vehicle effects. The effect of cromakalim was also determined in solutions containing different concentrations of $CaCl_2$ (0–2.5 mM) to ascertain the conditions necessary for expression of the contractile response. In addition, the effect of cromakalim was examined in bovine trachealis muscle strips in order to determine whether the mechanical changes obtained in the rabbit aorta were species- or tissue-specific.

Effects of glyceryl trinitrate, forskolin and nifedipine in Ca-free PSS In these studies we examined the ability of other smooth muscle relaxants to produce contraction in Ca-free conditions. Segments of aorta bathed in Ca-free PSS were exposed to either the guanylate cyclase stimulator, glyceryl trinitrate (0.3–10 μM), the adenylate cyclase stimulator, forskolin (0.01–1 μM), the Ca entry blocker nifedipine (300 nM) or appropriate vehicle by use of protocol described above. Changes in smooth muscle tension were measured.

Inhibitory effects of glibenclamide and high K-PSS Two characteristic effects of K-channel openers in normal PSS are their inability to relax contractions either produced by high K-PSS or by other agonists in the presence of high K-PSS and the ability of glibenclamide to antagonize K-channel opener-induced relaxations. Thus the effects of raising the [KCl] of a Ca-free PSS or the addition of glibenclamide to Ca-free PSS on the contractions produced by cromakalim were examined.

Following equilibration in Ca-free PSS, segments of rabbit aorta were pre-incubated in Ca-free PSS containing glibenclamide (0.1 μM, 0.3 μM or 1 μM), 60 mM additional KCl (ie total [KCl] 65.9 mM) or appropriate vehicle for a period of 20 min. Cumulative concentration-effect curves to cromakalim (1–30 μM) were then constructed in the continuing presence of the modifying condition. Only the upper part of the concentration-effect curve was determined in these experiments owing to the lengthy contact time (>30 min) which was allowed for each concentration of cromakalim.

Measurement of ^{42}K-efflux

The ability of cromakalim to increase the rate of ^{42}K-efflux under Ca-free conditions was investigated to characterize further the changes induced by cromakalim under Ca-free conditions. Strips of rabbit aorta with endothelium removed were impaled on syringe needles, attached to a perspex gassing manifold and placed over collection vials containing PSS bubbled with 100% O_2. The rack of vials was positioned in a thermostatically-controlled water bath at 37°C. Following 30–60 min equilibration, tissues were loaded with ^{42}K (1.57 μCi ml^{-1}) for 3.5 h. The ^{42}K was allowed to efflux from the tissues for fourteen 4-min collection periods (the first two of which were discarded). During these periods tissues were exposed either to PSS or Ca-free PSS alone, or to cromakalim (1 μM), glibenclamide (1 μM) plus cromakalim (1 μM) or vehicle for cromakalim (0.007% ethanol) each in the continuing presence of Ca-free PSS. At the end of the final collection period the tissues were blotted for 10 s between filter pads underneath a 1 kg weight and finally placed in vials. These, together with the tubes containing the collected incubation samples, were counted for gamma-emmissions over a 2-min period by use of a Packard gamma counter. Correction factors for both the background radiation and short half-life of ^{42}K (12.4 h) were automatically made. The efflux of ^{42}K is expressed as an efflux rate coefficient which indicates the efflux per min as a percentage of the radiolabel remaining in the tissue at the beginning of each particular efflux period (% per min).

Microelectrode recordings

Muscle strips obtained from rabbit aorta contain only a relatively small proportion of smooth muscle and impalements with sharp microelectrodes are made difficult by the large amount of elastin and connective tissue present. However, bovine trachealis strips are comparably rich in smooth muscle and are thus much easier to study electrophysiologically. For this reason the present microelectrode studies were performed in the trachealis strips.

Strips of isolated bovine trachealis muscle, prepared as described above, were placed in a recording chamber through which oxygenated PSS was flowing at a rate of 5 ml min^{-1}. Each strip was secured onto the Sylgard floor of the chamber by fine pins and allowed to equilibrate for 30 min. Glass microelectrodes (tip resistance approximately 50 MΩ) were inserted into the smooth muscle layer and the resting membrane potential was determined by sampling the potential of a number of different cells within the muscle strip. In some experiments the strips remained in PSS, whilst in others the tissues were then exposed to Ca-free PSS and the new resting membrane potential determined after 10 min equilibration. During subsequent exposure to cromakalim, most notably in the tissues bathed in Ca-free PSS, it was difficult to maintain electrode impalements for more than 10 min. Therefore, in order to sample the membrane potential over a longer period of 120 min (corresponding with the time course of the contractile response), cell-sampling techniques were employed. The electrode was thus deliberately withdrawn from the cell following attainment of the maximum initial change in potential produced by cromakalim. The membrane potential of the muscle strip was then monitored by impaling cells at varying times over the remaining experimental period.

In one series of experiments performed in tissues equilibrated in Ca-free PSS, two strips of trachealis muscle were placed in parallel in the recording chamber. The first strip was used for membrane potential recordings as above. The second strip, which was attached to a force transducer at one end and secured to the Sylgard base of the chamber at the other end, was used to monitor the simultaneous changes in tension.

Chemicals and solutions

The following chemicals were used in this study: cromakalim and BRL 38226 (+)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol; SmithKline Beecham), EGTA and forskolin (Sigma), glibenclamide (Hoechst), glyceryl trinitrate (ICI), minoxidil sulphate and Ro 31-6930 (2-(6-cyano-2,2-dimethyl-2H-1-benzopyran-4-yl) pyridine 1-oxide; Roche Products), pinacidil (Leo) and $^{42}K_2CO_3$ (University of Manchester, Risley reactor).

The normal composition of the MOPS-buffered PSS (PSS)

was (mM): NaCl 129.7, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11.1, MOPS 10. Ca-free MOPS-buffered PSS (Ca-free PSS) was prepared as above but $CaCl_2$ was omitted and additional $MgCl_2$ (8.8 mM) and EGTA (2 mM) included. The total $MgCl_2$ content of the solution was thus 10 mM. In one series of experiments (as indicated in the text) both EGTA (2 mM) and $MgCl_2$ (8.8 mM) were omitted from the Ca-free PSS. When tissues were loaded with ^{42}K, KCl was excluded from the loading PSS and replaced with $^{42}K_2CO_3$ to give a final [K] of 5.9 mM. All of the solutions were adjusted to pH 7.4 with 4 M NaOH.

Data analysis

Where appropriate, data were analysed by Analysis of Variance with Student's range test or unpaired *t* test. Probability levels (P) < 0.05 were taken to indicate a significant difference between mean responses.

Results

Isometric tension studies

Effects of K-channel openers in Ca-free PSS Cromakalim (0.1–10 μM) produced a concentration-dependent contraction of both rabbit aortic and bovine trachealis smooth muscle strips bathed in Ca-free PSS. The contraction was delayed in onset by 15–20 min as shown in the original tension recording from rabbit aorta (Figure 1a) and reached a plateau contraction after approximately 120 min. The mean size of contraction produced by 1 μM cromakalim was 1.8 ± 0.2 g ($n = 5$) in aortic segments and 1.1 ± 0.1 g ($n = 4$) in bovine trachealis strips. In rabbit aortic strips bathed in Ca-free PSS, significant contractions were produced by all the K-channel openers tested (Figure 1b). Taking into consideration the failure to attain maximal contractions with both pinacidil and BRL 38266, approximate EC_{50} values were obtained by assigning an arbitrary value of 100% to the maximum effect produced in this study. In order of potency, the estimated EC_{50} values were as follows:- Ro 31-6930 (0.08–0.01 μM, $n = 6$), cromakalim (0.22 ± 0.09 μM, $n = 6$), minoxidil sulphate (1.47 ± 0.37 μM, $n = 5$), pinacidil (1.77 ± 0.37 μM, $n = 5$) and BRL 38226 (54.00 ± 7.44 μM, $n = 5$).

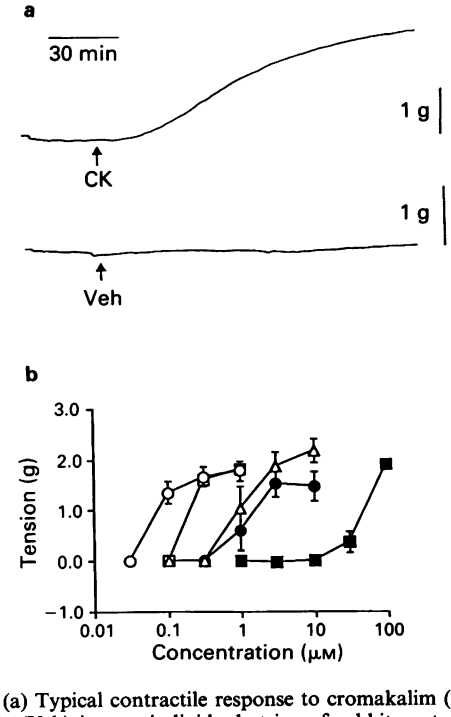

Figure 1 (a) Typical contractile response to cromakalim (CK, 1 μM) and vehicle (Veh) in two individual strips of rabbit aorta bathed in Ca-free PSS. (b) The contractile responses following exposure to increasing concentrations of Ro 31-6930 (○), cromakalim (□), pinacidil (△), minoxidil sulphate (●) and BRL 38226 (■) in rabbit aortic strips bathed in Ca-free PSS. These data have been corrected for changes occurring in the equivalent vehicle controls. The points show the mean values ($n = 5$ or 6) ± s.e.mean (vertical bars).

Cromakalim-induced contractions were not dependent on the presence of EGTA or of a raised [$MgCl_2$] in the Ca-free PSS. Thus, cromakalim still produced contraction of rabbit aortic strips bathed in a nominally Ca-free PSS containing neither elevated $MgCl_2$ levels nor EGTA. However, no contraction was produced in vehicle-treated control tissues or following cromakalim addition to strips which were bathed in solutions containing 1 mM or 2.5 mM Ca (Figure 2).

Effects of other smooth muscle relaxants in Ca-free PSS In contrast to the K-channel openers, smooth muscle relaxants believed to act through mechanisms which do not involve K-channels failed to produce any contraction of rabbit aortic segments bathed in Ca-free PSS (data not shown). No tension increments were seen with either forskolin (0.01–1 μM), glyceryl trinitrate (0.3–10 μM) or a maximally-effective relaxant concentration of nifedipine (300 nM).

Inhibition of responses to cromakalim by high [KCl] and by glibenclamide The effect of pre-incubating rabbit aortic strips in Ca-free PSS containing an elevated [KCl] is shown in the pair of original tension recordings (Figure 3a,b). Addition of 60 mM KCl produced a small sustained contraction in Ca-free PSS (total [KCl] = 65.9 mM). No such tension increase was evoked following exposure of control tissues to the vehicle for KCl (Ca-free PSS containing 5.9 mM KCl). Subsequent addition of 1 μM cromakalim produced a contraction in the control tissue pretreated with vehicle. In contrast, no contraction was produced by cromakalim in the tissue pre-treated with additional 60 mM KCl. Maximum tension increments produced by cromakalim in the two groups of tissues were 2.1 ± 0.4 g, $n = 6$ (3 μM cromakalim; [KCl] = 5.9 mM) and 0.0 ± 0.1 g, $n = 6$ (10 μM cromakalim; [KCl] = 65.9 mM).

When compared to vehicle (maximum of 0.01% ethanol), glibenclamide (0.1–1 μM) produced a significant concentration-dependent inhibition of the contractile response to cromakalim in Ca-free PSS (Figure 3c). The response to cromakalim (1 μM), which usually produced a near-maximal contraction, was fully inhibited. However, the maximum contraction produced by raising the concentration of cromakalim remained unchanged.

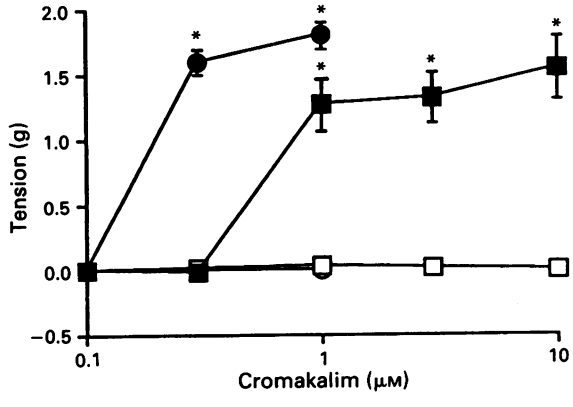

Figure 2 The response to cromakalim in rabbit aortic strips bathed in 2.5 mM Ca-PSS (○), 1 mM Ca-PSS (□), Ca-free PSS (ie containing 2 mM EGTA and a total [$MgCl_2$] of 10 mM) (●) and nominally Ca-free PSS (ie containing no EGTA and a total [$MgCl_2$] of 1.2 mM) (■). The points show the mean values ($n = 4$ or 5) ± s.e.mean (vertical bars). *Indicates a significant contractile effect of cromakalim in the Ca-free solutions compared to normal PSS ($P < 0.05$).

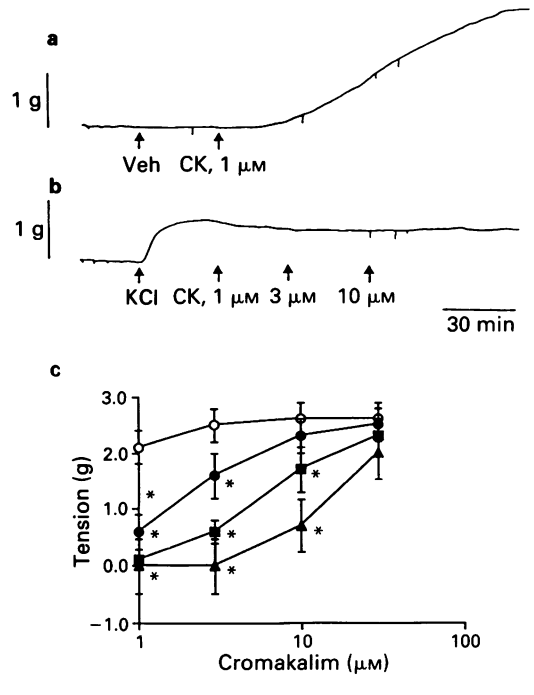

Figure 3 The contractile response produced by cromakalim (CK) in individual strips of rabbit aorta bathed in Ca-free PSS following pretreatment with (a) vehicle (Veh) for KCl (Ca-free PSS alone) or (b) 60 mM KCl. (c) The effect of pretreatment with glibenclamide, 0.1 μM (●), 0.3 μM (■) and 1 μM (▲) or vehicle (○) on the response to cromakalim in rabbit aortic strips bathed in Ca-free PSS. The points show the mean values ($n = 6$) ± s.e.mean (vertical bars). *Indicates a significant difference between the effects of glibenclamide and its vehicle.

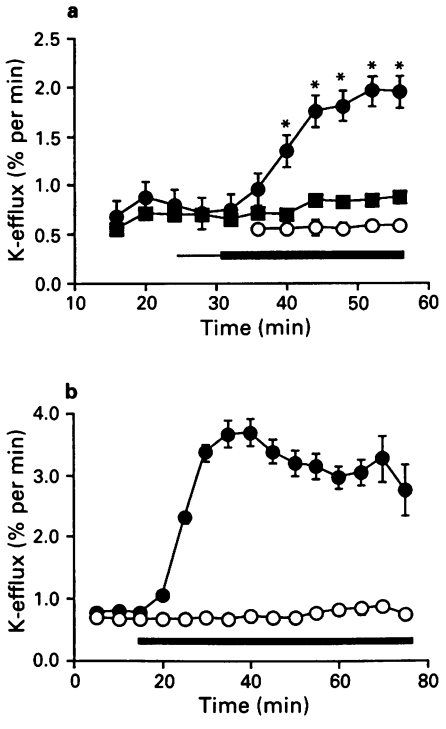

Figure 4 (a) The effect of cromakalim (1 μM) on the rate of ^{42}K efflux from rabbit aortic strips bathed in Ca-free PSS in the absence (●) and presence (■) of glibenclamide (1 μM). The basal efflux rate is also shown (○). The points show the mean values ($n = 6$) ± s.e.mean (vertical lines). The thin and thick horizontal bars indicate the periods of exposure to glibenclamide (or vehicle) and glibenclamide (or vehicle) plus cromakalim, respectively. *Indicates a significant difference between cromakalim-induced efflux compared to both basal efflux and cromakalim-induced efflux in the presence of glibenclamide ($P < 0.05$). (b) The effect of prolonged exposure to cromakalim, 1 μM (●) or vehicle (○) on the rate of ^{42}K-efflux from strips of rabbit aorta bathed in Ca-free PSS. The points show the mean values ($n = 6$) ± s.e.mean (vertical lines). The horizontal bar represents the period of exposure to cromakalim.

^{42}K-efflux studies

The basal rate of ^{42}K-efflux from rabbit aortic strips was increased from a maximum of 0.38% per min to a maximum of 0.59% per min following incubation in Ca-free PSS. Despite this elevated baseline, cromakalim produced an approximate three fold increase in the ^{42}K efflux rate and this increase was fully inhibited by pretreatment with glibenclamide (1 μM for 15 min; Figure 4a). In tissues bathed in Ca-containing PSS, cromakalim (1 μM) produced a more modest two fold increase in ^{42}K-efflux rate which was likewise inhibited by pretreatment with glibenclamide (data not shown). Glibenclamide (1 μM) alone produced no change in the basal efflux for ^{42}K in tissues bathed either in PSS or in Ca-free PSS. In a separate experiment, the maximal cromakalim-induced increase in ^{42}K-efflux rate from aortic strips bathed in Ca-free PSS was found to persist over a time period consistent with that of the cromakalim-induced contraction (Figure 4b).

Microelectrode studies

The resting membrane potential of trachealis strips bathed in PSS was -62.5 ± 1.4 mV ($n = 20$ impalements from 4 tissues). Cromakalim (10 μM) produced a shift in membrane potential towards negative values (Figure 5a) and the maximum hyperpolarization obtained was 22.5 ± 2.6 mV ($n = 4$ impalements from 4 tissues). The membrane hyperpolarization was well-maintained throughout the period of exposure to cromakalim (approximately 160 min; Figure 5c). The mean resting membrane potential in the second group of trachealis strips was -63.7 ± 1.2 mV ($n = 39$ impalements from 7 tissues). Following equilibration of these strips in Ca-free PSS, the membrane potential decreased to a new resting level of -47 ± 2.0 mV ($n = 11$ impalements from 6 tissues). Subsequent exposure to cromakalim (10 μM) produced an initial membrane hyperpolarization (maximum hyperpolarization 24.4 ± 0.9 mV; $n = 3$ impalements from 3 tissues; Figure 5b). In contrast to the sustained hyperpolarization observed with cromakalim in PSS, the cromakalim-induced hyperpolarization in Ca-free PSS was not sustained. Over a period of approximately 80 min, the membrane potential returned to pre-cromakalim levels and after longer exposure, a mean depolarization of about 10 mV was observed (Figure 5c).

During the contact period with cromakalim (10 μM) in Ca-free PSS the tension in trachealis strips, mounted in parallel to the strip used for electrical recording, increased to a maximum of 2.2 ± 0.1 g ($n = 3$). This period of increasing tension followed the time-course of the fade in membrane hyperpolarization (Figure 6). Correlation coefficients determined from the scatter plots related these two variables (ie fade in membrane hyperpolarization and increase in tension) with a mean correlation coefficient of 0.76 ± 0.01 which did not deviate significantly from linearity.

Discussion

In the present study, a contractile response was always evoked when segments of rabbit aorta bathed in Ca-free, MOPS-buffered PSS were exposed to K-channel openers. Segments of bovine trachealis muscle bathed in Ca-free PSS

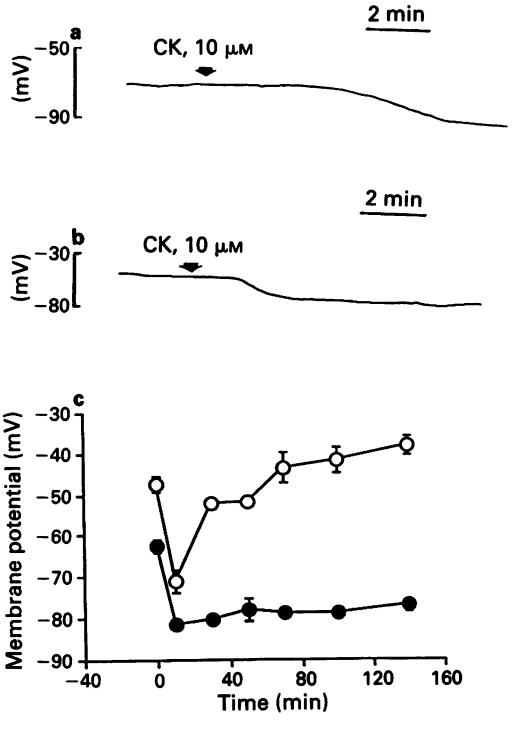

Figure 5 The early hyperpolarizing response to cromakalim (CK, 10 μM) in bovine trachealis strips bathed in PSS (a) or in Ca-free PSS (b). The effects of prolonged exposure to cromakalim on membrane potential in strips bathed in PSS (●) or in Ca-free PSS (○) are shown in (c). The points show the mean values ($n = 2$ to 6) ± s.e.mean (vertical bars).

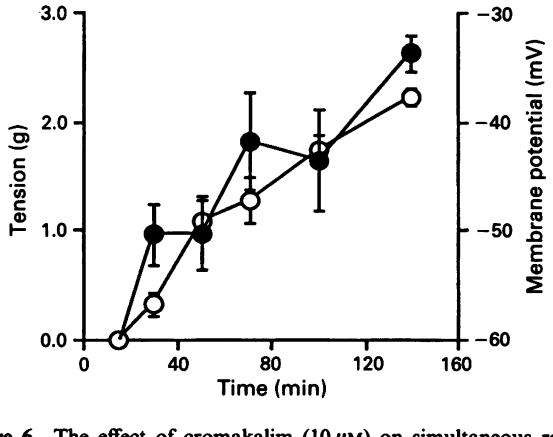

Figure 6 The effect of cromakalim (10 μM) on simultaneous recordings of membrane potential (●) and tension (○) in trachealis strips in Ca-free PSS. The points show the mean values ($n = 3$) ± s.e.mean (vertical bars).

produced a similar contraction in response to the prototype compound, cromakalim. Although identical Ca-free solutions were used in an earlier investigation of the modulatory role of K-channel openers in intracellular Ca storage and release experiments (Bray *et al.*, 1991), a K-channel opener-induced increase in tension has not been previously demonstrated. However, in the experiments of Bray *et al.* (1991), K-channel openers were in contact with tissues bathed in Ca-free PSS for a maximum period of 15 min, before the addition of potent spasmogens. Since there was at least a 15 min delay before the onset of the K-channel opener-induced contractions under these experimental conditions it is not surprising that such effects remained undetected in the previous study.

The cromakalim-induced contraction was dependent on the absence of Ca from the bathing solution. However, the response did not require the presence of either the elevated levels of $MgCl_2$ or of EGTA in the Ca-free PSS since the contractions were also obtained in a nominally Ca-free PSS which contained neither of these modifications. There was some decrease in the potency of cromakalim in the nominally Ca-free PSS compared with the Ca-free PSS, (containing EGTA and raised Mg). It thus seems likely that inclusion of EGTA which is known to produce smooth muscle membrane destabilization even in very low concentrations (50 μM; Guan *et al.*, 1988), may in some way potentiate the contractile response to cromakalim.

The contractile response in Ca-free PSS was not a feature unique to cromakalim. Rather the response was observed with all the structurally-disparate K-channel openers tested and was elicited at concentrations similar to those which produce the relaxant responses in PSS. The rank order of potency of these agents (Ro 31-6930 > cromakalim > minoxidil sulphate > pinacidil > BRL 38226) was also similar to that found for the relaxant effects of the K-channel openers in a variety of other experimental systems (Duty *et al.*, 1990; Newgreen *et al.*, 1990; Paciorek *et al.*, 1990; Piper *et al.*, 1990). The observed low potency of BRL 38226 (the (+)-enantiomeric component of cromakalim) is consistent with previous findings that this enantiomer is the less potent relaxant and hypotensive component of cromakalim (Buckingham *et al.*, 1986; Hof *et al.*, 1988).

To verify that the contractile response was a specific feature related to K-channel opening, the ability of other smooth muscle relaxants, devoid of K-channel opening activity, to initiate a similar contractile response in Ca-free PSS was investigated. These studies revealed that neither forskolin, glyceryl trinitrate nor nifedipine produced any contraction under these experimental conditions. This indirectly suggests that the contractile response to cromakalim does not involve stimulation of either adenylate or guanylate cyclases or inhibition of Ca entry through dihydropyridine-sensitive Ca channels. From these findings it is concluded that the observed contraction is a unique feature of the K-channel openers and which is not shared by the other classes of smooth muscle relaxant tested.

Having established that this response was a characteristic of K-channel openers, we examined whether the contraction could be modified by procedures known to inhibit the relaxant effects of these agents. The first of these, which is a diagnostic feature of agents like cromakalim (Buckingham *et al.*, 1989; Murray *et al.*, 1989, Newgreen *et al.*, 1990; Piper *et al.*, 1990) was the ability of glibenclamide, a blocker of the ATP-sensitive K-channel in cardiac myocytes (Escande *et al.*, 1988) and pancreatic β cells (Zünkler *et al.*, 1988), to inhibit the response. Pretreatment with this agent produced a competitive-like inhibition of the K-channel opener induced contraction to cromakalim, consistent with an association between this event and the opening of glibenclamide-sensitive K-channels.

Relaxations induced by K-channel openers are also inhibited in PSS containing high concentrations of KCl (usually ≥ 40 mM). Elevation of extracellular K depolarizes the smooth muscle cells and shifts the potassium equilibrium potential (E_K) to a less negative value (Hamilton *et al.*, 1986). Under these experimental conditions the relaxant efficacy of cromakalim is markedly reduced or even abolished as has been widely reported for a variety of smooth muscles (Allen *et al.*, 1986; Hamilton *et al.*, 1986; Hollingsworth *et al.*, 1987; Foster *et al.*, 1989). Likewise, in the present study cromakalim failed to produce any contractile response in rabbit aortic strips following pre-incubation in Ca-free PSS containing an elevated [KCl] of 65.9 mM. This finding adds further to the similarities between the pharmacological characteristics of the contractile response and those of other K-channel opener-induced responses in normal PSS. The tension increment produced in the present study by elevating the [KCl] of

Ca-free PSS *per se* may result from depolarization-induced release of intracellular Ca (Kobayashi *et al.*, 1986).

The ^{42}K-efflux studies showed that cromakalim produced an approximately 3 fold increase in the rate of ^{42}K-exchange from strips of rabbit aorta bathed in Ca-free PSS. Consistent with the ability of glibenclamide to inhibit the cromakalim-induced contraction, this rise in ^{42}K-efflux was fully inhibited following pre-treatment with glibenclamide. Sulphonylurea-sensitive K-efflux in normal Ca-containing PSS has been observed both in the present study (data not shown) and on many previous occasions (Buckingham *et al.*, 1989; Quast & Cook, 1989; Newgreen *et al.*, 1990). In all these cases, the increase in K-efflux was lower than that produced by cromakalim under the present Ca-free conditions. In Ca-free solutions there was an elevated basal efflux rate which is thought to reflect destabilization of the plasmalemma. This elevated K-permeability is often experimentally restricted by the inclusion of raised levels of Mg in the bathing solution (Martin & Gordon, 1983; Coldwell & Howlett, 1988). Although this procedure was adopted in the present study, there still remained an increased basal level of ^{42}K efflux, in agreement with more recently published data (Post *et al.*, 1989; Cox, 1990). Clearly this destabilizing effect of Ca-free solution may explain why the increments in efflux produced by cromakalim were higher in Ca-free compared to Ca-containing PSS.

Complex electrical changes occurred when bovine trachealis strips bathed in Ca-free PSS were exposed to cromakalim. Initially, a significant membrane hyperpolarization was observed in both Ca-containing and Ca-free PSS on exposure to cromakalim. Such an increase in membrane potential is a well-documented effect of cromakalim in Ca-containing PSS (Allen *et al.*, 1986; Hamilton *et al.*, 1986; Foster *et al.*, 1989; Bray *et al.*, 1991). However, in contrast to the well-maintained hyperpolarization observed in Ca-containing PSS, the cromakalim-induced hyperpolarization in Ca-free PSS was found to fade gradually leading eventually to a depolarization relative to pre-cromakalim levels. The reason for this phenomenon is not understood although a similar effect has been described for adrenaline-induced hyperpolarization of guinea-pig taenia caeci. Like the response to cromakalim in trachealis, these adrenaline-induced hyperpolarizations of taenia caeci are well-maintained in Ca-containing PSS yet exhibit fading in Ca-free PSS. Intracellular Ca release and the subsequent opening of Ca-dependent K channels are thought to be responsible for the adrenaline-induced hyperpolarization and the non-sustained nature of the response in Ca-free PSS may reflect depletion of internal Ca stores (Den Hertog & Van Den Akker, 1987). Whether the present fade also reflects such a depletion of Ca stores cannot be determined from the present data. Indeed, the Ca-dependency of the cromakalim-sensitive K-channel is still subject to controversy (e.g. Beech & Bolton, 1989; Gelband *et al.*, 1989; Noack *et al.*, 1992) and the state of the intracellular Ca stores during the K-channel opener-induced contractile response remains unknown. Clearly further work is needed to clarify this matter.

Whatever the cause of the non-sustained hyperpolarization, it is arguably an important event considering the temporal correlation between this response and the gradual rise in tension observed with cromakalim in Ca-free PSS. The possibility remains, therefore, that this reduction in the hyperpolarization is of paramount significance in the expression of the contractile response. Such a fall in membrane potential may indeed initiate some secondary intracellular event associated with the resultant tension generation. However, as yet no causal relationship has been demonstrated between these two parameters. Furthermore, the lack of Ca together with the presence of EGTA in the PSS makes it unlikely that residual extracellular Ca entering through voltage-dependent Ca-channels is responsible for the contraction.

Although the time course of the cromakalim-induced contraction differs markedly from typical intracellular Ca release phenomena, the absence of extracellular Ca during the response argues in favour of a contributory role of such Ca release. For some time links have been proposed between intracellular Ca release and K-channel modulation in the sarcoplasmic reticular membrane of skeletal muscle (Somlyo *et al.*, 1981). In functional studies in a diversity of preparations, Ca release from internal stores such as those in the sarcoplasmic and endoplasmic reticulum of skeletal muscle has been shown to require a countercurrent flow of K^+ in order to maintain electroneutrality across the membrane of the store (Muallem *et al.*, 1985; Fink & Stephenson, 1987; Shah & Pant, 1988). In the study of Fink & Stephenson (1987), K-channel blockade in skeletal muscle increased the loading and subsequent agonist-induced release of Ca from internal stores. Consistent with this effect, K-channel opening (induced by cromakalim) reduced subsequent release and/or refilling of intracellular Ca stores in rabbit renal artery and aorta (Wilson & Cooper, 1989; Bray *et al.*, 1991). Since K-channel blockade was shown to inhibit leakage of Ca from the internal store in skeletal muscle by reducing transmembrane K-conductance, it follows that K-channel opening may induce leakage from the Ca store in smooth muscle by enhancing the K-conductance of this membrane. Such an increased K-movement across the membrane could in turn induce an enhanced leak of Ca from the store leading to its eventual depletion. The outcome of such a response could have produced the effects described by Bray *et al.* (1991) and Wilson & Cooper (1989). In direct connection to the present study, this speculative Ca leak may feasibly account for both the non-maintained hyperpolarization and the tension generation observed with cromakalim in Ca-free solution. However, recently-published data in smooth muscle cells isolated from rabbit trachea suggest that BRL 38227 does not release intracellular calcium *per se* although it does inhibit release and re-uptake of this calcium source (Chopra *et al.*, 1992). Clearly further work is required to clarify these issues and to determine the potential significance of these findings with respect to the present contractile response.

Smooth muscle contractions associated with the release of Ca from intracellular stores generally exhibit a much faster time-course than that of the cromakalim-induced increase in tension (Iino, 1990). In contrast, the time-dependency of the response to cromakalim was similar to that of the smooth muscle contractions produced by phorbol esters (under both Ca-containing and Ca-free conditions) via activation of protein kinase C (Sybertz *et al.*, 1986; Khalil & van Breemen, 1988). Whether a similar mechanism involving activation of this enzyme accounts for the cromakalim-induced contraction is the subject of current investigations.

S.D. held an SERC Case Award in conjunction with Roche Products Ltd.

References

ALLEN, S.L., BOYLE, J.P., CORTIJO, J., FOSTER, R.W. & SMALL, R.C. (1986). Electrical and mechanical effects of BRL 34915 in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **89**, 395–405.

BEECH, D.J. & BOLTON, T.B. (1989). Properties of the cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. *Br. J. Pharmacol.*, **98**, 851–864.

BRAY, K.M., DUTY, S. & WESTON, A.H. (1989). Analysis of the spasmogenic effects of cromakalim in rabbit isolated aorta. *J. Physiol.*, **417**, 67P.

BRAY, K.M., WESTON, A.H., DUTY, S., NEWGREEN, D.T., LONGMORE, J., EDWARDS, G. & BROWN, T.J. (1991). Differences between the effects of cromakalim and nifedipine on agonist-induced responses in rabbit aorta. *Br. J. Pharmacol.*, **102**, 337–344.

BUCKINGHAM, R.E., CLAPHAM, J.C., COLDWELL, M.C., HAMILTON, T.C. & HOWLETT, D.R. (1986). Stereospecific mechanism of action of the novel antihypertensive agent, BRL 34915. *Br. J. Pharmacol.*, **87**, 78P.

BUCKINGHAM, R.E., HAMILTON, T.C., HOWLETT, D.R., MOOTOO, S. & WILSON, C. (1989). Inhibition by glibenclamide of the vasorelaxant action of cromakalim in the rat. *Br. J. Pharmacol.*, **97**, 57–64.

CHOPRA, L.C., TWORT, C.H.C. & WARD, J.P.T. (1992). Direct action of BRL 38227 and glibenclamide on intracellular calcium stores in cultured airway smooth muscle of rabbit. *Br. J. Pharmacol.*, **105**, 259–260.

COLDWELL, M.C. & HOWLETT, D.R. (1988). Potassium efflux enhancement by cromakalim (BRL 34915) in rabbit mesenteric artery: an indirect effect independent of calcium? *Biochem. Pharmacol.*, **37**, 4105–4110.

COX, R.H. (1990). Effects of a putative K^+ channel activator, BRL 34915 on arterial contraction and ^{86}Rb efflux. *J. Pharmacol. Exp. Ther.*, **252**, 51–59.

DEN HERTOG, A. & VAN DEN AKKER, J. (1987). The action of procainamide and quinidine on the alpha 1-receptor-operated channels in smooth muscle cells of guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **137**, 233–239.

DUTY, S., PACIOREK, P.M., WATERFALL, J.F. & WESTON, A.H. (1990). A comparison of the regional haemodynamic profiles of Ro 31-6930, cromakalim and nifedipine in anaesthetised normotensive rats. *Eur. J. Pharmacol.*, **185**, 35–42.

DUTY, S. & WESTON, A.H. (1991a). Effects of cromakalim on agonist-induced changes in tension and intracellular calcium levels in isolated strips of bovine trachealis. *Br. J. Pharmacol.*, **104**, 297P.

DUTY, S. & WESTON, A.H. (1991b). The effects of cromakalim on membrane potential and tension in isolated bovine trachealis in Ca-free and Ca-containing physiological salt solutions. *Br. J. Pharmacol.*, **102**, 173P.

ESCANDE, D., THURINGER, D., LEGUERN, S. & CAVERO, I. (1988). The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent K^+ channels in isolated cardiac myocytes. *Biochem. Biophys. Res. Commun.*, **154**, 620–625.

FINK, R.H.A. & STEPHENSON, D.G. (1987). Ca^{2+} movements in muscle modulated by the state of K^+ channels in the sarcoplasmic reticulum membrane. *Pflügers Arch.*, **409**, 374–380.

FOSTER, C.D., FUJII, K., KINGDON, J. & BRADING, A.F. (1989). The effect of cromakalim on the smooth muscle of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **97**, 281–291.

GELBAND, C.H., LODGE, N.J. & VAN BREEMEN, C. (1989). A Ca^{2+}-activated K^+ channel from rabbit aorta: modulation by cromakalim. *Eur. J. Pharmacol.*, **167**, 201–210.

GUAN, Y.Y., KWAN, C.Y. & DANIEL, E.E. (1988). The effects of EGTA on vascular smooth muscle contractility in calcium-free medium. *Can. J. Physiol. Pharmacol.*, **66**, 1053–1056.

HAMILTON, T.C., WEIR, S.W. & WESTON, A.H. (1986). Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity of rat portal vein. *Br. J. Pharmacol.*, **88**, 103–111.

HAMILTON, T.C. & WESTON, A.H. (1989). Cromakalim, nicorandil and pinacidil: Novel drugs which open potassium channels in smooth muscle. *Gen. Pharmacol.*, **88**, 103–111.

HOF, R.P., QUAST, U., COOK, N.S. & BLARER, S. (1988). Mechanism of action of systemic and regional haemodynamics of the potassium channel activator BRL 34915 and its enantiomers. *Circ. Res.*, **62**, 679–686.

HOLLINGSWORTH, M., AMÉDÉE, T., EDWARDS, D., MIRONNEAU, J., SAVINEAU, J.P., SMALL, R.C. & WESTON, A.H. (1987). The relaxant action of BRL 34195 in the rat uterus. *Br. J. Pharmacol.*, **91**, 803–813.

IINO, M. (1990). Calcium release mechanisms in smooth muscle. *Jap. J. Pharmacol.*, **54**, 345–354.

ITO, S., KAJIKURI, J., ITOH, T. & KURIYAMA, H. (1991). Effects of lemakalim on changes in Ca^{2+} concentration and mechanical activity induced by noradrenaline in the rabbit mesenteric artery. *Br. J. Pharmacol.*, **104**, 227–233.

KHALIL, R.A. & VAN BREEMEN, C. (1988). Sustained contraction of vascular smooth muscle: calcium influx or protein kinase C activation? *J. Pharmacol. Exp. Ther.*, **244**, 537–542.

KOBAYASHI, S., KANAIDE, H. & NAKAMURA, M. (1986). Complete overlap of caffeine- and K^+ depolarisation-sensitive intracellular calcium storage sites in cultured rat arterial smooth muscle cells. *J. Biol. Chem.*, **261**, 15709–15713.

MARTIN, W. & GORDON, J.L. (1983). Differential calcium dependence of contractile responses and ^{86}Rb efflux from rabbit aorta induced by vasoactive stimuli. *J. Cell. Physiol.*, **115**, 46–52.

MUALLEM, S., SCHOEFFIELD, M., PANDOL, S. & SACHS, G. (1985). Inositol trisphosphate modification of ion transport in rough endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.*, **82**, 4433–4437.

MURRAY, M.A., BOYLE, J.P. & SMALL, R.C. (1989). Cromakalim-induced relaxation of guinea-pig isolated trachealis: antagonism by glibenclamide and by phentolamine. *Br. J. Pharmacol.*, **98**, 865–874.

NEWGREEN, D.T., BRAY, K.M., MCHARG, A.D., WESTON, A.H., DUTY, S., BROWN, B.S., KAY, P.B., EDWARDS, G., LONGMORE, J. & SOUTHERTON, J.S. (1990). The action of diazoxide and minoxidil sulphate on rat blood vessels: a comparison with cromakalim. *Br. J. Pharmacol.*, **100**, 605–613.

NOACK, Th., DEITMER, P., EDWARDS, G. & WESTON, A.H. (1992). Characterization of potassium currents modulated by BRL 38227 in rat portal vein. *Br. J. Pharmacol.*, **106**, 717–726.

PACIOREK, P.M., COWLRICK, I.S., PERKINS, R.S., TAYLOR, J.C., WILKINSON, T.G.F. & WATERFALL, J.F. (1990). Ro 31-6930, a novel potassium channel opener with smooth muscle relaxant and antihypertensive properties. *J. Cardiovasc. Pharmacol.*, **15**, 188–197.

PIPER, I., MINSHALL, E., DOWNING, S.J., HOLLINGSWORTH, M. & SADRAEI, H. (1990). Effects of several potassium channel openers and glibenclamide on the uterus of the rat. *Br. J. Pharmacol.*, **101**, 901–907.

POST, J.M., SMITH, J.M. & JONES, A.W. (1989). BRL 34915 (cromakalim) stimulation of ^{42}K efflux from rabbit mesenteric arteries is modulated by calcium. *J. Pharmacol. Exp. Ther.*, **250**, 591–597.

QUAST, U. & COOK, N.S. (1989). In vitro and in vivo comparison of two K^+ channel openers, diazoxide and cromakalim, and their inhibition by glibenclamide. *J. Pharmacol. Exp. Ther.*, **250**, 261–271.

SHAH, J. & PANT, H.C. (1988). Potassium channel blockers inhibit inositol trisphosphate-induced calcium release in the microsomal fractions isolated from the rat brain. *Biochem. J.*, **250**, 617–620.

SOMLYO, A.V., GONZALEZ-SERRATOS, H., SHUMAN, H., MCCLELLAN, G. & SOMLYO, A.P. (1981). Calcium release and ionic changes in the sarcoplasmic reticulum of tetanized muscle: an electron probe study. *J. Cell Biol.*, **90**, 577–594.

SYBERTZ, E.J., DESIDERIO, D.M., TETZLOFF, G. & CHIU, P.J.S. (1986). Phorbol dibutyrate contractions in rabbit aorta: calcium dependence and sensitivity to nitrovasodilators and 8-Br-cyclic GMP. *J. Pharmacol. Exp. Ther.*, **239**, 78–83.

WILSON, C. & COOPER, S.M. (1989). Effect of cromakalim on contractions in rabbit isolated renal artery in the presence and absence of extracellular Ca^{2+}. *Br. J. Pharmacol.*, **98**, 1303–1311.

YANAGISAWA, T., TESHIGAWARA, T. & TAIRA, N. (1990). Cytoplasmic calcium and the relaxation of canine coronary arterial smooth muscle produced by cromakalim, pinacidil and nicorandil. *Br. J. Pharmacol.*, **101**, 157–165.

ZÜNKLER, B.J., LENZEN, S., MÉNNER, K., PANTEN, U. & TRUBE, G. (1988). Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic β cells. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, 225–230.

(Received June 2, 1992
Revised August 12, 1992
Accepted September 1, 1992)

British Journal of Pharmacology

VOLUME 107 (4) DECEMBER 1992

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.

Special Reports should normally occupy no more than two printed pages of the Journal; two illustrations (Figures or Tables, with legends) are permitted. As a guideline, with type face of 12 pitch and double-line spacing, a page of A4 paper could contain about 400 words. The absolute maximum length of the *Special Report* is 1700 words. For each Figure or Table, please deduct 200 words. The manuscript should comprise a Title page with key words (maximum of 10), a Summary consisting of a single short paragraph, followed by Introduction, Methods, Results, Discussion and References (maximum of 10). In all other respects, the requirements are the same as for Full Papers (see current 'Instructions to Authors').

Papers will be considered for publication on all aspects of pharmacology, including chemotherapy.

Manuscripts (two copies) should be sent to Editorial Office, British Journal of Pharmacology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE. Authors should consult the Instructions to Authors and the Nomenclature Guidelines for Authors in Vol. 107, January 1993. These Instructions and Guidelines also appear with the journal Index for volumes 105–107, 1992.

The *British Journal of Pharmacology* is published monthly by the Scientific & Medical Division, Macmillan Press Ltd.

The journal is covered by *Current Contents*, *Excerpta Medica* and *Index Medicus*.

All business correspondence and reprint requests should be addressed to the Scientific & Medical Division, Macmillan Press Ltd., Houndmills, Basingstoke, Hampshire RG21 2XS, UK. Telephone: (0256) 29242; Fax: (0256) 810526.

Annual subscription prices for 1993 EC £455, elsewhere £499/US$950 (sterling rate is definitive). Orders must be accompanied by remittance. Cheques should be made payable to Macmillan Press, and sent to: Macmillan Press Ltd., Subscription Department, Brunel Road, Houndmills, Basingstoke, Hampshire RG21 2XS, UK.

Overseas subscribers may make payments into UK Post Office Giro Account No. 5192455. Full details must accompany the payment.

Second Class postage paid at Rahway NJ. US Mailing Agent: Mercury Airfreight International Ltd, Inc., 2323 Randolph Avenue, Avenel, New Jersey, NJ 07001, USA.

Enquiries concerning advertising space or rates should be addressed to: Michael Rowley, Advertisement Manager, Macmillan Press Ltd., 4 Little Essex Street, London WC2R 3LF. Telephone: 071 836 6633; Fax: 071 379 0820.

ISSN 0007–1188

0007–1188/92 $3.50 + $0.00

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.

Special Reports should normally occupy no more than two printed pages of the Journal; two illustrations (Figures or Tables, with legends) are permitted. As a guideline, with type face of 12 pitch and double-line spacing, a page of A4 paper could contain about 400 words. The absolute maximum length of the *Special Report* is 1700 words. For each Figure or Table, please deduct 200 words. The manuscript should comprise a Title page with key words (maximum of 10), a Summary consisting of a single short paragraph, followed by Introduction, Methods, Results, Discussion and References (maximum of 10). In all other respects, the requirements are the same as for Full Papers (see current 'Instructions to Authors').

PREPARATION OF MANUSCRIPTS

Authors are strongly recommended to read the full *Instructions to Authors* and *Nomenclature Guidelines for Authors* (*Br. J. Pharmacol.* 1992, **105,** 245–254) 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. **Acknowledgments**—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*, 1992, **105,** 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)
    (c) the same material is neither 'in press' (i.e. is in proof or has definitely been accepted for publication) nor under consideration elsewhere. Furthermore it will not be submitted or published elsewhere before a decision has been reached by the Editorial Board of the *British Journal of Pharmacology* and will not be submitted elsewhere if accepted by the *British Journal of Pharmacology*.
    (d) Copyright assignment is included.

12. **Cover letter**—To state clearly
    (a) Corresponding author's full postal address, telephone, telex or Fax number
    (b) where appropriate, that *either* ethical approval has been given for investigation *or* Company or Institutional permission to publish work has been received.

13. **Reminder**—Packaging to be sufficiently robust to protect Figures and to withstand mailing.

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., 1992,* **105,** 245–254.

BRITISH JOURNAL OF PHARMACOLOGY VOLUME 107 (4) 1992

Typeset by Elite Typesetting Techniques, Southampton, Hampshire
and printed in Great Britain by Unwin Brothers Ltd., The Gresham Press, Old Woking, Surrey