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SPECIAL REPORT

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Nitric oxide is involved in the regulation of angiogenesis

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The *in vivo* model of the chick embryo chorioallantoic membrane (CAM) was used to study the involvement of nitric oxide (NO) in angiogenesis. The nitrovasodilator sodium nitroprusside (NaNP) and the amino acid, L-arginine, inhibited angiogenesis, assessed as both collagenous protein biosynthesis and vascular density. N^G-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor, increased both collagenous protein biosynthesis and vascular density, indicating that this agent promotes angiogenesis. These results suggest that NO may participate in the regulation of angiogenesis. Manipulation of NO synthesis therefore, may prove to be another approach for controlling angioproliferative diseases.

Keywords: Chick chorioallantoic membrane (CAM); nitric oxide (NO); N^G-mono-methyl-L-arginine (L-NMMA); angiogenesis; collagenous protein biosynthesis; sodium nitroprusside (NaNP)

Introduction Angiogenesis is, with few exceptions, a very slow process in the adult (Denekamp, 1982). It is accelerated however in a variety of proliferative states, including tumour growth and wound healing (Folkman, 1985; Leibovich, 1984). Although many angiogenic mediators have been identified, the exact sequence of events involved and the regulation of this process remain unclear. Major steps in angiogenesis include invasion of the vascular wall of the 'parent vessel' by activated endothelial cells which then proliferate, migrate and form the lumen of the new vessel. Nitric oxide (NO), is an endogenous vasoactive molecule with multiple properties (Moncada *et al.*, 1991). Recently, it was shown that nitrovasodilators including sodium nitroprusside (NaNP), inhibit endothelial cell proliferation via stimulation of guanylate cyclase (Garg & Hassid, 1989) suggesting that this effect was probably due to spontaneous NO formation by NaNP. Angiogenesis is intimately linked to endothelial cell proliferation. It was of interest therefore to investigate whether NO formation is involved in this process. In the present study the effects of N^G-monomethyl-L-arginine (L-NMMA) (Rees *et al.*, 1989), NaNP and L-arginine, were examined on the chick chorioallantoic membrane (CAM), an *in vivo* model of angiogenesis (Folkman, 1985). It is reported here that NO may be involved in the maintenance of the non-angiogenic status of the vascular endothelium and as such may be a target for the control of angioproliferative diseases.

Methods The *in vivo* CAM angiogenesis model, initially described by Folkman (1985) and modified as previously reported (Maragoudakis *et al.*, 1988) was used. Briefly, fresh fertilized eggs were incubated for 4 days at 37°C when a window was opened on the egg shell, exposing the CAM. The window was covered with tape and the eggs were returned to the incubator until day 9 when the test materials were applied. The test materials or vehicle and 0.5 μCi [U-^{14}C]-labelled proline, were placed on sterile plastic discs and were allowed to dry. The control discs (containing vehicle and radiolabelled proline) were placed on the CAM 1 cm away from the disc containing the test material. A solution of cortisone acetate (100 μg/ disc) was routinely incorporated in all discs in order to prevent an inflammatory response. The discs were inverted and placed on the CAM, the windows were covered and the eggs incubated until day-11 when assessment of angiogenesis took place.

Biochemical evaluation of newly formed vessels was performed by determining the extent of collagenous protein biosynthesis in the CAM lying directly under the discs (Maragoudakis *et al.*, 1988). Briefly, the area under the disc was cut off, placed in an appropriate buffer and protein biosynthesis was stopped. Non protein bound radioactivity was removed by washing with trichloroacetic acid. Pellets containing protein-bound radioactivity were resuspended and subjected to collagenase digestion. The resulting tripeptides corresponding to basement membrane collagen and other collagenous material synthesized by the CAM from [U-^{14}C]-proline, were counted and expressed as c.p.m. mg^{-1} protein.

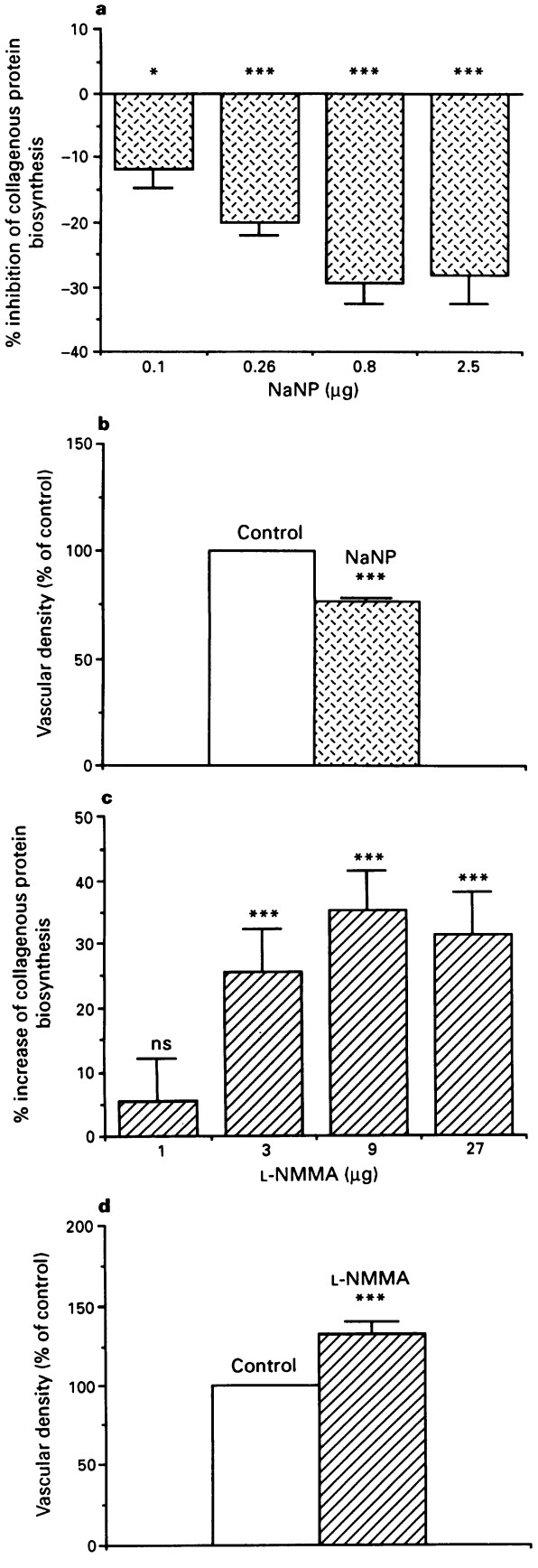
For morphological evaluation, eggs were treated as above in the absence of radiolabelled proline. At day 12, eggs were flooded with 10% buffered formalin and the plastic discs removed. An area around the disc was cut off, placed on a glass slide and the vascular density index was measured by the method of Harris-Hooker *et al.* (1983).

For each egg, total collagenous protein biosynthesis and vascular density under the disc containing the test material were expressed as a percentage of the control disc containing vehicle. Results were compared by paired *t* test.

Materials Fertilized eggs were obtained from Ioannina, Greece. Collagenase type VII from clostridium histolyticum, cortisone acetate, L-arginine and sodium nitroprusside were purchased from SIGMA Chem. Co. (Poole). L-[U-^{14}C]-proline, specific activity 273 mCi mmol^{-1} was purchased from New England Nuclear Inc. N^G-monomethyl-L-argine (L-NMMA) was a gift from Dr S. Moncada.

Results Sodium nitroprusside from 0.1–7.5 μg/ disc caused a dose-dependent decrease in collagenous protein biosynthesis (Figure 1a) which ranged from 11.8 ± 3.1% to 29.5 ± 4.4% of control (n = 5-22). Furthermore, morphological evaluation of the effect of NaNP (2.5 μg/ disc) on the CAM showed that this compound caused a 23.4 ± 1.9% (n = 10) decrease in vascular density (Figure 1b). The NO synthase inhibitor L-NMMA (Rees *et al.*, 1989) from 1.0–27.0 μg/ pellet caused a dose-related increase in collagenous protein biosynthesis (Figure 1c) ranging from 5.6 ± 5.5% to 32.0 ± 8.4% of control (n = 7–37). A similar result was observed when the effect of L-NMMA (27 μg/disc) on the CAM was assessed morphologically, i.e. a 30.8 ± 5.8%, (n = 10) increase in vascular density was observed (Figure 1d). The inactive analogue D-NMMA, when present at the same doses, had no effect (data not shown). L-Arginine, the natural precursor of NO, at a dose of 2.3 μg/ disc, caused a decrease in collagenous protein

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biosynthesis of 22.9 ± 5.0%, compared to control, ($n = 10$, $P < 0.01$). The same amount of L-arginine caused a 9.8 ± 3.0%, ($n = 5$, $P < 0.05$), decrease in vascular density. Larger doses (up to 21 μg) did not cause a further decrease (data not shown) in either parameter.

Discussion In the present study it was shown that NaNP, a vasolidator which generates NO spontaneously (Ignarro *et al.*, 1981), caused a significant depression of angiogenesis as shown by decreases in collagenous protein biosynthesis and vascular density in the CAM. The amino acid L-arginine, the endogenous precursor for NO formation (Moncada *et al.*, 1991), had a similar albeit smaller effect. L-NMMA a specific inhibitor of NO synthase (Rees *et al.*, 1989) (but not the inactive analogue D-NMMA) caused a significant increase in collagenous protein biosynthesis and vascular density. These results suggest that NO negatively regulates the angiogenic process. The modest antiangiogenic effect caused by L-arginine (particularly when vascular density was assessed) is not unexpected since exogenous L-arginine is unable to compete with the endogenous amino acid which may be presented to the enzyme more efficiently (Rees *et al.*, 1989). The extent of increase of angiogenesis caused by L-NMMA, was around 30% and may reflect the extent to which NO may participate in the angiogenic process in the CAM under the present experimental conditions. The experiments described here were performed in the presence of cortisone in order to prevent an inflammatory response in the CAM, leading to artifacts. However, corticosteroids have been shown to inhibit the inducible form of the NO synthase (O'Connor & Moncada, 1991). This may lead to an underestimation of the role of NO of angiogenesis. Efforts are being made to devise experiments where the contribution of this form of the enzyme will be studied, without compromising the reproducibility of the system.

Angiogenesis is a phenomenon of pathophysiological importance which proceeds in a sequence of distinct steps involving proliferative and migratory phenomena (Folkman, 1985). NO may act directly in the CAM to inhibit endothelial cell proliferation as it does in messangial cells (Garg & Hassid, 1989). On the other hand NO may act indirectly by preventing endogenous mediators of cell growth to be released and/or act as mitogens. In conclusion, modulation of NO formation appears to have an effect on angiogenesis and under normal conditions, may play a role in maintaining the vascular endothelium in a quiescent state. It is possible that in 'angiogenic' disease states where excessive angiogenesis is evident, the synthesis or availability of NO is compromised, thus, tipping the balance towards the initiation of proliferative events in the vessel wall. This new role for NO may offer an alternative way of controlling angiogenesis-associated disease.

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Figure 1 Effect of sodium nitroprusside (NaNP) and of N^G-monomethyl-L-arginine (L-NMMA) on angiogenesis in the chick chorioallantoic membrane (CAM) expressed as (a,c) collagenous protein biosynthesis (CPB) and (b,d) vascular density. Results are expressed as mean ± s.e. of the mean% of control and are compared by Student's paired t test. $^*P < 0.05$; $^{**}P < 0.02$ and $^{***}P < 0.01$. The number of observations n is indicated in the text.

References

DENECAMP, J. (1982). Endothelial cell proliferation as a novel approach to targetting tumour therapy. *Br. J. Cancer*, **45**, 136–139.

FOLKMAN, J. (1985). Tumor angiogenesis. *Adv. Cancer Res.*, **43**, 175–203.

GARG, U.C. & HASSID, A. 1989). Inhibition of rat messangial cell mitogenesis by nitric oxide-generating vasodilators. *Am. J. Physiol.*, **257**, F60–F66.

HARRIS-HOOKER, S.A., GAJDUSEC, S.M., WIGHT, T.N. & SWARTZ, S.M. (1983). Neovascularization responses induced by cultured aortic endothelial cells. *J. Cell Physiol.*, **114**, 302–310.

IGNARRO, L.J., LIPPTON, H., BARICOS, W.H., HYMAN, A.L. KADOWITZ, P.J. & GRUETTER, C.A. (1981). Mechanisms of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide. Evidence for involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.*, **218**, 739–749.

LEIBOVICH, S.J. (1984). Mesenchymal cell proliferation in wound repair. The role of macrophages. In *Soft and Hard Tissue Repair*. ed. Hunt, T.K., Heppelston, R.B., Pines, E. & Rovee, D. pp. 329–351. New York: Praeger Press.

MARAGOUDAKIS, M.E., PANOUTSAKOPOULOU, M. & SARMONIKA, M. (1988). Rate of basement membrane biosynthesis as an index to angiogenesis. *Tissue Cell*, **20**, 531–539.

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

O'CONNOR, K.J. & MONCADA, S. (1991). Glucocorticoids inhibit the induction of nitric oxide synthase and the related cell damage in adenocarcinoma cells. *Biochim. Biophys. Acta*, **1097**, 227–231.

REES, D.D., PALMER, R.M.J., HODSON, H.H. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

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Inhibition by N^{ω}-nitro-L-arginine methyl ester of the electrocortical arousal response in rats

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In rats chronically implanted with cannulae into one lateral cerebral ventricle and recording electrodes onto the fronto-parietal cortex, the effects of systemic or intraventricular administration of the nitric oxide (NO) synthesis inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), on electrocortical (ECoG) arousal response evoked by sound stimulation were studied. In control animals, a single acoustic stimulation (80 dB for 15 s) produced a significant decrease in ECoG total voltage power lasting approximately 25 s. No tolerance developed after repeating the same sound stimulation at 15, 30, 60 min and 24 h intervals. Under these experimental conditions, pretreatment with L-NAME, given systemically (10 mg kg^{-1}, i.p.) or intracerebroventricularly (300 μg), significantly reduced the sound-evoked arousal response 1 h and 15 min later, respectively. In conclusion, the present data are in favour of a physiological role of NO in the control of arousal mechanisms.

Keywords: Nitric oxide (NO); electrocortical arousal; N^{ω}-nitro-L-arginine methyl ester

Introduction Nitric oxide (NO) has been shown to occur in the mammalian CNS (Bredt *et al.*, 1991) where it has messenger properties (Garthwaite, 1991; Moncada *et al.*, 1989). Despite its involvement as a putative trans-synaptic messenger in long-term depression (Shibuki & Okada, 1991) and potentiation (O'Dell *et al.*, 1991), the physiological significance of NO in the brain remains obscure. *In vitro* experiments have shown that receptor agonists (e.g. N-methyl-D-aspartate, NMDA) which enhance intracellular Ca^{2+}, can activate NO synthase to produce NO (see Garthwaite, 1991) by converting the endogenous substrate, L-arginine, into citrulline (Moncada *et al.*, 1989). Recently, it has been reported that intracerebroventricular (i.c.v.) microinfusion of L-arginine produces a marked fall in the amplitude of electrocortical (ECoG) activity and that this is abolished by previous microinfusion of N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Mollace *et al.*, 1991), an inhibitor of NO-synthase (Moncada *et al.*, 1991). This suggests that NO formed from L-arginine may produce ECoG arousal. In addition, the short half-life of NO (Moncada *et al.*, 1991) led us to hypothesize that it may act in the brain as a signalling molecule mediating rapid and short-lasting cortical neuronal activation. Under normal conditions, discrete peripheral stimuli produce a widespread, quantifiable ECoG desynchronization (arousal or alert response) (Kiloh *et al.*, 1972). Therefore, to test our hypothesis, we have studied the effects of the NO synthesis inhibitor, L-NAME, on sound-evoked ECoG desynchronization in rats.

Methods Adult male Wistar rats (250–280 g), housed in a temperature- (22 ± 2°C) and humidity- (60 ± 5%) controlled colony room, with tap water and laboratory food available *ad libitum*, were used for the study. Under chloral hydrate (400 mg kg^{-1}, i.p.) anaesthesia, surface cortical electrodes ($n = 4$) were implanted onto each fronto-parietal region (Bagetta *et al.*, 1988) to permit ECoG recording. The animals were allowed one week for recovery before testing. In awake, freely moving animals ECoG activating response was evoked by an acoustic stimulus (80 dB) of 15 s duration, delivered at 0, 15, 30 and 60 min and then at 24 h. The sound-evoked response, occurring in the domain of the total ECoG spectrum power (0.25–16 Hz) was monitored each second after the stimulus was delivered; computerized quantitation of the sound-evoked decrease in the ECoG signal amplitude (μV) was obtained with the aid of a Berg-Fourier analyzer (OTE Biomedica, Florence). Online, qualitative assessment of the ECoG activity was obtained with an 8-channel EEG machine interfaced with the Berg-Fourier analyzer. The data are expressed as mean ± s.e.mean.

Results The background ECoG activity in an awake rat is shown in Figure 1 (upper panel, trace a). Application of a 15 s acoustic stimulus (80 dB) induces ECoG desynchronization which is reproducible (Figure 1, upper panel, traces b and c). The evoked desynchronization started 6.6 ± 1.8 s after the delivery of the stimulus had been completed and this yielded a 69.6 ± 5.1% decrease in the ECoG signal amplitude. The reduction in amplitude reached its maximum 18.6 ± 4.2 s later and complete recovery to baseline level was obtained 23.0 ± 6.9 s after the stimulation had been completed. Repeated application of the acoustic stimulus (15 min, 30 min, 60 min and 24 h after the first stimulus; $n = 12$ experiments) did not produce significant changes in the latency to onset of ECoG response, time to peak or recovery time (Figure 2). Under these experimental conditions, systemic administration of L-NAME (10 mg kg^{-1}, i.p.; $n = 8$ rats) significantly reduced the sound-evoked ECoG response from 75.2 ± 5.6% to 39.7 ± 15.5% ($P < 0.05$; Student's t test) 60 min later, in comparison to controls (vehicle-treated) (Figures 1 and 2). No significant effects were evident 15 min and 30 min after L-NAME administration. In addition, this treatment doubled the latency of ECoG response onset (13.0 ± 2.5 s vs control 6.7 ± 2.2 s; $P < 0.05$) and significantly shortened the recovery time (15.0 ± 6.7 s vs control 30.0 ± 10.2 s; $P < 0.05$). A dose of 1.0 mg kg^{-1}, i.p. L-NAME was ineffective ($n = 6$). A similar inhibition of sound-evoked ECoG response was obtained with a shorter latency (15 min) after microinfusion of L-NAME (300 μg; $n = 6$) into one lateral cerebral ventricle (Figure 2).

Discussion The present data demonstrate that sound-evoked ECoG arousal is prevented by systemic injection of L-NAME, a potent inhibitor of NO synthesis. This effect does

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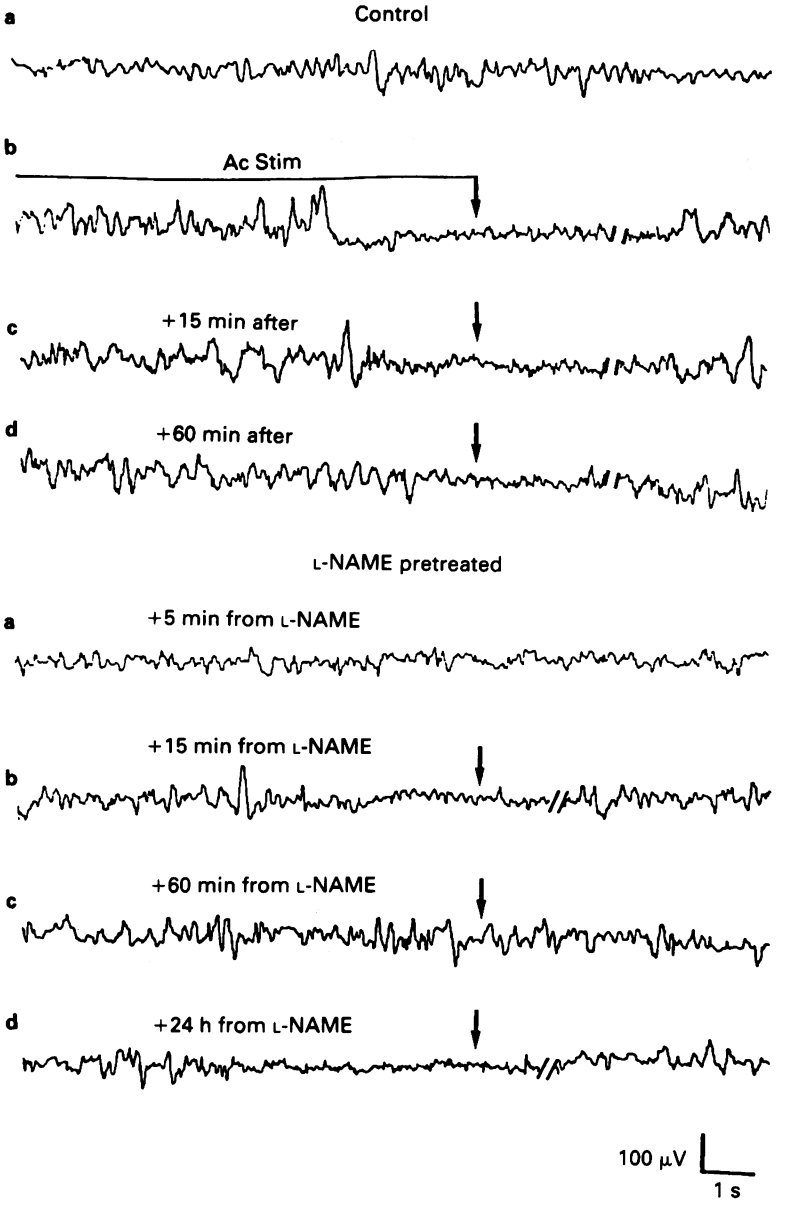


Figure 1 Electrocortical (ECoG) records from a control rat (upper panel) showing the desynchronizing ECoG response to a 15 s acoustic stimulus (Ac Stim) (80 dB; trace b); similar responses, are obtained 15 min (trace c) and 60 min (trace d) after the first stimulus has been delivered. Trace (a) shows the background ECoG activity. Systemic treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}, i.p.) prevents the sound-evoked ECoG response and this is evident 60 min after acoustic stimulation (lower panel; trace c) but not 5 min (trace a) or 15 min (trace b) after. Complete recovery is obtained 24 h after L-NAME administration (trace d). The arrowheads indicate the end of the acoustic stimulation. It is evident that ECoG arousal response starts before the end of acoustic stimulation (b, c and d of control experiments).

not seem to be due to the increase in systemic blood pressure eventually produced by L-NAME (see Moncada *et al.*, 1991) since pretreatment with adrenaline at a dose producing increase in blood pressure (1 mg kg^{-1}, s.c., given 5 min before sound stimulation) did not affect the ECoG arousal response (data not shown). The inhibitory effects induced by L-NAME are due to an action at the CNS level; in fact, i.c.v. injection of L-NAME produced a similar inhibition of sound-evoked ECoG response, though after a shorter (15 min) latency. Previous experiments have shown that intracerebral injection of nanomolar doses of NO or NO-releasing agents, e.g. SNAP (see Moncada *et al.*, 1991), produces ECoG desynchronization (Bagetta *et al.*, 1992). These effects were similar in duration and intensity to those produced by intracerebral 8-bromo-cyclic GMP and could be prevented by the NO-trapping agent, haemoglobin, given into the same site or by systemic pretreatment with methylene blue (Bagetta *et al.*, 1992), a soluble guanylate cyclase inhibitor (see Moncada *et al.*, 1991). The present experiments show that inhibition of NO synthesis in the rat brain leads to a significant reduction of the ECoG arousal response and, together with our previous findings (Mollace *et al.*, 1991; Bagetta *et al.*, 1992), suggest that NO may play a physiological role in the control of the arousal state.

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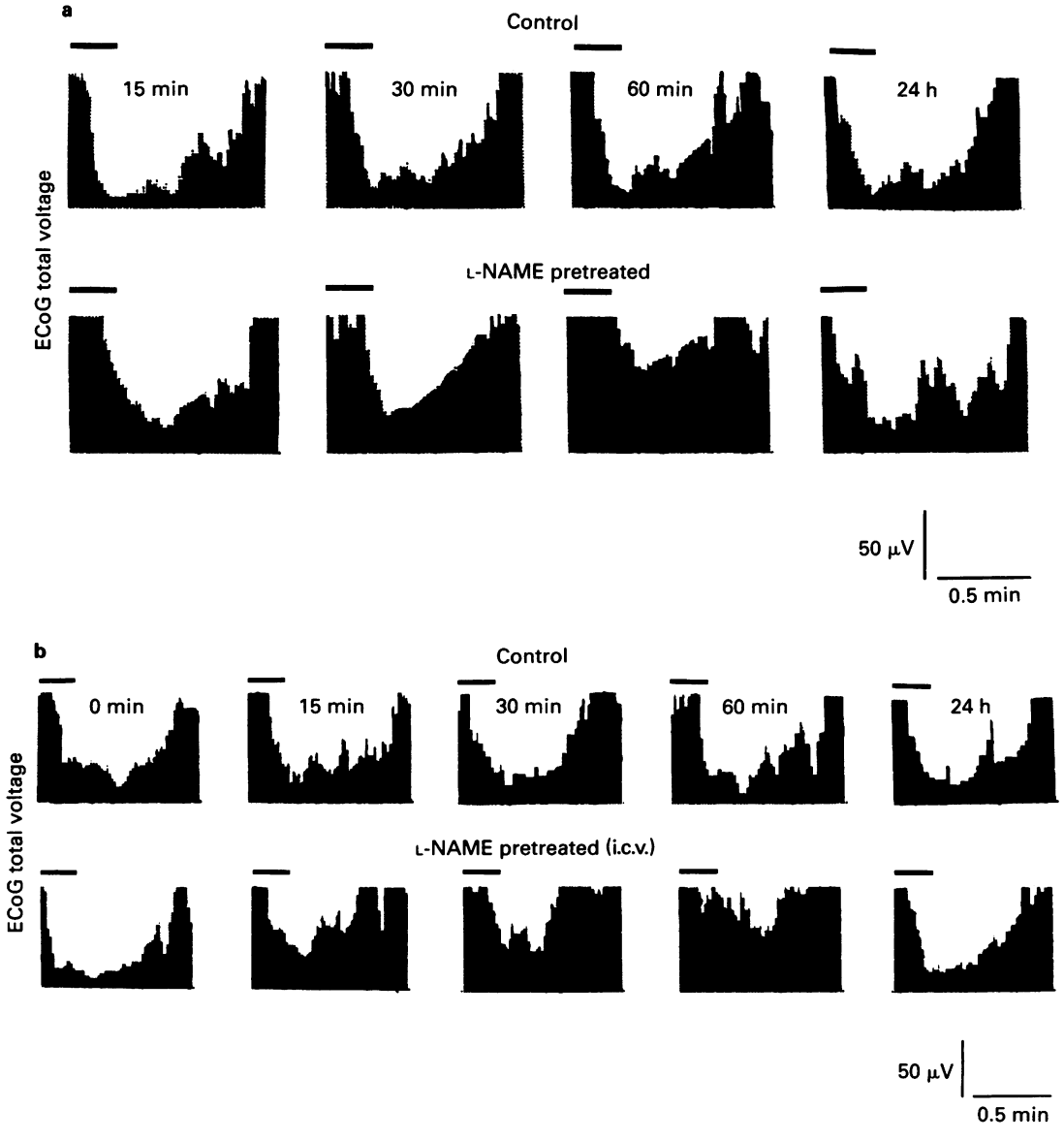


Figure 2 Computerized analysis of the electrocorticol (ECoG) desynchronizing response evoked by a 15 s acoustic stimulus (80 dB) in control and N^{ω}-nitro-L-arginine methyl ester (L-NAME) pretreated rats. In control rats ($n = 12$) sound stimulation evokes an approximately 80% decrease in ECoG signal amplitude with no reduction in the response intensity upon restimulation 15, 30 and 60 min and 24 h after the first stimulus (time zero) has been delivered. Significant reduction of the sound-evoked ECoG amplitude is observed 60 min and 15 min after systemic (10 mg kg^{-1}, i.p.; $n = 8$) (a) or intracerebroventricular (i.c.v; 300 μg; $n = 8$) (b) injection of L-NAME, respectively. The horizontal bar above individual ECoG records indicates the duration (see calibration) of the stimulus.

References

BAGETTA, G., DE SARRO, G.B., PRIOLO, E. & NISTICÒ, G. (1988). Ventral tegmental area: site through which dopamine D_2-receptor agonists evoke behavioural and electrocortical sleep in rats. *Br. J. Pharmacol.*, **95**, 860–866.

BAGETTA, G., IANNONE, M., LOMBARDI, U. & NISTICÒ, G. (1992). Evidence that nitric oxide is the mediator of the arousal electrocortical desynchronization in rats. International Congress on *Nitric Oxide: Brain and Immune System*, Paraelios (Calabria) Italy, 16–18 September, 1992. Abstract C12.

BREDT, D.S., GLATT, C.E., HWANG, P.M., FOTUHI, M., DAWSON, T.M. & SNYDER, S.H. (1991). Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron*, **7**, 615–624.

GARTHWAITE, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci.*, **14**, 60–67.

KILOH, L.G., COMAS, A.J. & OSSELTON, J.W. (1972). *Clinical Electroencephalography*. London: Butterworths.

MOLLACE, V., BAGETTA, G. & NISTICÒ, G. (1991). Evidence that L-arginine possesses proconvulsant effects mediated through nitric oxide. *Neuroreport*, **2**, 269–272.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1989). Biosynthesis of nitric oxide from L-arginine: a pathway for the regulation of cell function and communication. *Biochem. Pharmacol.*, **38**, 1709–1715.

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

O'DELL, T.J., HAWKINS, R.D., KANDEL, E.R. & ARANCIO, O. (1991). Tests of the role of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11285–11289.

SHIBUKI, K. & OKADA, D. (1991). Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature*, **349**, 326–328.

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Inhibitors of nitric oxide synthetase prevent castor-oil-induced diarrhoea in the rat

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1 Castor oil (2 ml orally) produced copious diarrhoea in rats 3 h after its administration.

2 Pretreatment (intraperitoneal, i.p.) of rats with the NO synthesis inhibitors N^G-nitro-L-arginine methyl ester (L-NAME, 1–25 mg kg^{-1}) and N^G-monomethyl-L-arginine (L-NMMA, 2.5–100 mg kg^{-1}) inhibited or prevented castor-oil-induced diarrhoea. L-Arginine (150–600 mg kg^{-1}, i.p.) administered to rats pretreated with L-NAME 10 mg kg^{-1}, drastically reduced the antidiarrhoeal activity of L-NAME in a dose-related manner. D-Arginine (900 mg kg^{-1}) did not modify the protection by L-NAME.

3 Pretreatment (i.p.) of rats with L-NAME (2.5–25 mg kg^{-1}) decreased the intestinal fluid accumulation and Na^+ secretion induced by castor oil. L-Arginine (600 mg kg^{-1}) but not D-arginine (900 mg kg^{-1}) counteracted the inhibitory effect of L-NAME (10 mg kg^{-1}).

4 L-NAME (10 and 25 mg kg^{-1}) had no significant effect on the intestinal transit in normal rats or those given castor oil.

5 These results provide evidence that nitric oxide (NO) could play an important role in castor-oil-induced diarrhoea.

Keywords: Castor oil; diarrhoea; NO; N^G-nitro-L-arginine methyl ester (L-NAME); N^G-monomethyl-L-arginine (L-NMMA); L-arginine

Introduction

Castor oil is a very effective laxative and, like bile salts and other laxatives, decreases absorption and increases secretion in the small intestine by multiple undetected mechanisms (for ref. see Donowitz & Welsh, 1987).

Castor oil and its active principle, ricinoleic acid, reduce active Na^+ and K^+ absorption and decrease Na^+, K^+-ATPase in the small intestine and colon (Gaginella & Phillips, 1975) but have not been clearly shown to increase adenosine 3':5'-cyclic monophosphate (cyclic AMP) content in the small intestine and even in the colon this effect is disputed (Simon *et al.*, 1978; Racusen & Binder, 1979). As with other laxatives, castor oil changes the intestinal permeability and the histology but it has not been established whether this contributes to the laxative effect (Gaginella & Phillips, 1976). Castor oil also increases platelet activating factor and some other autacoids throughout the rat intestinal tract (Capasso *et al.*, 1986; Pinto *et al.*, 1989; 1992) particularly in the small intestine. However, it remains to be determined rigorously whether this contributes to the laxative action. Castor oil also alters intestinal contractility (Gaginella & Bass, 1978) but the mechanism of this action, the type of response and its involvement in diarrhoea is still debated. Nitric oxide (NO), generated from L-arginine, but not from D-arginine, may modify gastrointestinal motility when released from non-adrenergic, non-cholinergic (NANC) neurones (Boeckxtaens *et al.*, 1990; Gustafsson *et al.*, 1990; Desai *et al.*, 1991).

In order to investigate whether NO plays a role in the diarrhoea caused by castor oil we have studied the effects of N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA), which inhibit the NO synthetase enzyme (Rees *et al.*, 1990; Gardiner *et al.*, 1990), and L-arginine, a natural substrate of NO synthetase (Moncada *et al.*, 1991).

Some of the results described here were presented at the First International Symposium on Natural Drugs and the Digestive Tract (Naples, 1992).

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Methods

Animals

Male Wistar (Morini) rats, weighing 150–170 g, were used after acclimatization for a week in their housing conditions (23 ± 2°C; 60% humidity). Standard food (Morini) was withheld 14 h before experiments but there was free access to drinking water.

Castor oil diarrhoea

Rats were treated intraperitoneally with L-NAME 1–25 mg kg^{-1}, L-NMMA 2.5–100 mg kg^{-1}, L-arginine 150–600 mg kg^{-1}, D-arginine 600 and 900 mg kg^{-1}; 15 min later 2 ml castor oil were administered by gavage. Control rats received the same volume (2 ml) of olive oil orally. Rats were scored (blind) for copious (++), mild (+) or lack (0) of diarrhoea 3 h after oil challenge. The activity score was calculated by taking the sum of the number of '+' rats and twice the number of '++' rats. Thus, for groups of 12 rats, the maximum score indicating severe diarrhoea was 24 and 0 was no diarrhoea.

Intestinal fluid volume and electrolyte secretion

Intraluminal fluid accumulation was determined by enteropooling (Robert *et al.*, 1976). L-NAME (2.5–25 mg kg^{-1}, i.p.) was injected 30 min before giving castor oil orally. Control rats received olive oil orally. After a further 30 min the animals were killed, the small intestine removed, its content collect in a test tube and its volume measured. Samples of the fluid were analysed for Na^+ concentrations by an electrochemical method after high performance liquid chromatography (h.p.l.c.) (Poole & Schuette, 1984).

Small intestinal transit

The method of Vischer & Casals-Stenzel (1983) was generally followed. Rats were given orally a 10% charcoal suspension in 5% gum arabic 30 min after they had received L-NAME

10 or 25 mg kg^{-1} i.p. or the vehicle. After 30 min the rats were killed and gastrointestinal tract was removed and the distance travelled by the marker was measured and expressed as a percentage of the total length of the intestine from the pylorus to caecum. L-NAME 10–25 mg kg^{-1} i.p. was also studied on intestinal transit in rats each giving 2 ml castor oil 30 min later and killed after a further 30 min.

Chemicals

N^G-nitro-L-arginine methyl ester (L-NAME) hydrochloride, N^G-monomethyl-L-arginine (L-NMMA) acetate, L-arginine hydrochloride, D-arginine hydrochloride, castor oil (all Sigma) and olive oil (Carapelli) were used.

Statistics

Diarrhoea was expressed as total score (Piercey & Ruwart, 1979) and a chi-squared test was used to determine the significance between groups. Fluid volume, Na^+ secretion and small intestinal transit were expressed as mean ± s.e; Student's unpaired or paired t test was used to determine the significance of differences between means and $P<0.05$ was taken as statistically significant.

Table 2 Diarrhoea induced by castor oil (CO) in rats treated with L- or D-arginine: effect of N^G-nitro-L-arginine methyl ester (L-NAME) 10 mg kg^{-1}

Treatment (mg kg^{-1}, i.p.)	*Diarrhoea score* ++	+	0	*Total score* (max = 24)
Control	0	0	12	0
CO (2 ml per rat)	12	0	0	24
CO + L-NAME	0	2	10	2
CO + L-NAME + L-arginine 150	0	3	9	3
CO + L-NAME + L-arginine 300	3	5	4	11
CO + L-NAME + L-arginine 600	10	1	1	21[a]
CO + L-NAME + D-arginine 900	0	2	10	2
CO + L-arginine 600	12	0	0	24
CO + D-arginine 900	12	0	0	24
L-Arginine 600	0	0	12	0
D-Arginine 900	0	0	12	0

Twelve animals were used for each group. N^G-nitro-L-arginine methyl ester (L-NAME), L-arginine or D-arginine were given i.p. 15 min before oral administration of castor oil. Results were analyzed by chi-squared test.
[a]$P<0.01$ vs castor oil (CO) + L-NAME.

Results

Castor oil diarrhoea

Three hours after castor oil administration all rats produced copious diarrhoea and a maximum score of 24 (Table 1). L-NAME 1 mg kg^{-1} had little effect while 2.5, 5 and 10 mg kg^{-1} reduced the diarrhoea score to 14, 8 and 2 respectively (Table 1). L-NAME 25 mg kg^{-1} gave complete protection. Lower doses of L-NMMA 2.5 and 25 mg kg^{-1} had little or no effect but 100 mg kg^{-1} caused a reduction. The highest dose of either L-NAME and L-NMMA or L-arginine did not alter the consistency or the number of faecal pellets of rats not given castor oil (Table 2). L-Arginine did not modify the castor-oil-induced diarrhoea, but it counteracted the effect of L-NAME 10 mg kg^{-1} in a dose-related manner (Table 2). In contrast, D-arginine 900 mg kg^{-1} did not modify the antidiarrhoeal effect of L-NAME (Table 2); it was also inactive in control rats, and did not modify diarrhoea in rats given castor oil.

Intestinal fluid accumulation

In preliminary experiments castor oil increased the volume of intestinal fluid, the effect being maximal at 30 min but absent after 60 min. In the following experiments the influence of L-NAME was therefore assessed at 30 min. Pretreatment with L-NAME dose-dependently reduced castor oil-induced intestinal fluid accumulation; L-NAME 25 mg kg^{-1} gave complete protection but did not alter intestinal fluid volume in control rats (Table 3). L-Arginine (600 mg kg^{-1}) but not D-arginine (900 mg kg^{-1}) counteracted the inhibitory effect of L-NAME (10 mg kg^{-1}). Neither L- nor D-arginine altered intestinal fluid volume in untreated (olive oil) rats or rats given castor oil.

Electrolyte secretion

Na^+ secretion induced by castor oil 30 min after its administration was inhibited by L-NAME (2.5–25 mg kg^{-1}) in a dose-related manner (Table 3). In rats given both 25 mg kg^{-1} of L-NAME and castor oil the values were similar to controls. L-NAME had a similar inhibitory effect on both Na^+ levels and fluid volume. L-Arginine (600 mg kg^{-1}) but not D-arginine (900 mg kg^{-1}) counteracted the L-NAME (10 mg kg^{-1}) effect.

Table 1 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) on diarrhoea induced by castor oil (CO)

Treatment (mg kg^{-1}, i.p.)	*Diarrhoea score* ++	+	0	*Total score* (max = 24)	*Protection* (%)
Control	0	0	12	0	–
CO (2 ml per rat)	12	0	0	24	–
CO + L-NAME 1.0	9	2	1	20	16.7
CO + L-NAME 2.5	6	2	4	14	41.6
CO + L-NAME 5.0	2	4	6	8[a]	66.7
CO + L-NAME 10.0	0	2	10	2[c]	91.7
CO + L-NAME 25.0	0	0	12	0[c]	100.0
CO + L-NMMA 2.5	11	1	0	23	4.2
CO + L-NMMA 25.0	8	2	2	18	25.0
CO + L-NMMA 100.0	6	0	6	12[b]	50.0
L-NAME 25.0	0	0	12	0	–
L-NMMA 100.0	0	0	12	0	–

Twelve animals were used for each group. N^G-nitro-L-arginine methyl ester and N^G-monomethyl-L-arginine were given i.p. 15 min before oral administration of 2 ml castor oil. Results were analyzed by chi-squared test.
[a]$P<0.05$; [b]$P<0.01$ and [c]$P<0.001$ vs castor oil (CO).

Table 3 Inhibition of castor oil (CO)-stimulated fluid and Na^+ secretion in rat small intestine by N^G-nitro-L-arginine methyl ester (L-NAME)

Treatment (mg kg^{-1}, i.p.)	*Volume* (ml)	*Na^+* (mM)	*Na^+ amount* (μmol)
Control	0.24 ± 0.05	152.3 ± 12.8	36.2 ± 3.5
CO	1.45 ± 0.15[a]	152.9 ± 13.0	220.4 ± 18.3[a]
CO + L-NAME 2.5	1.35 ± 0.32	160.7 ± 14.0	216.9 ± 13.6
CO + L-NAME 10.0	0.37 ± 0.10[b]	139.5 ± 9.4	51.6 ± 5.4[b]
CO + L-NAME 25.0	0.24 ± 0.07[b]	139.6 ± 10.0	33.5 ± 4.2[b]
CO + L-NAME 10.0 + L-arginine 600.0	1.40 ± 0.16[c]	150.6 ± 12.7	210.8 ± 11.4[c]
CO + L-NAME 10.0 + D-arginine 900.0	0.37 ± 0.11	140.7 ± 7.8	52.1 ± 4.7
CO + L-arginine 600.0	1.43 ± 0.13	152.5 ± 10.5	218.1 ± 16.4
CO + D-arginine 900.0	1.46 ± 0.15	151.7 ± 13.0	221.5 ± 15.0
L-NAME 25.0	0.26 ± 0.04	176.8 ± 15.5	46.0 ± 4.8
L-Arginine 600.0	0.27 ± 0.06	153.6 ± 11.5	41.5 ± 6.6
D-Arginine 900.0	0.25 ± 0.04	152.4 ± 10.7	38.1 ± 4.5

Results are expressed as means ± s.e. of 7–9 experiments.
[a]$P<0.001$ vs control; [b]$P<0.001$ vs castor oil (CO); [c]$P<0.001$ vs castor oil (CO) + N^G-nitro-L-arginine (L-NAME) 10.0 mg kg^{-1}.

Intestinal transit

In normal rats the marker usually travelled more than half of the total length of the small intestine in 30 min: the mean ± s.e. was 58.0 ± 3.4% ($n = 10$). In rats given castor oil the value was 47.2 ± 3.0% ($n = 10$) (18.6% less, $P > 0.05$). L-NAME had no significant effect on the intestinal transit in normal (L-NAME 10 mg kg^{-1}: 56.4 ± 2.7% transit $n = 10$; L-NAME 25 mg kg^{-1}: 55.5 ± 3.0%, $n = 10$) or castor-oil-treated rats (L-NAME 10 mg kg^{-1}: 48.3 ± 3.0%, $n = 10$; L-NAME 25 mg kg^{-1}: 51.7 ± 3.4%, $n = 8$).

Discussion

It has been reported that castor oil causes diarrhoea 1–2 h after administration in rats (Niemegeers *et al.*, 1972; Vischer & Casals Stenzel, 1983). We obtained a highly reproducible response 3 h after castor oil administration (Pinto *et al.*, 1989; and the present results) and we used this 3 h time-period because at this time all rats exhibited scored diarrhoea.

We have shown that L-NAME and L-NMMA, inhibitors of NO synthesis from L-arginine (Rees *et al.*, 1990; Gardiner *et al.*, 1990) prevented or reduced the diarrhoea induced when given 15 min before the castor oil. Both compounds at the doses used in the present study can inhibit the enzyme(s) forming NO both in peripheral tissues (for ref. see Moncada & Higgs, 1991) and in the central nervous system (Knowles *et al.*, 1989). The finding that L-NAME was more potent and effective than L-NMMA in inhibiting the diarrhoea is consistent with previous reports that L-NAME is more potent than L-NMMA (Mulsch & Bussel 1990; Moore *et al.*, 1990). The dose of L-arginine, a substrate of NO synthesis, able to counteract the L-NAME effect can be 3–100 fold higher than that of NO synthesis inhibitor, depending on tissues and species (Rees *et al.*, 1990). In the present study it was 60 fold higher. In addition, as has been reported (Palmer *et al.*, 1988; Sakuma *et al.*, 1988; Amezcua *et al.*, 1989) the effect of arginine was enantiomer-specific; D-arginine was without effect. These results suggest that nitric oxide could be involved in castor-oil-induced diarrhoea.

Castor oil and its active ingredient ricinoleic acid change the transport of water and electrolytes to a net hypersecretory response. Our results show that the stimulated fluid and Na^+ secretion induced by castor oil were inhibited by L-NAME in a dose-related manner, whereas control rats were unaffected. Castor-oil-induced diarrhoea might also result from changes in intestinal transit (Gaginella & Phillips, 1975). Most reports suggest that motor activity is decreased during castor oil diarrhoea (Gaginella & Bass, 1978; Sarna, 1991) while others indicate the opposite (Vischer & Casals-Stenzel, 1983; Melo *et al.*, 1988). Our results show a tendency of castor oil to delay the transit to a rate below normal but this effect was not significant. In the light of the accepted hypothesis that NO may mediate the relaxation of intestinal muscle (Bult *et al.*, 1990; Christinck *et al.*, 1990; Boeckxstaens *et al.*, 1991) it was expected that transit might be modified in rats given L-NAME. In the present results, L-NAME did not modify the effect of the castor oil or influence the intestinal transit in control rats. The ability of L-NAME given i.p. to inhibit diarrhoea at doses which had no effect on the transit rate strongly suggests that the antidiarrhoeal activity of L-NAME is related to antisecretory mechanism and not to inhibition of transit. The mechanism underlying the antisecretory effect of L-NAME is however unclear. Most active secretagogues are potent vasodilators (Granger *et al.*, 1987). The antidiarrhoeal activity of L-NAME is not, however, just due to its vasoconstrictor effect because L-NMMA in doses causing the same vasoconstrictor effect as L-NAME did not block the diarrhoea. On the other hand vasopressin, a well known vasoconstrictor agent, causes water and electrolyte secretion in the small intestine (Donowitz & Welsh, 1987). In our results the failure of L-NAME to affect normal defaecation (but also secretion and transit) in rats probably implies that NO is not synthesized normally in the intestine in amounts sufficient to play a role in defaecation but in response to stimuli (i.e. castor oil) and the supply of L-arginine for NO synthesis is not rate-limiting. Hutcheson *et al.* (1990) suggest that endotoxin, an intestinal secretagogue (Tsurumi & Fujimura, 1983), induces the formation of NO from L-arginine by endothelial cells or possibly activated leucocytes. Most probably during castor oil-induced diarrhoea there is in the gut a generation of NO and this could explain the antidiarrhoeal effect by L-NAME. NO, once generated, could modulate cell activation and consequently release endogenous secretagogues or alternatively act directly to influence water and electrolyte secretion. The inhibition of diarrhoea could also be achieved if the NO synthesis inhibitors possess anti-acetylcholine, anti-histamine or anti-5-hydroxytryptamine (5-HT) activity, substances that facilitate NO synthesis (Amezcua *et al.*, 1988; Garrison, 1990) and are involved (5-HT and histamine) in diarrhoea caused by castor oil (Capasso *et al.*, 1986).

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References

AMEZCUA, J.L., DUSTING, G.J., PALMER, R.M.J. & MONCADA, S. (1988). Acetylcholine induces vasodilatation in the rabbit isolated heart through the release of nitric oxide, the endogenous nitrovasodilator. *Br. J. Pharmacol.*, **95**, 830–834.

AMEZCUA, J.L., PALMER, R.M.J., DE SOUZA, B.M. & MONCADA, S. (1989). Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. *Br. J. Pharmacol.*, **97**, 1119–1124.

BOECKXSTAENS, G.E., PELCKMANS, P.A., BULT, H., DE MAN, J.G., HERMAN, A.G. & VAN MAERKE, Y.M. (1991). Evidence for nitric oxide as a mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut. *Br. J. Pharmacol,*. **102**, 434–436.

BOECKXSTAENS, G.E., PELCKMANS, P.A., RAMPART, M., VERBEUREN, T.J., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990). NANC mechanism in the ileocolonic junction. *Arch. Int. Pharmacodyn.*, **303**, 270–281.

BULT, H., BOEKXSTAENS, P.A., PELKMANS, 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.

CAPASSO, F., MASCOLO, N., AUTORE, G. & ROMANO, V. (1986). Laxatives and the production of autacoids by rat colon. *J. Pharm. Pharmacol.*, **38**, 627–629.

CHRISTINCK, F., JURY, J., CAYABYAB, F. & DANIEL, E.E. (1990). Nitric oxide may be the final mediator of nonadrenergic, noncholinergic inhibitory junction potentials in the gut. *Can. J. Physiol. Pharmacol,*. **69**, 1448–1458.

DESAI, K.M., SESSA, W.S. & VANE, J.R. (1991). Involvement of nitric oxide in the reflex relaxation of the stomach to accomodate food or fluid. *Nature*, **351**, 477–479.

DONOWITZ, M. & WELSH, M.J. (1987). Regulation of mammalian small intestine electrolyte secretion. In *Physiology of the Gastrointestinal Tract*. ed. Johnson, L.R. pp. 1351–1388. New York: Raven Press.

GAGINELLA, T.S. & BASS, P. (1978). Laxatives: an update on mechanism of action. *Life. Sci.*, **23**, 1001–1010.

GAGINELLA, T.S. & PHILLIPS, S.F. (1975). Ricinoleic acid: current view of an ancient oil. *Dig. Dis.*, **20**, 1171–1177.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1990). Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension*, **15**, 486–490.

GARRISON, J.C. (1990). Histamine, Bradykinin, 5-Hydroxytryptamine and their antagonists. In *The Pharmacological Basis of Therapeutics*. ed. Goodman Gilman, A., Rall, T.W., Nies, A.S. & Taylor, P. pp. 574–599. New York: Pergamon Press.

GRANGER, D.N., KVIETYS, P.R., PERRY, M.A. & BARROWMAN, J.A. (1987). The microcirculation and intestinal transport. In *Physiology of the Gastrointestinal Tract*. ed Johnson, L.R. pp. 1671–1697. New York: Raven Press.

GUSTAFSSON, L.E., WIKLUND, C.U., WIKLUND, N.P., PERSSON, M.G. & MONCADA, S. (1990). Modulation of autonomic neuroeffector transmission by nitric oxide in guinea pig ileum. *Biochem. Biophys. Res. Commun.*, **173**, 106–110.

HUTCHESON, I.R., WHITTLE, B.J.R. & BOUGHTON-SMITH, N.K. (1990). Endotoxin-induced intestinal damage in the rat is enhanced by L-NMMA, an inhibitor of nitric oxide formation. *Br. J. Pharmacol.*, **99**, 111P.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5159–5162.

MELO, M. DE F., THOMAS, G. & MUKHERYEE, R. (1988). Antidiarrhoeal activity of bisnordihydrotoxiferine isolated from the root bark of *Strychnos trinervis* (Vell.) Mart. (Loganiaceae). *J. Pharm. Pharmacol.*, **40**, 79–82.

MONCADA, S. & HIGGS, E.A. (1991). Endogenous nitric oxide: physiology, pathology and clinical relevance. *Eur. J. Clin. Inv.*, **21**, 361–374.

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

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., 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.

MULSCH, A. & BUSSE, R. (1990). N^G-nitro-L-arginine (N-[imino (nitro-amino) methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn Schmiedebergs Arch. Pharmacol.*, **341**, 143–147.

NIEMEGEERS, C.J.E., LENAERTS, F.M. & JANSSEN, P.A.S. (1972). Difenoxine (R15403), the active metabolite of difenoxilate (R1132). *Arzneim. Forsch.*, **22**, 516–518.

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

PIERCEY, M.F. & RUWART, M.J. (1979). Naloxone inhibits the antidiarrhoeal activity of loperamide. *Br. J. Pharmacol.*, **66**, 373–375.

PINTO, A., AUTORE, G., MASCOLO, N., SORRENTINO, R., BIONDI, A., IZZO, A.A. & CAPASSO, F. (1992). Time course of Paf formation by gastrointestinal tissue in rats after castor oil challenge. *J. Pharm. Pharmacol.*, **44**, 224–226.

PINTO, A., CALIGNANO, A., MASCOLO, N., AUTORE, G. & CAPASSO, F. (1989). Castor oil increases intestinal formation of platelet activating factor and acid phosphatase release in the rat. *Br. J. Pharmacol.*, **96**, 872–874.

POOLE, C.F. & SCHUETTE, S.A. (1984). *Contemporary Practice of Chromatography*. pp. 313–317. Amsterdam: Elsevier.

RACUSEN, L.C. & BINDER, H.J. (1979). Ricinoleic acid stimulation of active anion secretion in colonic mucosa of the rat. *J. Clin. Invest.*, **63**, 743–749.

REES, D.D., PALMER, R.M.J., SCHULTZ, 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.

ROBERT, A., NEZAMIS, J.E., LANCASTER, C., HANCHAR, A.J. & KLEPPER, M.S. (1976). Enteropooling assay: a test for diarrhoea produced by prostaglandins. *Prostaglandins*, **11**, 809–814.

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.

SARNA, S.K. (1991). Physiology and pathophysiology of colonic motor activity. *Dig. Dis. Sci.*, **36**, 998–1018.

SIMON, B., CYZGAN, P., STICHL, A. & KATHER, H. (1978). Human colon adenylate cyclase: effects of bile acids. *Eur. J. Clin. Invest.*, **8**, 321–323.

TSURUMI, K. & FUJIMURA, H. (1983). Effect of antiinflamatory drugs on endotoxin-induced diarrhoea in mice. *Jpn. J. Pharmacol.*, **33**, 165–173.

VISCHER, P. & CASALS-STENZEL, J. (1983). Influence of prostacyclin and indomethacin on castor oil-induced gastrointestinal effect in rat. *J. Pharm. Pharmacol.*, **35**, 152–156.

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Fendiline inhibits L-type calcium channels in guinea-pig ventricular myocytes: a whole-cell patch-clamp study

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1 Fendiline, a diphenylalkylamine type of antianginal drug, was examined for its effects on L-type calcium channels in guinea-pig ventricular myocytes by the whole-cell patch-clamp technique.

2 Fendiline (0.3–100 μM) applied extracellularly inhibited the calcium channel current (I_{Ca}) in a concentration- and time-dependent manner. The IC_{50} of fendiline was 17.0 ± 2.43 μM and the Hill slope was 1.39 ± 0.23.

3 Inhibition of I_{Ca} by fendiline appeared with an onset of less than 3 s.

4 Fendiline inhibited I_{Ca} at all the membrane potentials tested and shifted the current-voltage curve upwards. The overall calcium channel conductance (g_{Ca}) of the cell was reduced and conductance-voltage curve was shifted to the left in the presence of fendiline.

5 Isoprenaline (0.5–1 μM), a β-adrenoceptor agonist, partially reversed the inhibitory effect of fendiline on I_{Ca}.

6 It is suggested that fendiline applied extracellularly blocks L-type calcium channels and reduces calcium channel conductance of the cell. The calcium channels thus inhibited are, nevertheless, still available for β-adrenoceptor stimulation.

Keywords: Fendiline; diphenylalkylamine; isoprenaline; L-type Ca^{2+}-channel; ventricular myocytes; heart; patch-clamp

Introduction

Fendiline (Sensit: γ-phenyl-*N*-(1-phenylethylbenzenepropan-amine)), an antianginal drug, is reported to possess weak calcium antagonistic properties (Fleckenstein *et al.*, 1977). This proposal was supported by subsequent findings of inhibition of calcium-mediated cardiac action potentials by fendiline (Tritthart & Koidl, 1985). These and other observations (Spedding, 1982; Bayer & Mannhold, 1987) provided indirect evidence for calcium channel blocking effect of fendiline leaving the specificity of its action on L-type calcium channels unclear. Meanwhile, fendiline was, however, reported to differ from verapamil, diltiazem and nifedipine in its effects on smooth muscle contractility (Spedding, 1982). It was also found to inhibit the effects of calmodulin (Johnson *et al.*, 1982; Johnson & Fugman, 1983; Bayer & Mannhold, 1987) and to block α_2-adrenoceptors (Motulsky *et al.*, 1983). Consequently a lingering controversy continues about the site and mechanism of action of fendiline necessitating further investigations.

We undertook this study to assess effects of fendiline on L-type calcium channels in guinea-pig ventricular myocytes subjected to whole-cell patch-clamp recording. The findings show that fendiline inhibits L-type calcium channel activity and the effects could be reversed by isoprenaline. Part of these findings have been presented at meetings (Schreibmayer *et al.*, 1991; Tripathi *et al.*, 1991; 1992).

Methods

Preparation of ventricular myocytes

Guinea-pigs (dosed with heparin 3000 iu kg^{-1}, s.c.) were killed by a blow on the neck and the heart was rapidly removed and mounted on Langendorff's apparatus for retrograde intra-arterial perfusion with oxygenated physiological salt solution (Na^+-PSS; 37°C). The perfusion was later changed to collagenase-PSS. Subsequently, the atria were dissected away and the ventricles gently chopped with fine blades to help cellular dispersion (Schreibmayer *et al.*, 1985; 1992). The tissue suspension was filtered through a nylon filter. The filtrate was consecutively centrifuged and the final cell suspension prepared in the culture medium. This cell suspension was stored in a CO_2 incubator at 37°C for 3–72 h. Aliquots of cell suspension were removed for electrophysiological experiments and placed in the recording chamber surrounded by Peltier element for temperature control (Patch-Clamp Tower, List Electronic, Germany). The cells were superfused with Na^+-PSS or choline-PSS (20°C) for recording the whole-cell currents.

Recording of L-type calcium channel current (I_{Ca})

The whole-cell version of patch-clamp technique was employed for recording the calcium current (Hamill *et al.*, 1981). Voltage pulses of appropriate configuration were applied to the cell via an AD/DA converter (Model TL-1-125 Computer Interface, Axon Instruments, U.S.A.) coupled to a 386 based PC-AT (Steiner Corporation, Austria). A patch-clamp amplifier (Model 3900, Dagan Corporation, U.S.A.) was used to pick-up the resulting currents filtered by Bessel-filters at about 1/5th of the sampling frequency. Generation of voltage pulses and data acquisition and analysis were carried out by use of a software package (pClamp 5.5, Axon Instruments, U.S.A.). Patch-pipettes were prepared from soft glass capillaries (No. 564, Assistant, Germany) and filled with Cs^+- and ATP-containing pipette solution.

Calcium currents to study current-voltage relationships and concentration- and time-dependent effects of fendiline were elicited by two different voltage pulse protocols (Figures 1a and 3a respectively). In both cases, voltage pulses were applied every 5 s. The cells were depolarized from a holding potential of −90 mV (2 s) to prepotential of −40 mV (100 ms) in order to inactivate fast sodium channels (Trautwein *et al.*, 1975; Lee *et al.*, 1979) and T-type calcium

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channels (Bean, 1985; Nilius et al., 1985). Thereafter, command potentials of 250 ms and 5 mV step increments to a final value of + 110 mV were applied to generate the families of I_{Ca} required for construction of current-voltage curves. The concentration- and time-dependent effects of fendiline were examined on peak I_{Ca} elicited by repetitive voltage pulses of 0.2 Hz having similar basic configuration but constant command potential of + 20 mV.

To assess the relationship between overall calcium channel conductance (g_{Ca}) of the cell and membrane potential, the conductance values were calculated from that of I_{Ca} recorded for current-voltage curves as: $g_{Ca} = I_{Ca}/(E_m - E_{Ca})$. E_{Ca}, the reversal potential for I_{Ca}, was obtained from current-voltage curves. The relative overall calcium channel conductance (G_{Ca}) of the cell was derived as: $G_{Ca} = g_{Ca}/g_{Ca(max)}$, where $g_{Ca(max)}$ is the maximal conductance in a given family of I_{Ca} recordings. The conductance-voltage curves were prepared by plotting the relative conductance against membrane potentials (Figure 2).

I_{Ca} recorded in Na^+-PSS, tetrodotoxin (TTX, 10 μM)-containing Na^+-PSS, and in choline-PSS were found to be similar in magnitude (not illustrated). Moreover, the current recordings in choline-PSS did not show fast sodium and outward potassium currents due to elimination of sodium from the extracellular solution and block of potassium channels by Cs^+ in the pipette solution respectively. The effect of fendiline on I_{Ca} was, therefore, studied with choline-PSS as the extracellular solution and Cs^+-containing solution in the pipette to avoid contamination from other ionic channels.

Solutions used (composition in mM)

Na^+-containing physiological salt solution (Na^+-PSS): NaCl 137, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 2, glucose 10, HEPES 10; buffered with NaOH to pH 7.4.

Choline-PSS: choline chloride 137, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 2, HEPES 10; buffered with KOH to pH 7.4.

Collagenase-PSS: NaCl 137, KCl 5.4, $CaCl_2$ 0.125, $MgCl_2$ 2, glucose 10, HEPES 10, collagenase 100 u ml^{-1}; buffered with NaOH to pH 7.4.

Cs^+-containing pipette solution: CsCl 110, EGTA 11, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10, K-ATP 63 mg to 25 ml; buffered with KOH to pH 7.4

Culture medium: Foetal calf serum 4%, penicillin-G 0.05% and streptomycin 0.05% added to Medium 199.

Stock solutions (20 mM) of the drugs were prepared in dimethylsulphoxide (DMSO) subsequent dilutions being made in choline-PSS. To avoid photodegradation of nifedipine, experiments with this agent were performed in yellow light.

Chemicals

Medium 199 and foetal calf serum were from Flow Laboratories and Sigma Chemical Co. respectively. Collagenase (Type CI-28) was procured from Worthington. Tetrodotoxin (TTX), isoprenaline hydrochloride, and dimethylsulphoxide (DMSO) were purchased from Sigma Chemical Co. Nifedipine was a gift from Bayer A.G. Thiemann Arzneimittel GmbH provided fendiline hydrochloride. All other chemicals were of reagent grade. All the solutions were made in double glass distilled water unless otherwise stated.

Statistical analysis

Student's *t* test was employed for determining the level of significance of observations. The mean values with s.d. are given throughout the text.

Results

Characteristics of I_{Ca} (Figure 1)

I_{Ca} recorded in choline-PSS showed consistency in its characteristics with a low run-down during experiments extending up to 2 h at times. The current voltage relationship was a typical inverted bell-shaped curve (Figure 1c). The maximal value of I_{Ca} recorded with the two types of voltage pulse protocols was -0.77 ± 0.27 nA ($n = 7$). The maximal I_{Ca} appeared at membrane potentials of $+12.1 \pm 8.1$ mV ($n = 7$) and the half-maximal I_{Ca} was at -2.7 ± 3.9 mV ($n = 4$). E_{Ca}, the reversal potential of I_{Ca}, was $+63.9 \pm 6.8$ mV ($n = 7$).

Nifedipine (0.3–1 μM) applied extracellularly blocked the peak I_{Ca} in less than 5 s (not illustrated; but see Schreibmayer et al., 1992). DMSO, the solvent used for various drugs, had

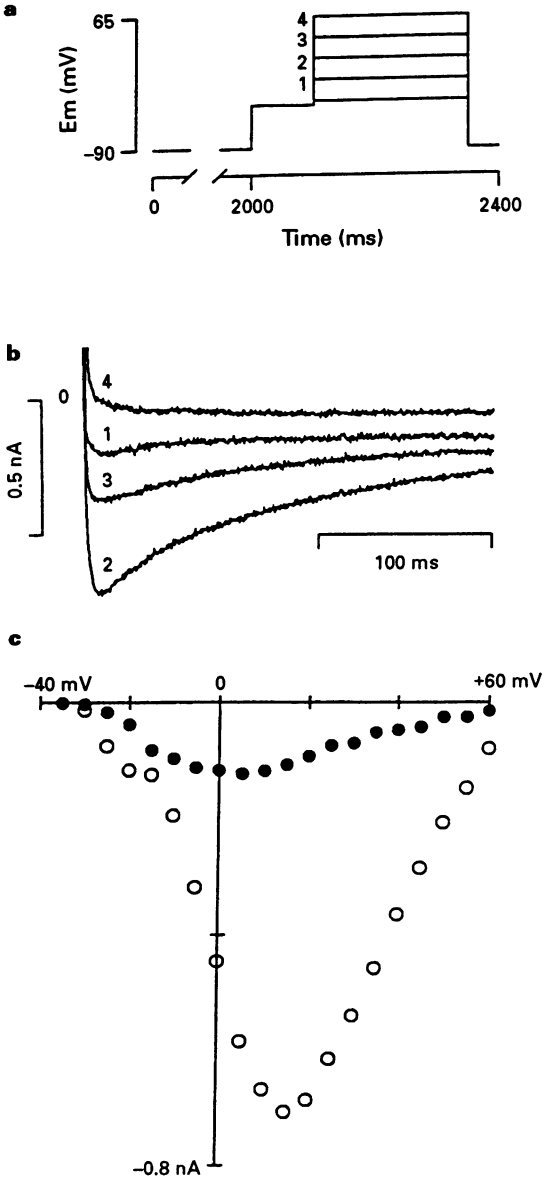


Figure 1 Effect of fendiline on whole-cell L-type calcium-channel current (I_{Ca}) in guinea-pig ventricular myocytes. (a) Voltage pulse protocol for recording I_{Ca}: holding potential −90 mV for 2 s, prepulse −40 mV for 100 ms, and command step potentials of 5 mV increments for 250 ms to +110 mV. The steps indicated by 1, 2, 3 and 4 represent −10, +15, +40 and +65 mV respectively. (b) Superimposed records of I_{Ca} corresponding to the step potentials numbered in (a). (c) Current (I_{Ca})-voltage (E_m) curves under control conditions (○) and in presence of fendiline 30 μM (●).

no effect on I_{Ca} in concentrations corresponding to the dilutions employed in this study (not illustrated). Since there was no residual transient inward calcium current (T-type) in the presence of nifedipine the inward current-recorded in this study was considered to be L-type (designated I_{Ca} here). The T-type calcium current, which is often reported in ventricular cells (Nilius *et al.*, 1985; Tytgat *et al.*, 1990), did not appear in our recordings despite a rather negative holding potential of − 90 mV due to inactivation of these channels by − 40 mV prepulse of 100 ms (Bean, 1985; Nilius *et al.*, 1985).

Effects of fendiline

Fendiline (0.3–100 μM), applied extracellularly, caused a concentration-dependent decrease in the amplitude of peak I_{Ca} elicited by repetitive voltage pulses. The submaximal and maximal I_{Ca} evoked for preparing current-voltage curves were greatly reduced by fendiline between − 30 to + 60 mV membrane potential as evident from Figure 1c which shows 50% to 90% inhibition of I_{Ca} at different potentials by 30 μM of the drug. This is mirrored in a severe attenuation of the entire current-voltage curve in the presence of fendiline (Figure 1c). It is clearly evident from Figure 1c that maximal I_{Ca} was strongly inhibited by fendiline and it now appeared at more negative potentials of − 5 mV instead of + 15 mV in this particular cell. The overall calcium channel conductance (g_{Ca}) was also reduced markedly by fendiline in the entire range of potentials and is reflected in a shift of conductance-voltage curve to the left (Figure 2).

The time-course and concentration-dependence of the effect of fendiline were studied on peak I_{Ca} elicited by continuous trains of constant voltage pulses. The inhibitory effect had a rapid onset, the first signs of decrease in peak I_{Ca} appearing in less than 3 s of drug application (Figure 3c). The log concentration-response curve for inhibition of the peak I_{Ca} by fendiline was characteristically sigmoidal (Figure 3c). The IC_{50} and Hill slope for this effect of fendiline were 17.0 ± 2.43 μM and 1.39 ± 0.23 respectively.

Inhibition of peak I_{Ca} by fendiline was partially reversed upon washout, the extent of recovery depending on the concentration and duration of application of the drug. A rapid washout recovery of 10 min was seen with the lower concentrations (≤ 30 μM; not illustrated). With the higher concentrations recovery was slow and partial up to 60 min of washout (Figure 3d). Prolonged application of higher concentrations (≥ 100 μM) of fendiline caused a sustained contracture and rounding off of the myocardiocytes as reported for calmidazolium (Klöckner & Isenberg, 1988).

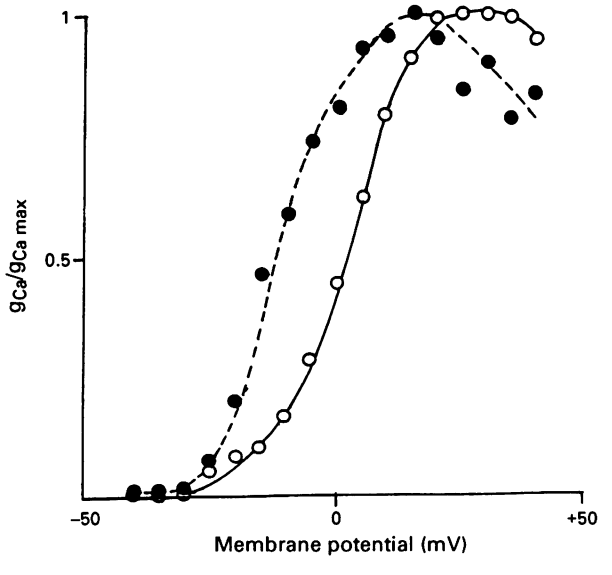


Figure 2 Effect of fendiline on overall L-type calcium channel conductance of the cell (g_{Ca}) in guinea-pig ventricular myocytes. The overall relative conductance (G_{Ca}) is expressed as a function of membrane potential (E_m). For details see text. Conductance-voltage curves under control conditions (○) and in presence of fendiline 30 μM (●). The values are derived from the records of current-voltage curves in Figure 1c.

Effects of isoprenaline on response to fendiline

Isoprenaline (0.5–1 μM), a β-adrenoceptor agonist, caused substantial increase in the amplitude of peak I_{Ca}. When applied in the presence of inhibitory concentrations of fendiline, isoprenaline induced a rapid and marked enhancement of calcium current antagonizing the blocking action of fendiline (Figure 3f; $n = 4$) suggesting the channels were still available for activation by phosphorylation.

Discussion

The present observation of inhibition of I_{Ca} by fendiline applied extracellularly to ventricular myocytes unequivocally demonstrates its blocking effect on cardiac L-type calcium channels. The specificity of this effect is evident from the fact that sodium, outward potassium and T-type calcium currents were eliminated and the observations represent drug effects on uncontaminated L-type calcium channel current. This consideration was of particular significance as fendiline inhibits cardiac fast sodium channels at high concentrations (Fleckenstein *et al.*, 1977). Moreover, calmodulin inhibitors alter sodium channel gating (Ichikawa *et al.*, 1991) and inhibit potassium channels (Klöckner & Isenberg, 1988). The present findings on calcium channel blocking activity of fendiline are supported by earlier reports of its calcium antagonistic property based on studies on myocardial and smooth muscle contractility and cardiac action potentials in multicellular preparations (Fleckenstein *et al.*, 1977; Spedding, 1982; Tritthart & Koidl, 1985; Bayer & Mannhold, 1987).

Concentration- and time-dependent inhibition of I_{Ca} with a rapid onset shows that fendiline has a direct blocking effect on L-type calcium channels by acting at the membrane level. The IC_{50} of 17.0 ± 2.43 μM found by us for this inhibitory effect is close to 14 μM (Fleckenstein *et al.*, 1977) and 10 μM (Tritthart & Koidl, 1985) reported for the calcium antagonistic effect of fendiline in multicellular preparations subjected to indirect measurement of calcium channel activity. The other types of calcium channel blockers, verapamil, nifedipine and diltiazem are reported to have IC_{50}s of 2.0, 0.3 and 50 μM respectively for inhibition of I_{Ca} in myocardium (Payet *et al.*, 1980; Uehara & Hume, 1985). In addition to these differences in the potency of cardiac L-type calcium channel blocking activity, fendiline is reported to differ from the other types of blockers in its effects on smooth muscle contractility (Spedding, 1982). However, overall pharmacological implications of these differences between the various types of calcium channel blockers need to be ascertained.

A quick onset of inhibition of I_{Ca} by fendiline and an early washout recovery from the effects of its lower concentrations strongly suggest that the drug blocks L-type calcium channels by acting at the membrane level and not necessarily via intracellular sites as implied from several reports (Spedding, 1982; Johnson *et al.*, 1982; Johnson & Fugman, 1983; Bayer & Mannhold, 1987). Radioligand binding studies have shown that fendiline binds to the dihydropyridine receptor sites on the calcium channel complex (Glossmann *et al.*, 1982; 1983; 1984) supporting this view. Although fendiline affects the binding of radiolabelled 1,4-dihydropyridines in membrane preparations differently from verapamil and diltiazem it has a Hill slope of 1 (Glossmann *et al.*, 1982; 1983; 1984). The inhibitory effect of fendiline on I_{Ca} was also found by us to have a Hill-slope in this range (1.39 ± 0.23). This fortuitous

parallelism between the two parameters of the effect of fendiline on L-type calcium channels also suggests its direct interaction with the calcium channel complex.

Calmodulin is another target site for the action of fendiline and its inhibition is implicated in pharmacological effects of the latter (Johnson & Fugman, 1982; Johnson *et al.*, 1983; Spedding, 1983; Bayer & Mannhold, 1987). Nevertheless, a direct involvement of calmodulin in the observed inhibition of L-type calcium channels by fendiline appears unlikely for two reasons. Firstly, the effects of fendiline on I_{Ca} had a rapid onset and significant washout recovery (at lower concentrations) which cannot be readily explained by effects on calmodulin, an intracellular calcium receptor. Secondly, and more importantly, calmidazolium and trifluoperazine, which are more potent calmodulin inhibitors than fendiline, are reported to inhibit I_{Ca} without the possibility of a role of calmodulin in it (Klöckner & Isenberg, 1988). A slow and incomplete recovery of I_{Ca} from the effects of higher concentrations of fendiline, however, could well be due to its intracellular accumulation and subsequent inhibition of calmodulin, as it has access to the cell interior following extracellular application (Weyhenmeyer *et al.*, 1989).

Inhibition of I_{Ca} by fendiline occurred at most of the potentials (-30 to $+60$ mV) as evident from the tendency of the current-voltage curve to flatten out in its presence. Despite a shift of the potential for maximal I_{Ca} to more negative voltages, the calcium current was found to be inhibited by fendiline throughout the entire range of membrane potentials albeit to varying magnitudes (Figure 1c). This effect is reflected in an almost parallel shift of the conductance-voltage curve to more negative potentials by fendiline (Figure 2), suggesting a substantial reduction of the overall calcium channel conductance of the cell.

It may be pointed out here that a Hill slope of 1 for

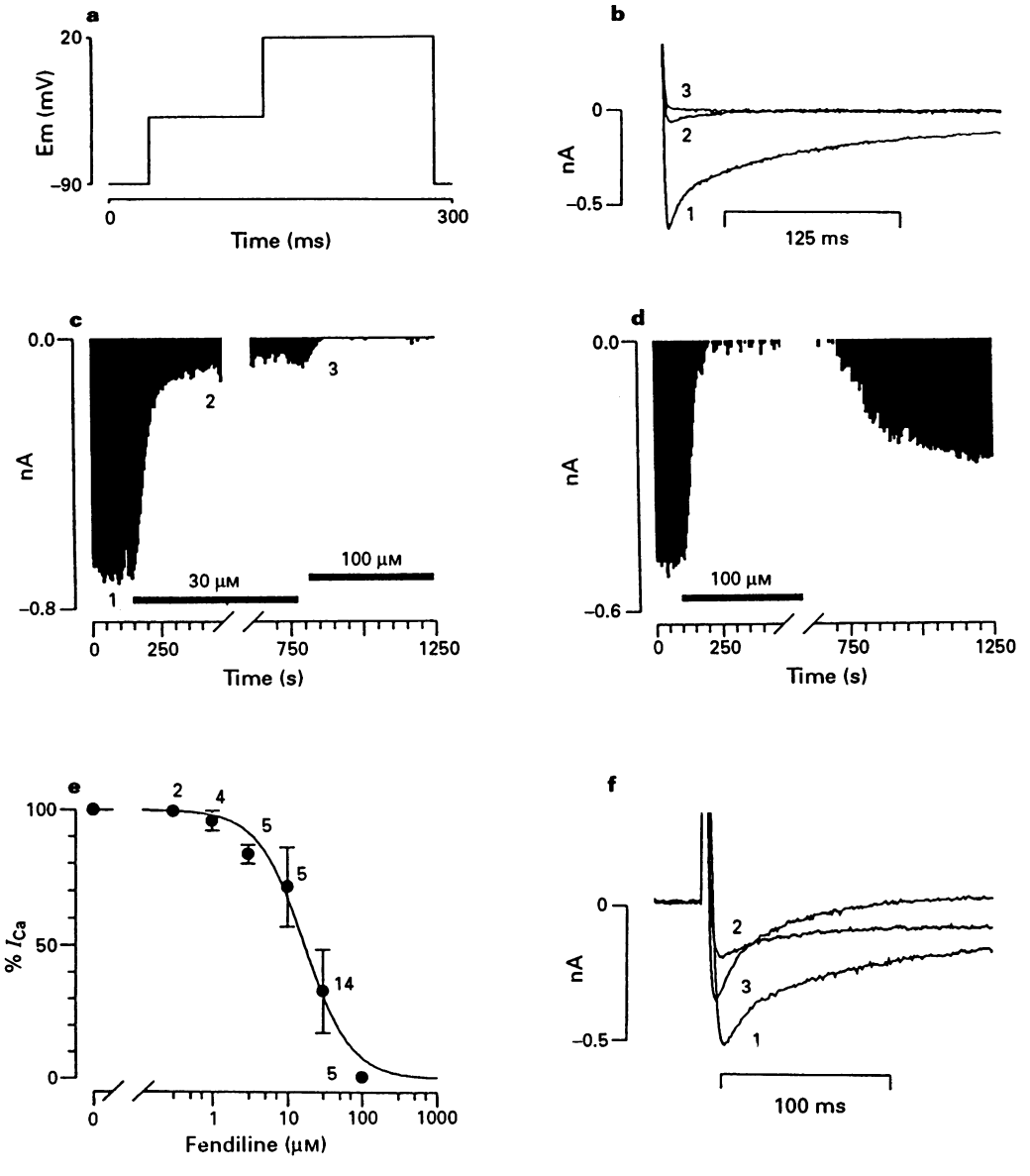


Figure 3 Effect of fendiline on peak I_{Ca} in guinea-pig ventricular myocytes elicited by recurring voltage pulses delivered at 0.2 Hz. (a) Voltage pulse protocol for recording peak I_{Ca}: holding potential -90 mV for 2 s, prepulse -40 mV, for 100 ms, and command potential of $+20$ mV for 250 ms. (b) Superimposed records of peak I_{Ca} control (1), and in presence of 30 μM (2) and 100 μM (3) fendiline, sampled from the records shown in (c). (c) Time-dependence of inhibitory effect of fendiline (30 μM and 100 μM). (d) Recovery of peak I_{Ca}, after washout of fendiline (100 μM). (e) Dose-response relationship of the effect of fendiline expressed as % of maximal peak I_{Ca}. (f) Superimposed records of peak I_{Ca}: control (1), following inhibition by 30 μM fendiline (2), and partially reversed by 1 μM isoprenaline in presence of 30 μM fendiline (3).

binding of fendiline to the 1,4-dihydropyridine receptor complex (Glossman *et al.*, 1982; 1983; 1984) and for its inhibitory effects on I_{Ca} (present study) in comparison to 1 for the effects of 1,4-dihydropyridines (Spedding, personal communication) may be construed as an indication of profound differences in molecular mechanism of action of these two types of agents. This conclusion is further supported by the reported failure of Bay K 8644, a calcium channel agonist, to neutralize the effects of fendiline on contractility in K^+-depolarized smooth muscle preparations (Spedding & Berg, 1984). That fendiline indeed acts at a site on the calcium channel complex different from the other types of calcium channel blockers is evident from a reversal of the stimulant effect of Bay K 8644 by fendiline unlike verapamil, diltiazem and nifedipine (Schreibmayer *et al.*, 1992) while isoprenaline, which modulates the channels differently from 1,4-dihydropyridines (Tsien *et al.*, 1986), effectively overcomes the block by fendiline. Taken together all these findings, when viewed in the light of Spedding's 1982 report of differential effect of fendiline vis-a-vis the other types of organic calcium channel blockers on smooth muscle contractility, indicate a novel specific binding site for this diphenylalkylamine agent on the L-type calcium channel complex.

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References

BAYER, R & MANNHOLD R. (1987). Fendiline: a review of its basic pharmacological and clinical properties. *Pharmacotherapeutica*, **5**, 103–136.

BEAN, B.P. (1985). Two kinds of calcium channels in canine atrial cells. Differences in kinetics, selectivity, and pharmacology. *J. Gen. Physiol.*, **86**, 1–30.

FLECKENSTEIN, A., FLECKENSTEIN-GRÜN, G. & BYON, Y.K. (1977). Fundamentale Herz- and Gefäßwirkungen des Ca-antagonistischen Koronartherapeuticums Fendilin (Sensit). *Drug Res.*, **27**, 652–671.

GLOSSMANN, H., FERRY, D., GOLL, A. & ROMBUSCH, M. (1984). Molecular pharmacology of the calcium channel: evidence for subtypes, multiple drug receptor sites, channel subunits, and the development of a radio-iodinated 1,4-dihyrdopyridine calcium channel label, (^{125}I) Iodipine. *J. Cardiovasc. Pharmacol.*, **6**, S608–S621.

GLOSSMANN, H., FERRY, D., LÜBBECKE, F., MEWES, R. & HOFMANN, F. (1982). Calcium channels: direct identification with radioligand binding sites. *Trends Pharmacol. Sci.*, **3**, 431–437.

GLOSSMANN, H., FERRY, D., LÜBBECKE, F., MEWES, R. & HOFMANN, F. (1983). Identification of voltage operated calcium channels by binding studies: differentiation of subclasses of calcium antagonist drugs with (3H)-nimodipine radioligand binding. *J. Receptor Res.*, **3**, 177–190.

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. *Pflugers Arch.*, **391**, 85–100.

ICHIKAWA, M., URAYAMA, M. & MATSUMOTO, G. (1991). Anti-calmodulin drugs block the sodium gating current of squid giant axons. *J. Membrane Biol.*, **120**, 211–222.

JOHNSON, J.D. & FUGMAN, D.A. (1983). Calcium and calmodulin antagonists binding to calmodulin and relaxation of coronary segments. *J. Pharmacol. Exp. Ther.*, **226**, 330–334.

JOHNSON, J.D., VAGHY, P.L., CROUCH, T.H., POTTER, J.D. & SCHWARTZ, A. (1982). A hypothesis for the action of some of the Ca-antagonist drugs: calmodulin as a receptor. *Adv. Pharmacol. Ther.*, **3**, 121–138.

KLÖCKNER, U. & ISENBERG, G. (1988). Calmodulin antagonists depress calcium and potassium currents in ventricular and vascular myocytes. *Am. J. Physiol.*, **253**, H1601–H1611.

LEE, K.S., WEEKS, T.A., KAO, R.L., AKAIKE, N. & BROWN, A.M. (1979). Sodium current in single heart muscle cells. *Nature*, **278**, 269–271.

MOTULSKY, H.J., SNAVELY, M.D., HUGHES, R.J. & INSEL, P.A. (1983). Interaction of verapamil and other calcium channel blockers with α-1 and α-2 adrenergic receptors. *Circ. Res.*, **52**, 226–231.

NILIUS, B., HESS, P., LANSMAN, J.B. & TSIEN, R.W. (1985). A novel type of cardiac calcium channel in ventricular cells. *Nature*, **316**, 443–446.

PAYET, M.D., SCHANNE, O.F. & RUIZ-CERETTI, E. (1980). Competition for slow channel of Ca^{2+}, Mn^{2+}, verapamil and D600 in rat ventricular muscle. *J. Mol. Cell. Cardiol.*, **12**, 635–638.

SCHREIBMAYER, W., TRIPATHI, O. & TRITTHART, H.A. (1991). Block of cardiac calcium channels by fendiline in guinea pig ventricular cells. *Biophys. J.*, **59**, 552a.

SCHREIBMAYER, W., TRIPATHI, O. & TRITTHART, H.A. (1992). Kinetic modulation of guinea pig cardiac L-type calcium channels by fendiline and reversal of the effects of Bay K8644. *Br. J. Pharmacol.*, **106**, 151–156.

SCHREIBMAYER, W., TRITTHART, H.A., ZERNIG, G. & PIPER, H.M. (1985). Single voltage dependent and outward rectifying potassium channels in isolated rat heart cells. *Eur. Biophys. J.*, **11**, 259–263.

SPEDDING, M. (1982). Assessment of 'Ca^{2+}-antagonist' effects of drugs in K^+ depolarized smooth muscle. Differentiation of antagonist subgroups. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **318**, 234–240.

SPEDDING, M. (1983). Direct inhibitory effects of some 'calcium antagonists' and trifluoperazine on the contractile proteins in smooth muscle. *Br. J. Pharmacol.*, **79**, 225–231.

SPEDDING, M. & BERG, C. (1984). Interaction between a 'calcium agonist', Bay K 8644, and calcium antagonists differentiate calcium antagonist subgroups in K^+-depolarized smooth muscle. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **328**, 69–75.

TRAUTWEIN, W., McDONALD, T.F. & TRIPATHI, O. (1975). Calcium conductance and tension in mammalian ventricular muscle. *Pflügers Arch.*, **354**, 55–74.

TRIPATHI, O., SCHREIBMAYER, W. & TRITTHART, H.A. (1991). Fendiline inhibits L-type Ca^{2+} channels and reverses the agonist effect of Bay K 8644 in guinea pig ventricular myocytes suggesting a novel site of action. *Proc. 5th International Symposium on 'Calcium Antagonists: Pharmacology and Clinical Research'.* Houston, Sept. 25–28, 1991, p. 112.

TRIPATHI, O., SCHREIBMAYER, W. & TRITTHART, O. (1992). A novel site of action on L-type calcium channels as evident from the effects of fendiline, a diphenylalkylamine agent, on guinea pig ventricular myocytes. *J. Mol. Cell. Cardiol.*, **24** (Suppl. I), S136.

TRITTHART, H.A. & KOIDL, B. (1985). Das Pharmakologische Workungsprinzip von Calciumantagonisten: Fendilin im Vergleich zu Nifedipin, Verapamil und Diltiazem. In *Calmodulin und Calciumantagonismus.* ed. Lüthy, E. *Drug Development and Evaluation*, No. 11, pp. 41–53. New York: Gustav Fischer Verlag.

TSIEN, R.W., BEAN, B.P., HESS, P., LANSMAN, J.B., NILIUS, B. & NOWYCKY, M.C. (1986). Mechanism of calcium channel modulation by beta-adrenergic agents and dihydropyrine calcium antagonists. *J. Mol. Cell. Cardiol.*, **18**, 691–710.

TYTGAT, J., VEREECKE, E. & CARMELIET, E. (1990). A combined study of sodium current and T-type calcium current in isolated cardiac cells. *Pflügers Arch.*, **417**, 142–148.

UEHARA, A. & HUME, J.R. (1985). Interactions of organic calcium channel antagonists with calcium channels in single frog atrial cells. *J. Gen. Physiol.*, **85**, 621–647.

WEYHENMEYER, R., GROß, M. & MAURER-SCHULTZE, B. (1989). Intracellular localization of the calcium- and calcodulin-antagonist Fendiline. *Drug Res.*, **39**, 199–201.

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Renal imidazoline preferring sites and solute excretion in the rat

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1 Moxonidine has been found to have an approximately 600 fold greater affinity for I_1 imidazoline preferring sites as compared to α_2-adrenoceptors in the rat kidney. The effects of an intrarenal infusion of moxonidine in an anaesthetized rat preparation were investigated and contrasted with the effects previously reported for α_2-adrenoceptor stimulation.

2 An intrarenal infusion of moxonidine (1, 3 and 10 nmol kg^{-1} min^{-1}) produced an increase in urine flow rate and sodium excretion. Moxonidine increased urine volume through an increase in osmolar clearance rather than an increase in free water clearance as previously reported for α_2-adrenoceptor stimulation.

3 The effects of moxonidine also appeared to be unique from the effects of α_2-adrenoceptor stimulation. An imidazoline preferring site specific blocking dose of idazoxan (0.3 mg kg^{-1}), but not an α_2-adrenoceptor specific blocking dose of rauwolscine (0.3 mg kg^{-1}) attenuated the renal effects of moxonidine (3 nmol kg^{-1} min^{-1}). Moreover, unlike α_2-adrenoceptor agonists, the effects of moxonidine were not altered by prior treatment with a V_2 vasopressin receptor antagonist.

4 These results indicate differences between stimulation of α_2-adrenoceptors and I_1 imidazoline preferring sites in the rat kidney and suggest a direct physiological function of renal imidazoline preferring sites.

Keywords: Moxonidine; clonidine; vasopressin (V2) antagonist; natriuresis; α_2-adrenoceptor

Introduction

Several studies (Strandhoy *et al.*, 1982; Blandford & Smyth, 1988; Gesek & Strandhoy, 1990) have suggested a heterogeneous response to α_2-adrenoceptor stimulation in the kidney. Studies in our laboratory have found a dose-selective dissociation of water and solute clearance for α_2-adrenoceptor stimulation in the anaesthetized rat (Blandford & Smyth, 1988). However, recent studies in the rat indicate that this dissociation, may in fact represent two distinct α_2-adrenoceptor subtypes and/or receptors (Smyth *et al.*, 1992). A similar discrepancy had been previously reported for α_2-adrenoceptors in the central nervous system. The antihypertensive effect of clonidine which had initially been thought to function through the α_2-adrenoceptor, was found to be mediated through stimulation of an imidazoline preferring site (IPS) (Bousquet *et al.*, 1984; Ernsberger *et al.*, 1988a). Thus, it appeared that many effects which were previously attributed to the α_2-adrenoceptor may have in fact been mediated through another receptor/site, the imidazoline preferring site.

Radioligand binding studies have also indicated a class of receptors which are distinct from α_2-adrenoceptors. Studies to characterize α_2-adrenoceptors, with the radiolabelled antagonists rauwolscine and idazoxan, indicated that in fact two distinct populations of receptors may be involved (Michel *et al.*, 1989; 1990). Similarly, clonidine was found to label two distinct populations of receptors (Ernsberger *et al.*, 1987; 1988b). Subsequent studies have demonstrated that these non-adrenoceptor binding sites have a low affinity for catecholamines and a greater affinity for imidazolines (Bricca *et al.*, 1989; Ernsberger *et al.*, 1990). The apparent heterogeneity of these imidazoline sites has resulted in the proposal of two subtypes, I_1 and I_2 (Ernsberger, 1992; Reis *et al.*, 1992). The I_1 subtype appears to predominate in the rat kidney (Ernsberger *et al.*, 1992).

Moxonidine was previously reported to act as a central α_2-adrenoceptor agonist in the treatment of hypertension (Planitz, 1984). However, recent studies have found that moxonidine has a very high affinity for I_1-imidazoline sites in the central nervous system with an approximately 70 fold selectivity for I_1 imidazoline sites as compared to α_2-adrenoceptors (Ernsberger *et al.*, 1992). Moreover, radioligand binding studies in the rat renal medulla have shown that moxonidine has a 600 fold higher affinity for the I_1-imidazoline preferring site (IPS) than for the α_{2B}-adrenoceptor (Ernsberger *et al.*, 1992). Conceivably, more specific stimulation of these imidazoline preferring sites may elicit different physiological responses from those previously found for α_2-adrenoceptor agonists. We therefore determined the effects of an intrarenal infusion of moxonidine on water and solute excretion in the intact anaesthetized rat.

Methods

The standard procedures have previously been reported by our laboratory (Blandford & Smyth, 1988; 1990). Briefly, male Sprague-Dawley rats (200–225 g) were obtained from The University of Manitoba (Charles River Breeding Stock) and cared for as per regional animal care standards protocol. They were fed a standard Purina rat chow diet, with free access to tap water, in cages at 22°C with a 12 h light/dark cycle.

The right kidney was removed via a flank incision under ether anaesthesia 7 to 14 days prior to experimentation. Rats were anaesthetized with pentobarbitone (Nembutal, BDH Chemicals, Poole, England, 50 mg kg^{-1}, i.p.). Anaesthesia was supplemented with additional doses of 5 mg kg^{-1} as required. The animal was placed on a Harvard Animal Blanket Control Unit with a rectal thermometer probe set for 37.5°C. A tracheotomy was performed leaving the rat to breathe spontaneously through PE-240 tubing. The left

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carotid artery was cannulated with PE-60 tubing and connected to a Statham pressure transducer (Model P23Dc) and Grass Model 5 Polygraph for the monitoring of blood pressure and heart rate. The left jugular vein was cannulated with PE-160 tubing for the infusion of normal saline at 97 $\mu l\ min^{-1}$. A left flank incision was performed and the remaining kidney exposed. The ureter was catheterized for the timed collection of urine into pre-weighed vials. Urine volume was determined gravimetrically. A 31 gauge stainless steel needle was inserted into the renal artery for the infusion of study drug or vehicle with a Harvard sage pump. Following surgery the rat was allowed to stabilize for 45 min before the collection of urine. A 15 min baseline control urine collection was obtained to ensure the surgical procedure had not altered renal function. An intrarenal infusion (3.4 $\mu l\ min^{-1}$) or moxonidine (1, 3 or 10 $nmol\ kg^{-1}\ min^{-1}$) or vehicle (saline) was started and maintained for the duration of the experiment during which 4 consecutive 15 min urine collections were obtained.

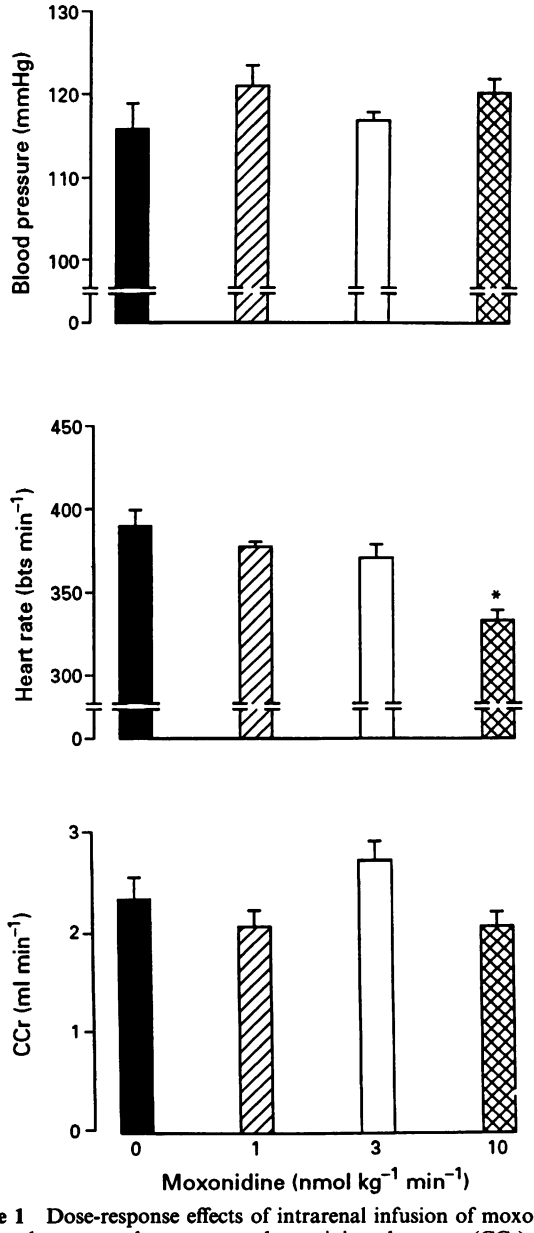


Figure 1 Dose-response effects of intrarenal infusion of moxonidine on blood pressure, heart rate and creatinine clearance (CCr) in the rat. Each group represents the mean ± s.e.mean (vertical bars) of at least 6 experiments. Moxonidine at 0 $nmol\ kg^{-1}\ min^{-1}$ represents vehicle (saline) infusion. $*P<0.05$ versus control vehicle.

V_2-vasopressin receptor antagonist

The role of vasopressin in the renal response to moxonidine was studied by administration of the specific V_2-vasopressin receptor antagonist, [d$(CH_2)_5$, D-Ile^2, Ile^4]-AVP. At the start of the second urine collection, the V_2 antagonist was administered as a slow intravenous bolus. A dose of 30 $nmol\ kg^{-1}$ was used as this dose had previously been demonstrated to block the renal effects of an α_2-adrenoceptor agonist, clonidine (Blandford & Smyth, 1990). Rats were randomly assigned to one of four study groups. Group 1 received an intrarenal infusion of saline (vehicle) at 3.4 $\mu l\ min^{-1}$. Group 2 received an intrarenal infusion of moxonidine at 3 $nmol\ kg^{-1}\ min^{-1}$. Group 3 received intrarenal saline with an intravenous administration of the specific V_2 antagonist. Group 4 received both the intrarenal moxonidine at 3 $nmol\ kg^{-1}\ min^{-1}$ following the intravenous dose of the V_2 antagonist.

Specific α_2-adrenoceptor and IPS antagonists

To determine the specificity of moxonidine, 3 $nmol\ kg^{-1}\ min^{-1}$ for the IPS over the α_2-adrenoceptor, the specific imidazoline site antagonist, idazoxan, or the specific α_2-adrenoceptor antagonist, rauwolscine, were given in a dose of 0.3 $mg\ kg^{-1}$ as a slow i.v. bolus at 30 min into the stabilization period. In the presence of these antagonists, the renal responses to moxonidine and clonidine (0.37 $nmol\ kg^{-1}\ min^{-1}$) were determined.

At the end of the experiment a sample of blood was collected through the carotid line. The plasma was frozen. Methylene blue was injected through the renal arterial line to confirm proper positioning of the needle. Urine and plasma

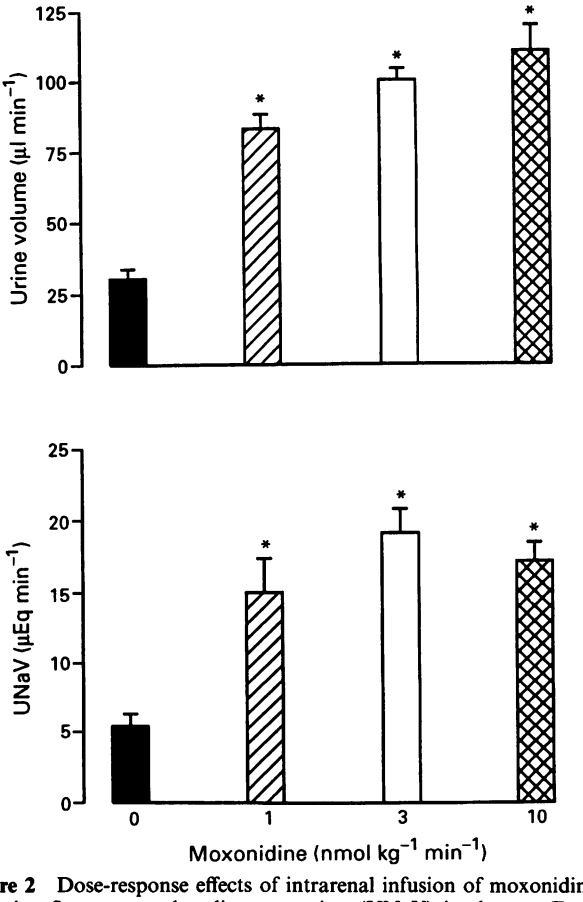


Figure 2 Dose-response effects of intrarenal infusion of moxonidine on urine flow rate and sodium excretion (UNaV) in the rat. Each group represents the mean ± s.e.mean (vertical bars) of at least 6 experiments. $*P<0.05$ versus control vehicle.

creatinine were measured with a Beckman Creatinine 2 Analyzer. Urine and plasma osmolality was measured with a Precision Systems Micro Osmometer. Sodium and potassium levels in urine and plasma were measured by a Beckman Klina Flame Photometer.

Statistical analysis

Each experimental group consisted of 6 rats. Data are presented as the mean ± standard error (s.e.) of the mean. Data were analyzed by repeated measures of analysis of variance. Significant interactions were further analyzed by a one way ANOVA Fisher's least squares difference multiple comparison test (Winer, 1971). A P value of <0.05 was deemed significant and is denoted by a * in the figures. For the purpose of presentation, data from the fifth collection period are shown since this is representative of differences observed between groups.

Drugs

Clonidine (Sigma Chemical Company, St. Louis, MO, U.S.A.); [d(CH_2)$_5$, D-Ile^2, Ile^4]-AVP (Penninsula Laboratories, Inc., Belmont, CA, U.S.A.); rauwolscine (Atomergic Chemicals Corp., Plainview, NY, U.S.A.); idazoxan (Research Biochemicals, Inc., Natick, MA, U.S.A.) and moxonidine (Supplied by Beiersdorf, AG, Hamburg, Germany) were used.

Results

The first urine collection for each experiment served as a preparation control (data not shown). This allowed evaluation of baseline renal function for each experiment and the determination of altered values secondary to the surgical procedure. In all groups studied, the measured parameters did not differ between groups during this first collection period prior to any experimental intervention. The fifth collection period is representative of the differences observed between groups and consequently these data are presented in detail.

In the present study, moxonidine failed to alter blood pressure and creatinine clearance at all three doses investigated (Figure 1). The highest infusion rate investigated, however, did produce a small but significant decrease in heart rate. Moxonidine increased urine flow rate and sodium excretion but not potassium excretion (data not shown) at all three doses investigated (Figure 2). The above increase in urine flow rate was secondary to an increase in solute excretion as reflected by an increase in osmolar clearance without a change in free water clearance (Figure 3).

The increase in osmolar clearance and not free water clearance reported above suggested that moxonidine, unlike α_2-adrenoceptor agonists, was mediating these effects independent of vasopressin. The significance of vasopressin in the above response to moxonidine was determined by the pretreatment of rats with a specific V_2 vasopressin receptor antagonist (Figure 4). As shown above in Figure 3, moxonidine alone increased urine volume primarily through an increase in solute (sodium) excretion. The V_2 antagonist alone increased urine volume by increasing free water clearance. When moxonidine was infused in the presence of the V_2 antagonist, a further increase in urine flow rate but not sodium excretion was observed. No significant differences in blood pressure, heart rate, creatinine clearance or potassium excretion were observed (data not shown).

A third series of experiments involved the use of the imidazoline antagonist, idazoxan, and the α_2-adrenoceptor antagonist, rauwolscine. As shown above, moxonidine increased urine flow rate and urine sodium excretion. Idazoxan (IPS antagonist) but not rauwolscine (α_2-adrenoceptor antagonist) significantly attenuated the renal effects of moxonidine (Figure 5). Conversely the same dose of rauwolscine, which failed to block the action of moxonidine, blocked the effects of clonidine (Figure 6). Consistent with two sites of action, idazoxan pretreatment at a dose that blocked the effects of moxonidine failed to alter the effect of clonidine.

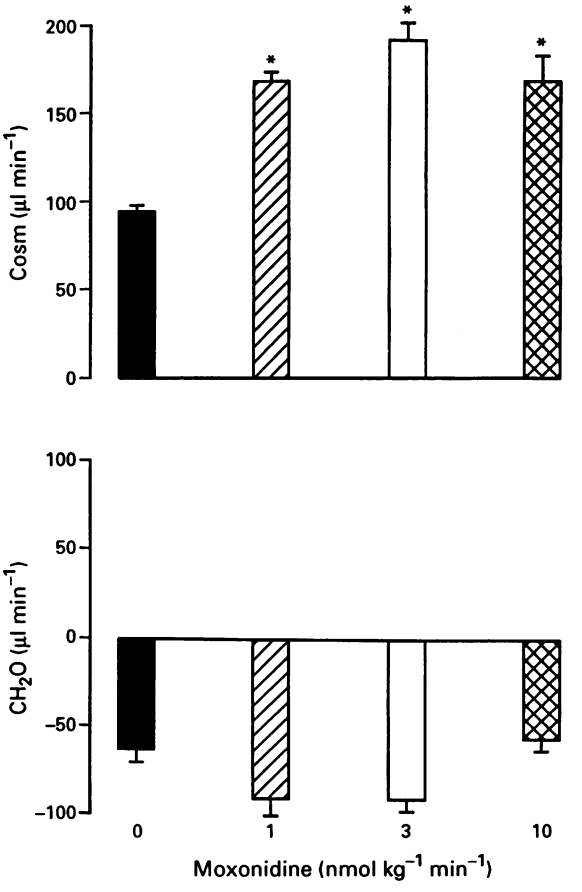


Figure 3 Dose-response effects of intrarenal infusion of moxonidine on osmolar clearance (Cosm) and free water clearance (CH_2O) in the rat. Each group represents the mean ± s.e.mean (vertical bars) of at least 6 experiments. *$P<0.05$ versus control vehicle.

Discussion

Both moxonidine and clonidine contain an imidazolidine ring structure and a phenylephrine catecholamine structure (Planitz, 1984). Despite this similarity in structure, recent radioligand binding studies suggest that moxonidine has a much greater affinity for the IPS than clonidine (Ernsberger *et al.*, 1992). The significance of the IPS was initially reported by Bousquet *et al.* (1984). These studies divided the effects of agonists, following microinjection into the nucleus reticularis lateralis, based on structure rather than previously reported specificity for α_2-adrenoceptors. They found that α_2-adrenoceptor agonists with a basic phenylethylamine structure, such as noradrenaline or α-methylnoradrenaline failed to lower the blood pressure. However, when agonists with an imidazoline moiety were injected into this same area, the blood pressure was found to drop significantly. These studies questioned whether the centrally mediated blood pressure lowering effects of many purported α_2-adrenoceptor agonists was in fact mediated at α_2-adrenoceptors.

The majority of functional studies have concentrated on the hypotensive effects of compounds with imidazoline structures as mediated through the central nervous system. However, imidazoline preferring sites have also been identified in the kidney (Michel & Insel, 1989; Parini *et al.*, 1989; Bidet *et*

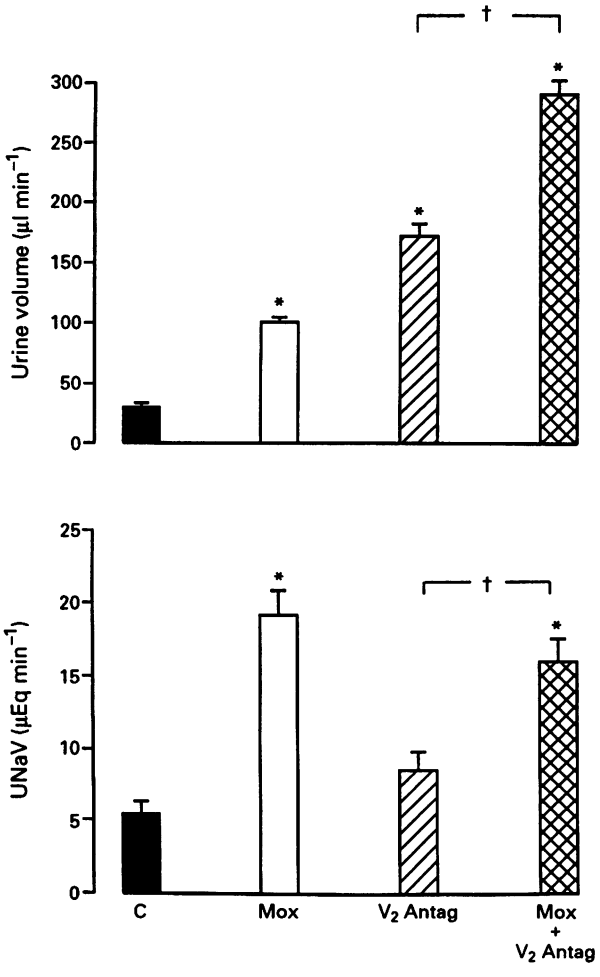


Figure 4 Effects of moxonidine in the presence and absence of a specific V_2 antagonist on urine volume and sodium excretion (UNaV) in the rat. C, control; Mox, moxonidine (3 nmol kg^{-1} min^{-1}); V_2 Antag, V_2 vasopressin receptor antagonist (30 nmol kg^{-1}); Mox + V_2 Antag, moxonidine following V_2 antagonist pretreatment. Each group represents the mean ± s.e.mean (vertical bars) of at least 6 experiments. $^*P<0.05$ versus control; $\dagger P<0.05$ between groups.

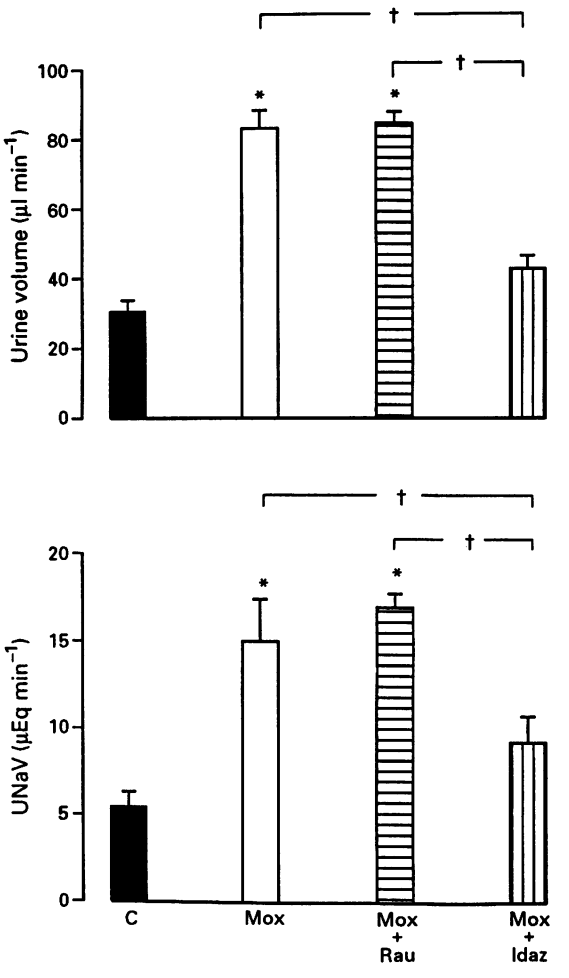


Figure 5 Effects of an intrarenal infusion of moxonidine in the absence and presence of a specific α_2-adrenoceptor antagonist (rauwolscine) and a specific IPS antagonist (idazoxan). C, vehicle control; Mox, moxonidine (3 nmol kg^{-1} min^{-1}); Mox + Rau, moxonidine (3 nmol kg^{-1} min^{-1}) + rauwolscine (0.3 mg kg^{-1}); Mox + Idaz, moxonidine (3 nmol kg^{-1} min^{-1}) + idazoxan (0.3 mg kg^{-1}). Each group represents the mean ± s.e.mean (vertical bars) of at least 6 experiments. $^*P<0.05$ versus control; $\dagger P<0.05$ between groups.

al., 1990). Studies with isolated renal tubular cells found that $^{22}Na^+$ entry could be blocked by idazoxan. A concurrent cellular alkalinization suggested that the IPS was associated with the $Na^+ - H^+$ antiporter. The effect of stimulating these sites on solute and water excretion in the intact animal had not been determined even though these sites had been identified in the kidney biochemically.

The above studies indicated that many effects previously attributed to activation of the α_2-adrenoceptor may in fact involve another site. Studies which have centred on the renal effects of purported α_2-adrenoceptor agonists have also indicated that more than one site and/or receptor may be involved. Strandhoy *et al.* (1982) first showed that a systemic intravenous infusion of clonidine in the dog produced a greater increase in urine flow rate and sodium excretion than a direct intrarenal infusion. Studies in our laboratory have focussed on the ability of a number of α_2-adrenoceptor agonists to increase urine flow rate based on changes in water (free water clearance) and/or solute (osmolar clearance) excretion. Results of a number of studies have been consistent with the effects of α_2-adrenoceptor agonists being mediated at more than one site and/or receptor. For example, low intrarenal infusion rates of clonidine increase free water clearance, while higher infusion rates also increase osmolar clearance (Blandford & Smyth, 1988). Although intrarenal infusion of clonidine produced an increase in urine flow rate secondary to an increase in free water clearance, intravenous infusions were found to increase osmolar clearance (Blandford & Smyth, 1989). Indomethacin pretreatment also had disparate effects, with an enhancement of the osmolar effects and an attenuation of the free water effects of clonidine (Blandford & Smyth, 1991). Thus, as with central administration of α_2-adrenoceptor agonists, the effects in the periphery also indicated other sites may be involved.

A recent study in our laboratory compared the ability of three purported α_2-adrenoceptor agonists to increase water (free water) and solute (osmolar) excretion (Smyth *et al.*, 1992). Clonidine and 2,6 dimethyl clonidine, two purported α_2-adrenoceptor agonists (Fondacaro *et al.*, 1989), displayed similar potencies in the ability to increase urine flow rate. However, the effects of clonidine were mediated primarily by an increase in free water clearance and the effects of 2,6 dimethyl clonidine were mediated primarily by an increase in osmolar clearance. Moreover, pretreatment with a specific V_2 vasopressin-specific antagonist completely attenuated the renal actions of clonidine but not of 2,6 dimethyl clonidine. These findings were consistent with the existence of two subtypes of α_2-adrenoceptors and/or two unique receptors. Although not previously documented, 2,6, dimethyl clonidine has been found to possess a high affinity for IPSs (Dr Paul Ernsberger, personal communication). Consequently, in the

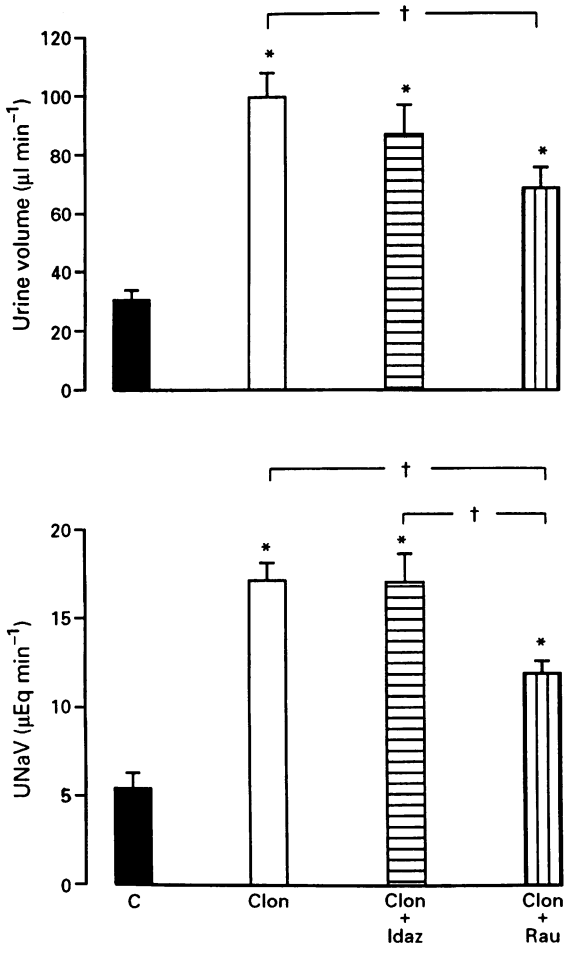


Figure 6 Effects of an intrarenal infusion of clonidine in the absence and presence of a specific α_2-adrenoceptor antagonist (rauwolscine) and a specific IPS antagonist (idazoxan). C, vehicle control; Clon, clonidine (0.37 nmol kg^{-1} min^{-1}); Clon + Idaz, clonidine (0.37 nmol kg^{-1} min^{-1}) + idazoxan (0.3 mg kg^{-1}); Clon + Rau, clonidine (0.37 nmol kg^{-1} min^{-1}) + rauwolscine (0.3 mg kg^{-1}). Each group represents the mean ± s.e.mean (vertical bars) of at least 6 experiments. *$P<0.05$ versus control; †$P<0.05$ between groups.

present study, we investigated the effects of a specific IPS agonist, moxonidine, on renal function.

The present studies provide three separate lines of evidence that stimulation of the IPS represents a distinct site from the α_2-adrenoceptor. Moxonidine produced a marked enhancement of sodium excretion and osmolar clearance at all 3 doses tested with no change in free water clearance. Studies with α_2-adrenoceptor agonists have shown an increase in urine flow rate associated with an increase in free water clearance (Stanton *et al.*, 1987; Blandford & Smyth, 1988). Only a very modest increase in osmolar clearance was reported following α_2-adrenoceptor stimulation. These different effects on free water clearance suggested that stimulation of α_2-adrenoceptors but not the IPS may be dependent on the renal effects of vasopressin. Previous studies in our laboratory demonstrated that pretreatment with a vasopressin V_2 receptor antagonist to block the effects of vasopressin completely attenuated the effect of α_2-adrenoceptor stimulation with low dose clonidine (Blandford & Smyth, 1990). These results were consistent with previous studies which had reported that the effects of renal α_2-adrenoceptor stimulation in the rat, both *in vivo* and *in vitro*, were dependent on the presence of vasopressin (Krothapalli *et al.*, 1983; Smyth *et al.*, 1985; Pettinger *et al.*, 1987). In contrast, administration of moxonidine in the presence of a V_2 antagonist increased urine flow rate further than that observed with the V_2 antagonist alone. The failure of the V_2 antagonist to attenuate the response to moxonidine is consistent with this site functioning independently of vasopressin.

Another line of evidence which suggests that the α_2-adrenoceptor and the IPS represent distinct sites and subserve distinct functions involved the use of idazoxan and rauwolscine at doses that were specific for the imidazoline preferring site and the α_2-adrenoceptor respectively. A number of studies had found that idazoxan bound with a high affinity to non-adrenoceptor sites and that these sites were different from α_2-adrenoceptors (Michel *et al.*, 1989; 1990). We therefore used idazoxan to antagonize these non-adrenoceptor sites, which were conceivably the sites at which the effects of moxonidine were mediated. Idazoxan but not rauwolscine blocked the enhancement of urine volume, sodium excretion and osmolar clearance caused by moxonidine. These studies in themselves did not support the specificity of the antagonists. However rauwolscine, at the same dose that failed to block the effects of moxonidine, attenuated the effects of clonidine. Similarly, idazoxan, at doses that blocked moxonidine, failed to alter the effects of clonidine. The selective blockade of these to agonists by rauwolscine and idazoxan provides support for the actions being mediated at two distinct sites.

These three lines of evidence provide strong support that stimulation of the I_1-imidazoline preferring site with moxonidine increases solute excretion at a site distinct from the α_2-adrenoceptor. As distinct from α_2-adrenoceptors, it appears that I_1-imidazoline preferring sites increase urine flow rate secondarily to an increase in solute excretion and this effect is independent of the renal action of vasopressin.

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References

BIDET, M., POUJEOL, P. & PARINI, A. (1990). Effect of imidazolines on Na^+ transport and intracellular pH in renal proximal tubule cells. *Biochim. Biophys. Acta*, **1024**, 173–178.

BLANDFORD, D.E. & SMYTH, D.D. (1988). Dose selective dissociation of water and solute excretion after alpha-2 adrenoceptor stimulation. *J. Pharmacol. Exp. Ther.*, **247**, 1181–1186.

BLANDFORD, D.E. & SMYTH, D.D. (1989). Enhanced natriuretic potency of intravenous clonidine: exta renal site of action? *Eur. J. Pharmacol.*, **174**, 181–188.

BLANDFORD, D.E. & SMYTH, D.D. (1990). Role of vasopressin in response to intrarenal infusions of alpha-2 adrenoceptor agonists. *J. Pharmacol. Exp. Ther.*, **255**, 264–270.

BLANDFORD, D.E. & SMYTH, D.D. (1991). Potentiation of the effects of renal α_2-adrenoceptor stimulation by indomethacin. *Can. J. Physiol. Pharmacol.*, **69**, 1196–1203.

BOUSQUET, P., FELDMAN, J. & SCHARTZ, J. (1984). Central cardiovascular effects of the alpha adrenergic drugs: differences between catecholamines and imidazolines. *J. Pharmacol. Exp. Ther.*, **230**, 232–236.

BRICCA, G., DONTENWILL, M., MOLINES, A., FELDMAN, J., BELCOURT, A. & BOUSQUET, P. (1989). The imidazoline preferring receptor: binding studies in bovine, rat and human brainstem. *Eur. J. Pharmacol.*, **162**, 1–9.

ERNSBERGER, P. (1992). Heterogeniety of imidazoline binding sites: proposed I1 and I2 subtypes. *Fundam. Clin. Pharmacol.*, **6** (suppl 1), 55s, (abstract).

ERNSBERGER, P., GIULIANO, R., WILLETTE, R.N., GRANATA, A.R. & REIS, D.J. (1988a). Hypotensive action of clonidine analogues correlates with binding affinity at imidazole and not alpha-2-adrenergic receptors in the rostral ventrolateral medulla. *J. Hypertens.*, **6** (suppl 4), S554–S557.

ERNSBERGER, P., GIULIANO, R., WILLETTE, R.N. & REIS, D.J. (1990). Role of imidazoline receptors in the vasodepressor response to clonidine analogs in the rostral ventrolateral medulla. *J. Pharmacol. Exp. Ther.*, **253,** 408–418.

ERNSBERGER, P., MEELY, M.P., MANN, J.J. & REIS, D.J. (1987). Clonidine binds to imidazoline binding sites as well as α_2-adrenoceptors in the ventrolateral medulla. *Eur. J. Pharmacol.*, **134,** 1–13.

ERNSBERGER, P., MEELY, M.P. & REIS, D.J. (1988B). An endogenous substance with clonidine-like properties: selective binding to imidazole sites in the ventrolateral medulla. *Brain Res.*, **441,** 309–318.

ERNSBERGER, P., WESTBROOKS, K.L., CHRISTEN, M.O. & SCHAFER, S.G. (1992). A second generation of centrally acting antihypertensive agents act on putative I_1-imidazoline receptors. *J. Cardiovasc. Pharmacol.*, **20** (suppl 4), (in press).

FONDACARO, J.D., MCCAFFERTY, G.P., KOLPAK, D.C. & SMITH, P.L. (1989). Antidiarrheal activity of alpha-2 adrenoceptor agonist SK&F 35886. *J. Pharmacol. Exp. Ther.*, **249,** 221–228.

GESEK, F.A. & STRANDHOY, J.W. (1990). Dual interactions between alpha-2 adrenoceptor agonists and the proximal Na-H exchanger. *Am. J. Physiol.*, **258,** F636–F642.

KROTHOPALLI, R.K., DUFFY, W.B., SENEKJIAN, H.O. & SUKI, W.N. (1983). Modulation of the hydroosmotic effect of vasopressin on the rabbit cortical collecting tubule by adrenergic agents. *J. Clin. Invest.*, **72,** 287–294.

MICHEL, M.C. & INSEL, P.A. (1989). Are there multiple imidazoline binding sites. *Trends Pharmacol. Sci.*, **10,** 342–344.

MICHEL, M.C., BRODDE, O.-E., SCHNEPEL, B., BEHRENDT, J., TSCHADA, R., MOTULSKY, H.J. & INSEL, P.A. (1989). [^{3}H]idazoxan and some other alpha-2 adrenergic drugs also bind with a high affinity to a non-adrenergic site. *Mol. Pharmacol.*, **35,** 324–330.

MICHEL, M.C., REGAN, J.W., GERHARDT, M.A., NEUBIG, R.R., INSEL, P.A. & MOTULSKY, H.J. (1990). Nonadrenergic [^{3}H]idazoxan binding sites are physically distinct from alpha-2 adrenergic receptors. *Mol. Pharmacol.*, **37,** 65–68.

PARINI, A., COUPRY, I., GRAHAM, R.M., UZIELLI, I., ATLAS, D. & LANIER, S.M. (1989). Characterization of an imidazoline/guanidinium receptive site distinct from the alpha-2 adrenergic receptor. *J. Biol. Chem.*, **264,** 11874–11878.

PETTINGER, W.A., UMEMURA, S., SMYTH, D.D. & JEFFRIES, W.B. (1987). Renal alpha-2 adrenoceptors and the adenylate cyclase-cAMP system: Biochemical and physiological interactions. *Am. J. Physiol.*, **252,** F199–F208.

PLANITZ, V. (1984). Crossover comparison of moxonidine and clonidine in mild to moderate hypertension. *Eur. J. Clin. Pharmacol.*, **27,** 147–152.

REIS, D.J., REGUNATHAN, S., WANG, H., FEINSTEIN, D.L. & MEELEY, M.P. (1992). Imidazoline receptors in the nervous system. *Fundam. Clin. Pharmacol.*, **6** (suppl 1), 23s–29s.

SMYTH, D.D., LI, P., BLANDFORD, D.E. & PENNER, S.B. (1992). Opposite rank order of potency for alpha-2 adrenoceptor agonists on water and solute excretion in the rat: Two sites and/or receptors. *J. Pharmacol. Exp. Ther.*, **261,** 1080–1086.

SMYTH, D.D., UMEMURA, S. & PETTINGER, W.A. (1985). Renal nerve stimulation causes alpha-1 adrenoceptor mediated sodium retention but not alpha-2 adrenoceptor antagonism of vasopressin. *Circ. Res.*, **57,** 304–311.

STRANDHOY, J.W., MORRIS, M. & BUCKALEW, JR., V. (1982). Renal effects on the antihypertensive, guanabenz in the dog. *J. Pharmacol. Exp. Ther.*, **221,** 347–352.

STANTON, B., PUGLISI, E. & GELLAI, M. (1987). Localization of alpha-2 adrenoceptor-mediated increase in renal Na^+, K^+, and water excretion. *Am. J. Physiol.*, **252,** F1016–1021.

WINER, B.J. (1971). *Statistical Principles in Experimental Design*, 2nd ed. New York: McGraw-Hill.

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The effect of cyclic AMP and cyclic GMP phosphodiesterase inhibitors on the superoxide burst of guinea-pig peritoneal macrophages

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1 The cyclic nucleotide phosphodiesterase (PDE) activity of guinea-pig peritoneal macrophages was partially characterized and the effects of selective and non-selective inhibitors of adenosine 3':5'-cyclic monophosphate (cyclic AMP PDE) and guanosine 3':5'-cyclic monophosphate (cyclic GMP PDE) phosphodiesterases on superoxide generation were investigated using peritoneal macrophages from horse-serum pretreated guinea-pigs.

2 The non-selective PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX) and the PDE I/V selective inhibitor, zaprinast, inhibited spontaneous superoxide generation with IC_{50}s of $30.7 \pm 11.3\,\mu M$ and $145 \pm 17\,\mu M$ respectively ($n = 6$ and 5). The concentration-response curves for the PDE IV selective inhibitors rolipram and Ro20-1724 were biphasic; mean maximum inhibitions were $56.9 \pm 5.9\%$ and $66.8 \pm 10.5\%$ respectively at $300\,\mu M$, but in 2 out of 6 (rolipram) and 2 out of 5 (Ro20-1724) experiments inhibition was $< 50\%$. The PDE III inhibitor SK&F 94120 was without effect. Spontaneous superoxide generation was reduced $57 \pm 10\%$ by $1\,\mu M$ prostaglandin E_2 (PGE_2) and $62.6 \pm 3.76\%$ by $1\,\mu M$ salbutamol.

3 The increase in superoxide generation elicited by FMLP (10^{-9}–10^{-5} M) was unaffected by any of the PDE inhibitors studied. Inhibition of FMLP-stimulated superoxide generation by PGE_2 was enhanced in the presence of $10\,\mu M$ IBMX.

4 Macrophages were found to contain a predominantly membrane bound cyclic AMP PDE (90% of total activity) which was unaffected by cyclic GMP or calcium/calmodulin. The cyclic AMP PDE activity in the cytosolic fraction was enhanced in the presence of calcium/calmodulin. Selective inhibitors of PDE IV inhibited the particulate cyclic AMP PDE activity (IC_{50}s rolipram $1.5 \pm 0.3\,\mu M$, Ro 20-1724 $4.1 \pm 0.6\,\mu M$) as did the non-selective inhibitor IBMX (IC_{50} $22 \pm 8\,\mu M$). The macrophage particulate PDE activity was resistant to inhibition by the PDE III inhibitor SK&F 94836 and the PDE I/V inhibitor, zaprinast. The cytosolic calcium/calmodulin stimulated cyclic AMP hydrolytic activity was inhibited by zaprinast (IC_{50} - calcium/calmodulin $123 \pm 39\,\mu M$; + calcium/calmodulin IC_{50} $17.7 \pm 6.3\,\mu M$).

5 The results indicate that guinea-pig peritoneal macrophages contain a type IV cyclic AMP PDE which is predominantly membrane associated and a predominantly cytosolic calcium/calmodulin stimulated cyclic AMP PDE. Functional studies suggest that both of these PDE activities contribute to cyclic AMP hydrolysis and regulation of superoxide generation in these cells. Inhibition of spontaneous superoxide generation, but not that stimulated by FMLP, suggests that the activity of PDE inhibitors is subject to functional antagonism but that this can be overcome by enhancing cyclic AMP formation.

Keywords: Macrophages; cyclic nucleotide phosphodiesterase isoenzymes; rolipram; Ro 20-1724; SKF 94120; 3-isobutyl-1-methylxanthine (IBMX); zaprinast; superoxide generation

Introduction

Raised levels of intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) exert an important inhibitory role in regulating the functional activity of a number of cell types involved in the inflammatory process including lymphocytes, mast cells, neutrophils and eosinophils (Torphy & Undem, 1991).

Intracellular levels of cyclic AMP are regulated by the degree of activation of adenylate cyclase which catalyzes the synthesis of cyclic AMP from adenosine 5'-triphosphate and its rate of breakdown by phosphodiesterase enzymes which hydrolyse cyclic AMP to the biologically inactive adenosine 5'-monophosphate. It is now well established that phosphodiesterase activity is exhibited by a heterogeneous group of distinct isoenzymes making up at least five different families as distinguished by their kinetic characteristics, substrate specificity, sensitivity to endogenous regulators and susceptibility to inhibition by selective agents (Beavo 1988; Beavo & Reifsnyder, 1990). In inflammatory cells a number of ligands are reported to elevate cyclic AMP levels, the increase in intracellular cyclic AMP generally mediating inhibitory responses (Yukawa *et al.*, 1989; Dent *et al.*, 1990; Marone *et al.*, 1980; Wirth & Kierszenbaum 1982; Cronstein *et al.*, 1985). More recently much attention has focused on the nature and the role of cyclic nucleotide phosphodiesterases (PDE's) in inflammatory cells. A number of studies have demonstrated that the phosphodiesterase catalysing cyclic AMP hydrolysis in a number of inflammatory cells including eosinophils, (Souness *et al.*, 1991; Dent *et al.*, 1991), neutrophils (Wright *et al.*, 1990; Nielson *et al.*, 1990), and monocytes (White *et al.*, 1990) is almost exclusively the type IV enzyme having a high affinity for cyclic AMP (low K_m) being unaffected by guanosine 3':5'-cyclic monophosphate (cyclic GMP) or calcium-calmodulin and being inhibited by the PDE IV-selective inhibitors rolipram and Ro 20-1724.

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In neutrophils and eosinophils, inhibition of the phosphodiesterase is associated with inhibition of the respiratory burst (Wright *et al.*, 1990; Nielson *et al.*, 1990; Souness *et al.*, 1991; Dent *et al.*, 1991), whilst in monocytes non-selective inhibitors reduce arachidonate metabolism stimulated by opsonized zymosan (Godfrey *et al.*, 1987). In macrophages a number of agonists, including isoprenaline, E-series prostaglandins and forskolin are known to elevate intracellular cyclic AMP (Liggett, 1989; Fuller *et al.*, 1988; Okonogi *et al.*, 1991). In contrast, there is a relative paucity of information concerning the phosphodiesterase activity in these cells or the effects of selective PDE inhibitors on macrophage function. Okonogi *et al.* (1991) have reported that the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) as well as the selective type IV inhibitor Ro20-1724 produce a synergistic elevation of cyclic AMP in prostaglandin E_2 (PGE_2)-treated murine macrophages. Whilst these authors did not study any functional consequence, Fuller *et al.* (1988) have reported that although incubation of human alveolar macrophages with isoprenaline and Ro20-1724 caused an increase in cyclic AMP accumulation there was no effect of either agent, alone or in combination, on macrophage superoxide generation, degranulation or thromboxane production in response to opsonized zymosan. Thus the the role of PDE isozymes in regulating macrophage function remains unclear.

Macrophages play a central role in a number of immune responses and have the capacity to release a wide range of pro-inflammatory stimuli including reactive oxygen species, lysosomal enzymes, lipid mediators and cytokines (Fels & Cohn, 1986). The therapeutic potential of isoenzyme selective PDE inhibitors has been the subject of a number of recent reviews (Weishaar *et al.*, 1985; Nicholson *et al.*, 1991; Torphy & Undem, 1991) and the identification of distinct isoenzyme families with variable tissue distribution has raised the premise that different PDE isoenzymes may be responsible for cyclic AMP or cyclic GMP hydrolysis in different tissues and cells. In addition the identification of selective inhibitors of these enzymes may enable specific pharmacological control of cyclic nucleotide regulated processes without the spectrum of side effects seen with non-selective inhibitors.

In order to determine whether selective PDE inhibition may have a role in regulating macrophage function, the aim of the present study was to investigate the effects of non-selective or isozyme selective PDE inhibitors, alone or in combination with stimuli to adenylate cyclase, on spontaneous and agonist-induced superoxide generation from guinea-pig peritoneal macrophages. In addition we have partially characterized the PDE isoenzymes present in these cells.

Methods

Macrophages were elicited in male Dunkin Hartley guinea-pigs (400–800 g) by pretreatment with horse serum (1 ml kg^{-1}, i.p.) twice weekly for 2–3 weeks. One week after the last injection the animals were killed by carbon dioxide narcosis and cells harvested from the peritoneal cavity by lavage with 30 ml Hanks balanced salts solution (HBSS without Ca^{2+} or Mg^{2+}) containing 0.38% (w/v) sodium citrate. Following centrifugation (150 *g* for 10 min) and lysis of contaminating red cells with 0.83% ammonium chloride (w/v), the peritoneal cells were resuspended in HBSS (without Ca^{2+} or Mg^{2+}) containing 5% (v/v) foetal calf serum (FCS) and overlayered onto a discontinuous Percol gradient (55%, 65%, 70% v/v in the resuspension buffer). The gradients were centrifuged at 150 *g* for 30 min at 20°C and macrophages, 84 ± 0.9% purity >95% viability as assessed by trypan blue exclusion, recovered from the surface of the 55% gradient stage (1.067 g ml^{-1}); the principal contaminating cell was the eosinophil (13 ± 0.8%).

Measurement of superoxide generation

Macrophages were washed with HBSS (without Ca^{2+} or Mg^{2+}) containing 5% FCS, resuspended at 20×10^6 cells ml^{-1} in HBSS (with Ca^{2+} and Mg^{2+}) and dispensed into 96-well microtitre plates, containing *p*-iodonitrotetrazolium violet (INTV), at 10^6 cells per well. Following a 15 min pre-incubation at 37°C with vehicle (0.1% dimethylsulphoxide DMSO) or drugs, the cells were challenged with FMLP (10^{-9}–10^{-5} M) or bovine serum albumin (BSA) for a further 30 min at 37°C. All control and drug treatment experiments were performed in parallel, using the same cell preparation. Superoxide generation was measured by reduction of *p*-iodonitrotetrazolium violet (INTV; Podczasy & Wei 1988; Souness *et al.*, 1990). INTV (50 mg) was dissolved in 0.5 ml DMSO and diluted to 1 mg ml^{-1} in HBSS. The final concentration of INTV used in the assay was 0.5 mg ml^{-1} (125 µl 1 mg ml^{-1} stock adjusted to 250 µl by additions to the well). Following incubation at 37°C the microtitre plates were centrifuged at 900 *g* for 10 min the supernatant aspirated and the cell pellet solubilized in DMSO containing 5% (v/v) conc HCl. The absorbance of the reduced dye in the solubilized cell pellet was measured at 492 nm in a Titertek Multiskan MCC/340 plate reader and the results expressed in absolute absorbance units or as the % increase in absorbance above basal levels. IC_{50}s were calculated as the concentrations causing 50% inhibition of spontaneous superoxide generation.

The iodonitrotetrazolium formazan (the reduced species of INTV) calibration curve, constructed by dilution of an 0.5 mg ml^{-1} stock (which represents maximum possible conversion of the initial species) is linear over the absorption range of 0.04–2.00 Abs. units corresponding to concentrations of the formazan of 0.001–0.06 mg ml^{-1}. The slope of the calibration curve was 0.97. The linearity of the absorption measurements over the 0–2 Abs. units range of the plate reader was ± 3% or 0.01 Abs. units which ever is the greater.

Preparation of macrophage subcellular fractions

Cells from the surface of the 55% gradient stage were incubated in 75 cm^2 plastic tissue culture flasks for 30 min in HBSS. Non-adherent cells were aspirated and the macrophage enriched adherent cell population removed by scraping. Macrophages ($100–200 \times 10^6$) were resuspended in HBSS, centrifuged at 250 *g* for 10 min at 4°C the supernatant decanted and the cell pellet resuspended in 5 ml homogenization buffer consisting of Tris-HCl pH 7.5, 20 mM, $MgCl_2$ 2 mM, dithiothreitol 1 mM, EDTA 5 mM, sucrose 250 mM, *p*-tosyl-L-lycine-chloromethyl ketone (TLCK) 20 µM, leupeptin 10 µg ml^{-1}, and 2000 units ml^{-1} aprotinin. Cells were homogenized on ice with a Dounce homogenizer (12 strokes). The homogenate was centrifuged at 105,000 *g* for 60 min at 4°C the supernatant collected and the pellet resuspended in an equal volume of homogenization buffer.

Solubilization and partial purification of particulate cyclic AMP PDE activity

The membrane bound cyclic AMP PDE was solubilized by homogenizing freshly prepared membranes with a Dounce homogenizer (12 strokes) in 2 ml of homogenization buffer containing 1% Triton-X 100. The homogenate was centrifuged at 100,000 *g* for 30 min and the supernatant containing the solubilized activity removed and the pellet resuspended in an equal volume of homogenization buffer. The solubilized sample was applied to a DEAE-trisacryl column (0.7 × 1 cm) pre-equilibrated with column buffer (20 mM Tris/HCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1% Triton-X 100, 20 µM *p*-tosyl-L-lycine-chloromethyl ketone pH 7.5.) The column was washed with 10 ml of column buffer and PDE activities eluted with a linear gradient of NaCl (0–0.5 M, 25 ml) in column buffer. The flow rate was 1 ml min^{-1} and 1 ml fractions were col-

lected. Assays on the eluting fractions were performed immediately after elution from the column.

Measurement of PDE activity

PDE activity in the soluble and particulate fractions as well as on column fractions was determined by the two-step radioisotope method of Thompson *et al.* (1979). The reaction mixture contained 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 0.2 mM EGTA and 0.05 mg ml^{-1} bovine serum albumin. Unless otherwise stated the substrate concentration was 1 μM. The IC_{50} values (concentrations which produced 50% inhibition of substrate hydrolysis) for the compounds examined were determined from concentration-response curves in which inhibitor concentrations ranged from 2.56 nM to 1 mM.

Protein concentrations were determined as described by Bradford (1976) with bovine serum albumin used as standard.

The nomenclature for the different cyclic nucleotide PDE's adopted in this paper is based on that of Beavo & Reifsnyder (1990).

Materials

HBSS with or without Ca^{2+} and Mg^{2+}, FCS and New born calf serum (NCS) were from Gibco. Chemicals were obtained from the following sources:- Percol (Pharmacia); aprotinin, BSA, calmodulin; cyclic AMP, cyclic GMP, dithiothreitol, DMSO, EDTA, EGTA, formyl-methionyl-leucyl-phenylalanine (FMLP), INTV, isobutylmethylxanthine (IBMX), leupeptin, mercaptoethanol, 5'-(N-ethylcarboxamido)-adenosine (NECA), prostaglandin E_2 (PGE_2), salbutamol, superoxide dismutase, TLCK, Tris-HCl and vasoactive intestinal polypeptide (VIP) Sigma Chemical Company, Poole, Dorset, UK). Cyclic [2,8-^{3}H]AMP (41 Ci $mmol^{-1}$) and cyclic [8-^{3}H]GMP (13.8 Ci $mmol^{-1}$) were from Amersham International (Little Chalfont Bucks, U.K.). Rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl) -2-pyrrolidone] and zaprinast were synthesized by the Department of Pharmaceutical Chemistry, Rhone-Poulenc Rorer Ltd., Dagenham Research Centre, U.K. Ro-20-1724 [1-4-(3-butoxy-4-methoxybenzyl)-2-imidozolidinone] was obtained from Roche Products Ltd. (Welwyn Garden City, U.K.). SKF 94120 (5-(4-acetamidophenyl)pyrazin-2-(1H)one) and SKF 94836 (**R,S**-2-cyano-1-methyl-3-[4-(1,4,5,6-tetra-hydro-4-methyl-6-oxo-3-pyridazinyl) phenyl]guanidine) were a gift from SmithKline Beecham Research Ltd., (Welwyn Garden City, U.K.). Drugs (rolipram, Ro 20-1724, SKF 94120, IBMX, NECA, VIP, salbutamol and PGE_2) were made up as a stock solution at 1 or 10 mM in DMSO and diluted 1: 100 in HBSS and then further 1:10 dilutions were made in HBSS containing 1% v/v DMSO. Stock solutions of FMLP (10 mM) were in HBSS containing 0.1% w/v BSA and serial dilutions made in the same solvent.

Statistics

Results are expressed as mean ± s.e.mean. Data were compared by Wilcoxon signed-ranks test; for inhibition of superoxide generation a one-tailed probability of $P<0.05$ was considered significant, in studies of the effects of calcium + calmodulin on cyclic AMP hydrolysis a two-tailed probability of $P<0.05$ was considered significant.

Results

The cell population purified by percol density centrifugation and harvested from the surface of the 55% gradient stage comprised 84 ± 0.9% macrophages and 13 ± 0.8% eosinophils the remainder being mononuclear cells. These cells were used for functional studies. Studies of macrophage PDE activity were carried out on the macrophage population enriched by adherence to plastic tissue culture flasks, the non-adherent eosinophils being removed prior to resuspension and homogenization of the adherent cells (>95% macrophages).

Effect of selective and non-selective PDE inhibitors on the macrophage respiratory burst

Incubation of macrophages at 37°C in 96-well microtitre plates was associated with a spontaneous rate of reduction of INTV that was linear with time over 30 min and attenuated (62 ± 3%, $n = 10$) by superoxide dismutase (SOD, 100 μg ml^{-1}, data not shown).

The effect of selective and non-selective PDE inhibitors on the SOD inhibited reduction of INTV (i.e. that attributable to superoxide generation) is shown in Figure 1; IBMX and zaprinast caused concentration-related reductions in superoxide generation with IC_{50}s of 30.7 ± 11.3 μM and 145 ± 17 μM respectively. In the case of the PDE IV inhibitors, rolipram and Ro20-1724, the concentration-inhibition relationship was biphasic with an initial phase of inhibition between 0.01 and 10 μM and the second between 10 and 300 μM. The inhibition of 'spontaneous' superoxide generation by rolipram and Ro20-1724 at 300 μM was 56.9 ± 5.9% and 66.8 ± 10.5% $n = 6$ and 5 respectively. In each case however, in two experiments inhibition failed to reach 50%. The selective PDE III inhibitor, SKF 94120, was ineffective at concentrations up to 300 μM.

FMLP (10^{-9}–10^{-5} M) elicited a concentration-dependent increase in reduction of INTV that was abolished in the presence of 100 μg ml^{-1} superoxide dismutase (Figure 4) and was therefore due to superoxide anion generation. In paired experiments incubation with IBMX, rolipram and Ro20-1724 but not SKF 94120 (all 10 μM) inhibited 'spontaneous' superoxide generation; 48.4 ± 5.1% by IBMX ($n = 10$, $P<0.05$),

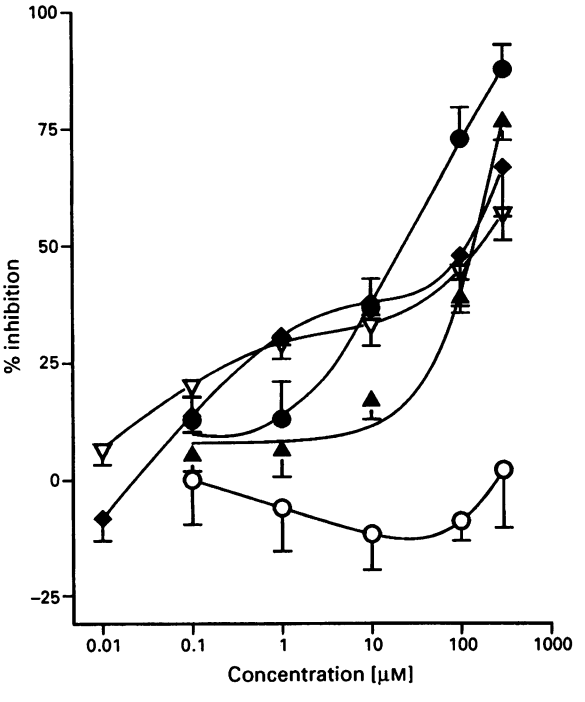


Figure 1 The effect of phosphodiesterase (PDE) inhibitors on spontaneous superoxide generation by elicited guinea-pig peritoneal macrophages: 10^6 cells were incubated for 30 min with increasing concentrations of PDE inhibitor:- (◆) Ro 20-1724; (▽) rolipram; (●) IBMX; (▲) zaprinast; (○) SKF 94120. Inhibition of superoxide generation is expressed as the % inhibition of the superoxide dismutase (100 μg ml^{-1}) inhibitable reduction of INTV measured at 492 nm in vehicle (0.1% DMSO)-treated cells. Data represent mean ± s.e.mean (vertical bars) from 5 or 6 experiments.

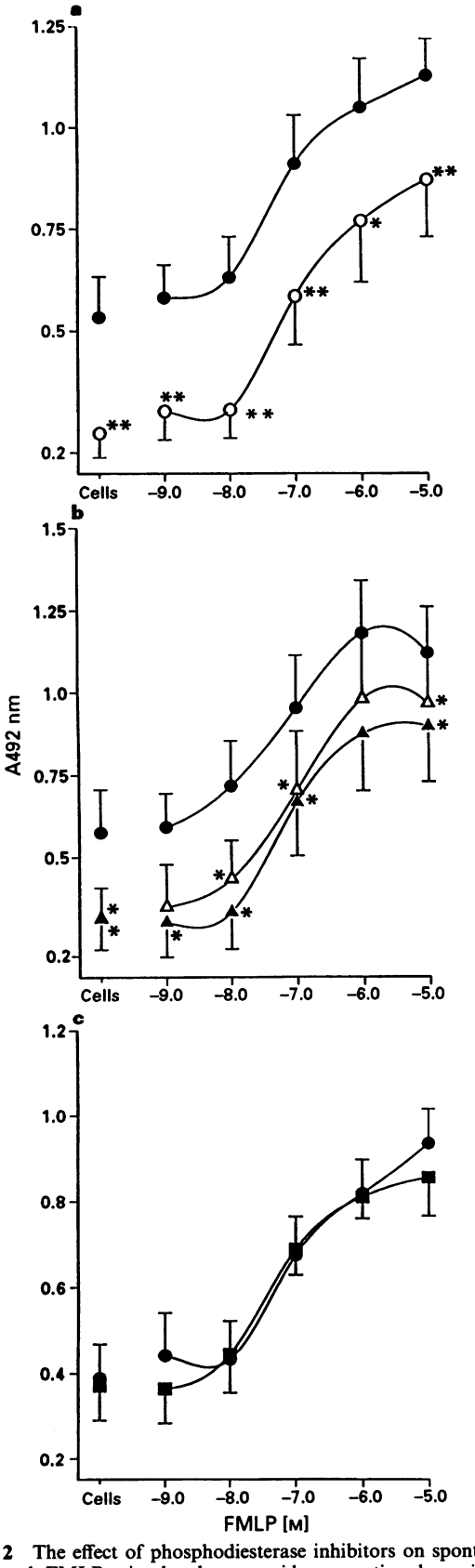


Figure 2 The effect of phosphodiesterase inhibitors on spontaneous (cells) and FMLP stimulated superoxide generation by guinea-pig elicited peritoneal macrophages: 10^6 cells were incubated for 15 min with 0.1% DMSO vehicle (●) or (a) 10 μM 3-isobutyl-1-methylxanthine (IBMX) (○); (b) 10 μM Ro 20-1724 (△), or 10 μM rolipram (▲); (c) 10 μM SK&F 94120 (■), prior to stimulation with FMLP for a further 30 min. Superoxide generation was measured as the superoxide dismutase (100 μg ml^{-1}) inhibitable reduction of INTV at 492 nm as described in the Methods. Results are expressed as the mean ± s.e.mean (vertical bars) of 5–7 paired experiments. $*P<0.03$; $**P<0.01$ compared to vehicle controls.

46.9 ± 6.05% by Ro 20-1714 ($n=5$, $P<0.05$) and 45.06 ± 7.66% by Rolipram ($n=5$, $P<0.05$) and as a consequence suppressed the SOD inhibited reduction of INTV in response to FMLP causing a downwards displacement of the FMLP dose-response curve (Figures 2a–c). The increase in superoxide generation stimulated by FMLP, following subtraction of spontaneous generation, however was unaffected by any of the inhibitors (Figure 3).

Effect of compounds which activate adenylate cyclase on the macrophage respiratory burst

At 1 μM, vasoactive intestinal polypeptide (VIP) and 5′-(N-ethylcarboxamido)-adenosine (NECA) were without effect on the resting rate of superoxide generation by these cells and had no effect on superoxide generation stimulated by FMLP. In contrast, salbutamol (1 μM) or PGE_2 (1 μM) significantly reduced spontaneous superoxide generation by 62.6 ± 3.76% ($n=5$, $P<0.05$) and 57.62 ± 10% ($n=5$, $P<0.05$), respectively, but like the PDE inhibitors were without effect on the increase stimulated by FMLP.

The inhibition of FMLP-stimulated superoxide generation by 1 μM PGE_2 or 10 μM IBMX was significantly greater when the two agents were combined, consistent with a synergistic interaction (Figure 4).

Characterization of macrophage PDE activity

Almost 90% of the cyclic AMP PDE activity of freshly prepared macrophages was membrane-bound. The PDE specific activity (SA) in the supernatant (SA = 66.8 ± 12.8 pmol min^{-1} mg^{-1} protein, $n=9$) was greater than that in the particulate fractions (SA = 34.8 ± 2.75 pmol min^{-1} mg^{-1} protein, $n=6$). (Table 1).

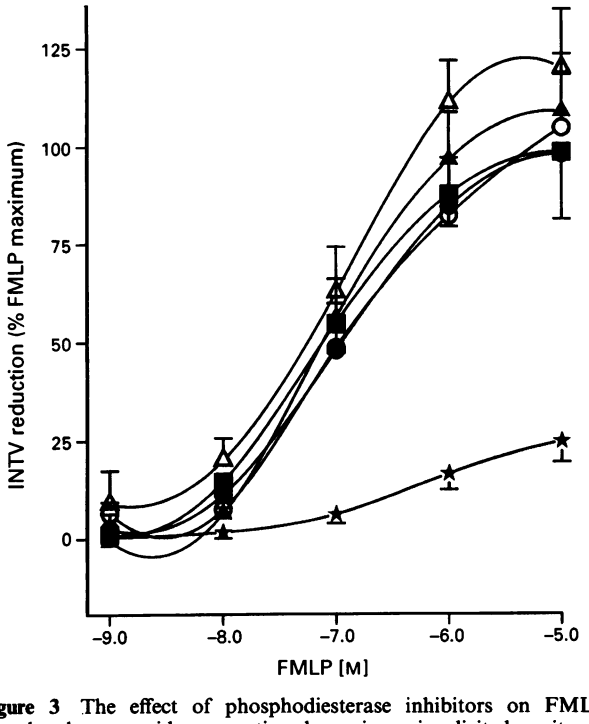


Figure 3 The effect of phosphodiesterase inhibitors on FMLP-stimulated superoxide generation by guinea-pig elicited peritoneal macrophages: 10^6 cells were incubated for 15 min with 0.1% dimethylsulphoxide (DMSO) vehicle (●) or 10 μM 3-isobutyl-1-methylxanthine (IBMX) (○); 10 μM Ro 20-1724 (△), 10 μM rolipram (▲) or 10 μM SK&F 94120 (■), prior to stimulation with FMLP for a further 30 min. (★) Shows the effects of superoxide dismutase (100 μg ml^{-1}) on the FMLP response. The data are expressed as a % of the maximum response to FMLP following subtraction of spontaneous reduction ('cells' in Figure 2). Results are expressed as the mean ± s.e.mean (vertical bars) of 5–7 paired experiments.

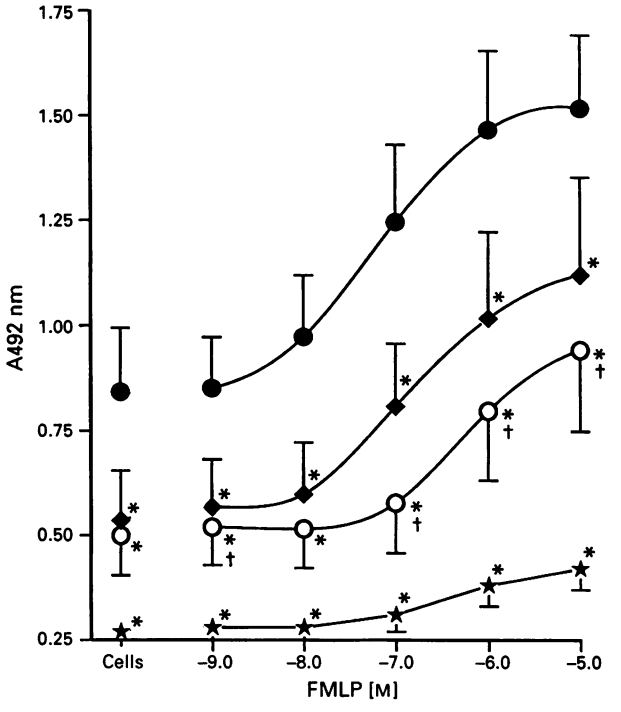


Figure 4 The effect of 1 μM prostaglandin E_2 (PGE_2) alone (◆) or in combination with 10 μM 3-isobutyl-1-methylxanthine (IBMX) (○) on spontaneous and FMLP stimulated INTV reduction by guinea-pig elicited peritoneal macrophages. Cells were pre-incubated with drugs or vehicle (●) for 15 min prior to stimulation with FMLP for a further 30 min and remained in contact with the cells for the total incubation time. 'Cells' is the spontaneous rate of INTV reduction in the absence of FMLP; (★) is the effect of 100 μg ml^{-1} superoxide dismutase on the spontaneous reduction of INTV and that stimulated by FMLP. Data represent mean ± s.e.mean of 5 separate paired experiments . *$P<0.03$ compared to vehicle; †$P<0.03$, compared to PGE_2.

Table 1 Subcellular distribution of cyclic AMP phosphodiesterase (PDE) activity in guinea-pig elicited peritoneal macrophages

	Specific activity (pmol min^{-1} mg^{-1} protein)	
Addition	*Supernatant*	*Pellet*
Cyclic AMP PDE:		
None	66.8 ± 12.8	34.8 ± 2.75
Ca^{2+} + CaM	86.9 ± 14.8*	37.9 ± 3.92
Cyclic GMP	64.8 ± 11.53	32.0 ± 2.99

Freshly prepared macrophages from horse-serum pretreated guinea-pigs were homogenized with a Dounce homogenizer (12 strokes) in 2 ml homogenization buffer. The homogenate was centrifuged at 100,000 *g* for 30 min at 4°C. The supernatant was removed and the pellet resuspended in an equal volume of homogenization buffer. Cyclic AMP PDE activity was measured in the absence (none) or presence of 2 mM calcium chloride + 10 u ml^{-1} calmodulin (Ca^{2+} + CaM) or 10 μM cyclic GMP. Cyclic nucleotide hydrolysis was measured in the presence of 1 μM substrate. The results represent means ± s.e.mean of 9 separate experiments. *$P<0.01$ compared to no addition (Two-tailed Wilcoxon signed-ranks test).

Cyclic AMP PDE activity in the soluble or particulate fractions was unaffected by cyclic GMP (10 μM) but addition of 2 mM $CaCl_2$ plus 10 u ml^{-1} calmodulin significantly increased activity in the cytosolic fraction ($P<0.01$; Table 1). Rolipram (IC_{50} 1.5 ± 0.3 μM), Ro-20-1724 (IC_{50} 4.1 ± 0.6 μM) and IBMX (IC_{50} 22.1 ± 8 μM) inhibited the bound, particulate cyclic AMP PDE (Table 2). In contrast, SK&F 94836, a selective PDE III inhibitor and zaprinast, a selective PDE I/V inhibitor only weakly inhibited the particulate PDE activity (IC_{50} > 500 μM). The cyclic AMP PDE activity in the cytosolic fraction was inhibited by zaprinast (IC_{50} 123.3 ± 39 μM, $n = 3$) and the potency of zaprinast was increased in the presence of calcium + calmodulin (IC_{50} 17.7 ± 6.2 μM, $n = 3$).

Table 2 Inhibitor potencies against guinea-pig elicited macrophage particulate cyclic AMP phosphodiesterase (PDE) activity

	IC_{50} (μM)
Rolipram	1.52 ± 0.32 (6)
Ro 20-1724	4.15 ± 0.65 (4)
IBMX	22.1 ± 8.0 (3)
SKF 94836	> 500 (3)
Zaprinast	> 500 (3)

Membrane-bound PDE was prepared by homogenization of 100–200 × 10^6 cells in 5 ml of homogenization buffer, on ice with a Dounce homogenizer (12 strokes). The homogenate was centrifuged at 105,000 *g* for 30 min and the pellet resuspended in an equal volume of homogenization buffer. PDE activity (1 μM substrate) was measured in the presence of increasing concentrations (2.56–1000 μM) of inhibitor, by the method of Thompson *et al.* (1973). Results are expressed as means ± s.e.mean, values in parentheses are the number of observations.

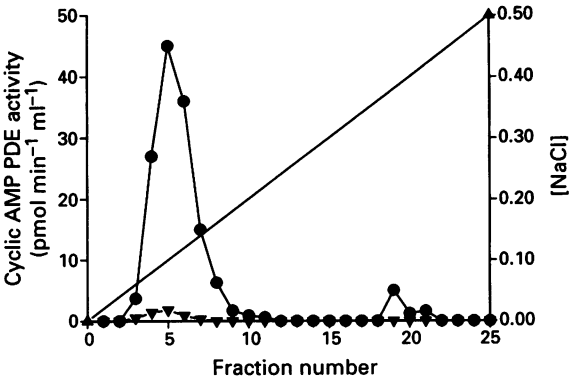


Figure 5 DEAE-trisacryl chromatography of solubilized cyclic AMP phosphodiesterase (PDE) activity. Preparations of the solubilized cyclic AMP PDE and its chromatography on DEAE trisacryl is described in Methods. Fractions were assayed for cyclic AMP PDE activity (1 μM) substrate in the presence of 200 μM EGTA. Recovery of activity was greater than 98%; (▼) is the effect of 4 μM rolipram on the eluting cyclic AMP PDE activity.

Gentle homogenization of macrophage membranes in 1% Triton-X 100 solubilized 80% of the particulate cyclic AMP PDE. Partial purification of this by DEAE-Tris acryl chromatography revealed a major peak of activity eluting with 0.1 M NaCl, which was completely inhibited by 4 μM rolipram. A second, minor peak of cyclic AMP PDE activity eluting with 0.4 M NaCl was also inhibited by 4 μM rolipram (Figure 5).

Discussion

Both cyclic AMP and cyclic GMP are known to mediate smooth muscle relaxation and five distinct PDE hydrolytic activities have been identified in airway smooth muscle which all differ in their kinetic characteristics and sensitivities to activators and inhibitors (Torphy & Cieslinski, 1990). In contrast the regulation of inflammatory cell function appears to be mediated by cyclic AMP alone (see Torphy & Undem, 1991, for references). The profile of PDE isoenzymes in inflammatory cells however has only recently been charac-

terized. We (Souness *et al.*, 1991) and others (Dent *et al.*, 1991) have shown that the PDE responsible for hydrolysis of cyclic AMP in eosinophils is predominantly a cyclic AMP specific, low K_m, rolipram and Ro 20-1724 inhibited isoenzyme defined as type IV. Similarly, Wright *et al.* (1990) and Nielson *et al.* (1990) have reported that the enzyme responsible for cyclic AMP hydrolysis in neutrophils is also almost exclusively the type IV PDE.

In the present study, in common with human neutrophils and guinea-pig eosinophils, the bulk of the cyclic AMP PDE hydrolytic activity in freshly prepared peritoneal macrophages (ca. 90%) was membrane-bound, although its exact location remains unknown. This activity was unaffected by calcium/calmodulin or cyclic GMP and was inhibited by rolipram and Ro 20-1724 but not selective inhibitors of the type III or V isoenzymes and thus is characteristic of the type IV PDE. Partial purification of the solubilized, particulate cyclic AMP PDE activity by DEAE Tris-acryl chromatography further resolved this activity into major and minor peaks, both of which were completely inhibited by rolipram. Multiple peaks of PDE IV activity have also been reported in the supernatant fraction of bovine tracheal smooth muscle (Shahid *et al.*, 1991) and rat hepatocytes (Lavan *et al.*, 1989) but have not been further characterized. Thus the nature of this second type IV-like activity remains unknown but it may represent a proteolytic breakdown product, aggregates of the monomeric type IV PDE or may be a PDE IV isoform.

In the present experiments analysis of PDE activity was carried out on an enriched macrophage population following adherence to a plastic surface with removal of non-adherent eosinophils. The resulting cell population was >95% macrophages compared to 84 ± 0.9% of the cell population intially recovered from the surface of the 55% gradient stage. Eosinophils (the major contaminant in the original cell population) however also possess a single membrane-bound cyclic AMP PDE IV (Souness *et al.*, 1991) and this may make a contribution, albeit small, to total cyclic AMP hydrolysis by the particulate fraction of the cell homogenate in the present experiments. The PDE IV activity in eosinophil membranes however is not solubilized by 1% Triton X-100 (Souness *et al.*, 1991; Dent *et al.*, 1991) whereas under identical conditions 80% of the membrane-associated PDE activity from the cells in the present study was solubilized. Furthermore this solubilized PDE activity was inhibited by rolipram (Figure 5). Thus the PDE IV activities identified on DEAE chromatography, accounting for the total solubilized activity and reflecting at least 80% of the total particulate cyclic AMP PDE activity are derived from the macrophage population. Furthermore significant hydrolysis of cyclic AMP by the soluble fraction of eosinophil homogenates has not been detected (Souness *et al.*, 1991; Dent *et al.*, 1991). Thus the calcium/calmodulin stimulated cyclic AMP PDE activity in the soluble fraction of the cell homogenates in the present experiments is derived from macrophages.

Interestingly the sensitivity of the particulate macrophage PDE IV to inhibition by rolipram or Ro 20-1724 was approximately 10 fold less than we and others have reported for guinea-pig peritoneal eosinophils (Dent *et al.*, 1991; Souness *et al.*, 1991) but is similar to that of the type IV PDE of human monocytes (rolipram 2.7 μM, Elliot & Leonard, 1989), canine trachea (Ro 20-1724 5.2 μM, Torphy & Ceislinski, 1990), human cardiac ventricle (rolipram 3.0, Ro 20-1724 3.8 μM, Reeves *et al.*, 1987) and dog kidney (Ro 20-1724 9 μM, rolipram 2 μM, Beavo, 1988). Whilst this difference in the sensitivity of the type IV PDE of eosinophils and macrophages to selective inhibitors cannot be explained at present it may indicate the existence of multiple isoforms of the PDE IV differing in their sensitivity to inhibition by rolipram or Ro 20-1724.

Consistent with the profile of particulate PDE activity in these cells, functional studies showed that the PDE IV inhibitors rolipram and Ro 20-1724, as well as the non-selective PDE inhibitor IBMX, but not an inhibitor of the PDE III enzyme (SKF 94120) inhibited spontaneous superoxide generation from guinea-pig peritoneal macrophages. The inhibition by the PDE IV inhibitors however was relatively poor, indeed in some experiments inhibition of superoxide generation by both rolipram or Ro20-1724 failed to reach 50%, at 300 μM. This relative insensitivity of the macrophage respiratory burst to PDE IV inhibitors is in marked contrast to their potent inhibition of eosinophil (IC_{50} ca. 0.1 μM) and neutrophil superoxide generation (Souness *et al.*, 1991; Dent *et al.*, 1990; Wright *et al.*, 1990). Nevertheless it is in agreement with the findings of Fuller *et al.* (1988) who have reported that human alveolar macrophage function is unaffected by PDE IV inhibition (Ro 20-1724, at concentrations up to 50 μM).

The magnitude of the SOD inhibited reduction of INTV by unstimulated guinea-pig peritoneal macrophages is similar to that caused by equivalent numbers of peritoneal eosinophils; it is possible therefore that up to 13% of INTV reduction in the present study is a consequence of free radical generation by the eosinophil contamination. Eosinophil superoxide generation however is relatively poorly inhibited by IBMX, is unaffected by zaprinast but is almost completely inhibited by 10 μM rolipram (Dent *et al.*, 1991; Souness *et al.*, 1991). The poor activity of rolipram in the present experiments coupled with the inhibitory activity of IBMX and zaprinast suggests therefore that the eosinophil contribution to total superoxide generation is small and that the principal source of reducing species is the macrophage. Furthermore since eosinophils do not produce a superoxide burst in response to FMLP (our unpublished observations, Shute *et al.*, 1990) the responses to FMLP in the present study further confirms that the macrophage is the principal source of superoxide generation in the present experiments.

Interestingly the rolipram and Ro20-1724 concentration-response curves were biphasic; the initial phase of the inhibition occurring over a concentration-range that has selectivity for the type IV enzyme (0.1–10 μM) whilst the second phase was seen at concentrations that may be expected to be non-selective (100 and 300 μM). Furthermore, whereas IBMX was apparently more effective than rolipram and Ro 20-1724 in inhibiting spontaneous superoxide generation, rolipram more potently inhibited the crude particulate PDE activity. This latter observation is not surprising as the particulate PDE activity is predominantly the type IV isoenzyme. The greater potency of IBMX in inhibiting superoxide generation together with the biphasic nature of the rolipram and Ro20-1724 concentration-response curves however, suggests that more than one cyclic AMP hydrolytic activity is involved in regulating macrophage superoxide generation. In this regard, Okonogi *et al.* (1991) have previously reported that IBMX is more potent than either rolipram or Ro 20-1724 as an inhibitor of total soluble PDE activity in murine peritoneal macrophages *in vitro*, and have reported evidence for the presence of a soluble calcium/calmodulin-dependent cyclic AMP PDE in these cells. Similarly in our present experiments we have identified a cyclic AMP PDE activity in the cytosolic fraction from guinea-pig peritoneal macrophages, albeit a minor component of the total cellular cyclic AMP PDE activity, that is enhanced in the presence of calcium plus calmodulin and inhibited by zaprinast,indicating the presence of PDE I activity. The inhibition of spontaneous superoxide generation by zaprinast therefore is most likely to be due to inhibition of this calcium/calmodulin stimulated activity.

A number of reports have indicated that resident macrophages exhibit negligible respiratory burst activity (Hartung *et al.*, 1983), whilst elicited macrophages release up to 12 times more superoxide anion in respone to stimulation than resident cells (Hartung *et al.*, 1983; Johnston *et al.*,1978). Thus in the present experiments, spontaneous superoxide anion generation is most likely to result from a combination of an enhanced capacity to generate superoxide anions as a result of their induction by horse-serum and adhesion of the cells to the plate. The functional capacity of

the cells to generate superoxide anions above the basal rate of spontaneous production being demonstrated by the macrophage response to FMLP and its almost complete suppresson by superoxide dismutase. However, whilst spontaneous superoxide generation was attenuated by IBMX, rolipram or RO20-1720 the incremental increase above the basal rate stimulated by FMLP was refractory. Thus, as with airway smooth muscle (Torphy *et al.*, 1983), the inhibition of superoxide generation by guinea-pig peritoneal macrophages is subject to functional antagonism. Nevertheless it is central to Sutherland's initial concept of cyclic AMP as a second messenger (Sutherland & Rall, 1960) that the effects of agents that elevate cyclic AMP levels should be enhanced in the presence of a PDE inhibitor. Thus the observation of a synergistic interaction between PGE_2 and IBMX, in the present experiments, as seen by their combined inhibition of FMLP stimulated superoxide generation, indicates that cyclic AMP concentrations in these cells can be raised to levels that can functionally overcome the effects of stimulation.

In conclusion the results indicate that the majority of the cyclic AMP PDE activity in guinea-pig peritoneal macrophages is a membrane associated type IV cyclic AMP PDE, the cell also possess a predominantly cytosolic calcium/calmodulin stimulated PDE which is a minor component of the total activity. Functional studies however suggest that both of these PDE activities contribute to regulation of superoxide generation in these cells. Inhibition of spontaneous superoxide generation, but not that stimulated by FMLP, suggests that the activity of PDE inhibitors is subject to functional antagonism but that this can be overcome by stimulating cyclic AMP formation in the presence of PDE inhibition.

References

BEAVO, J.A. (1988). Multiple isoenzymes of cyclic nucleotide phosphodiesterase. *Adv. Second Messenger Phosphoprot. Res.*, **22**, 1–38.

BEAVO, J.A. & REIFSNYDER, D.H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isoenzymes and the design of selective inhibitors. *Trends Pharmacol. Sci.*, **11**, 150–155.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein using the principal of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

CRONSTEIN, B.N., ROSENSTEIN, E.D., KRAMER, S.B., WEISSMANN, G. & HIRSCHORN, R. (1985). Adenosine: a physiologic modulator of superoxide anion generation by human neutrophils. Adenosine acts via an A_2 receptor on human neutrophils. *J. Immunol.*, **135**, 1366–1371.

DENT, G., GIEMBYCZ, M.A. & BARNES, P.J. (1990). Inhibition of eosinophil oxygen radical production by type IV-but not type III-selective cAMP phosphodiesterase inhibitors. *Br. J. Pharmacol.*, **99**, 163P.

DENT, G., GIEMBYCZ, M.A., RABE, K.F. & BARNES, P.J. (1991). Inhibition of eosinophil cyclic nucleotide PDE activity and opsonized zymosan stimulated respiratory burst by type IV selective PDE inhibitors. *Br. J. Pharmacol.*, **103**, 1339–1346.

ELLIOT, K.R.F. & LEONARD, E.J. (1989). Interactions of formylmethionyl-leucyl-phenylalanine, adenosine and phosphodiesterase inhibitors in human monocytes. Effets on superoxide release, inositol phosphates and cAMP. *FEBS Letts.*, **254**, 94–98.

FELS, A.O.S. & COHN, Z.A. (1986). The alveolar macrophage. *J. Appl. Physiol.*, **60**, 353–369.

FULLER, R.W., O'MALLEY, G., BAKER, A.J. & MACDERMOT, J. (1988). Human alveolar macrophage activation: inhibition by forskolin but not β-adrenoceptor stimulation or phosphodiesterase inhibition. *Pulm. Pharmacol.*, **1**, 101–106.

GODFREY, R.W., MANZI, R.M., GENNARO, D.E. & HOFFSTEIN, S.T. (1987). Phospholipid and arachidonic acid metabolism in zymosan-stimulated human monocytes: modulation by cAMP. *J. Cell. Physiol.*, **131**, 384–392.

HARTUNG, H.-P., PARNHAM, M.J., WINKLEMANN, J., ENGLEBERGER, W. & HADDING, U. (1983). Platelet activating factor (PAF) induces the oxidative burst in macrophages. *Int. J. Pharmacol.*, **5**, 115–121.

JOHNSTON, R.B., GODZIK, C.A. & COHN, Z.A. (1978). Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J. Exp. Med.*, **148**, 115–127.

LAVAN, B.E., LAKEY, T. & HOUSLAY, M.D. (1989). Resolution of soluble cyclic nucleotide phosphodiesterase isoenzymes, from liver and hepatocytes, identifies a novel IBMX-insensitive form. *Biochem. Pharmacol.*, **38**, 4123–4136.

LIGGETT, S.B. (1989). Identification and characterization of a homogenous population of β_2-Adrenergic receptors on human alveolar macrophages. *Am. Rev. Respir. Dis.*, **139**, 552–555.

MARONE, G., THOMAS, L.L. & LICHTENSTEIN, L.M. (1980). The role of agonists that activate adenylate cyclase in the control of cAMP metabolism and enzyme release by human polymorphonuclear leukocytes. *J. Immunol.*, **125**, 2277–2283.

NIELSON, C.P., VESTAL, R.E., STURM, R.J. & HEASLIP, R. (1990). Effects of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte respiratory burst. *J. Allergy Clin. Immunol.*, **86**, 801–808.

NICHOLSON, C.D., CHALLISS, R.A.J. & SHAHID, M. (1991). Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.*, **12**, 19–27.

OKONOGI, K., GETTYS, T.W., UHING, R.J., TARRY, W.C., ADAMS, D.O. & PRPIC, V. (1991). Inhibition of Prostaglandin E_2-stimulated cAMP accumulation by lipopolysaccharide in murine peritoneal macrophages. *J. Biol. Chem.*, **266**, 10305–10312.

PODCZASY, J.J. & WEI, R. (1988). Reduction of iodonitrotetrazolium violet by superoxide radicals. *Biochem. Biophys. Res. Commun.*, **150**, 1294–1301.

REEVES, M.L., LEIGH, B.K. & ENGLAND, P.J. (1987). The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. *Biochem. J.*, **241**, 535–541.

SHAHID, M., VAN AMSTERDAM, R.G.M., DE BOER, J., TEN BERGE, R.E., NICHOLSON, C.D. & ZAAGSMA, J. (1991). The presence of five cyclic nucleotide phosphodiesterase isoenzyme activities in bovine tracheal smooth muscle and the functional effects of selective inhibitors. *Br. J. Pharmacol.*, **104**, 417–477.

SHUTE, J.K., RIMMER, S.J., AKERMAN, M.K., CHURCH, M.K. & HOLGATE, S.T. (1990). Studies of cellular mechanisms for the generation of superoxide by guinea-pig eosinophils and its dissociation from granule peroxidase release. *Biochem. Pharmacol.*, **40**, 2013–2021.

SOUNESS, J.E., CARTER, C.E., DIOCEE, B.K., HASSAL, G.A., WOOD, L.J. & TURNER, N.C. (1991). Characterization of guinea-pig eosinophil phosphodiesterase activity. Assessment of its involvement in regulating superoxide generation. *Biochem. Pharmacol.*, **42**, 937–945.

SUTHERLAND, E.W. & RALL, T.W. (1960). The relation of adenosine-3′,5′-phosphate and phosphorylase to the actions of catecholamines and other hormones. *Pharmacol. Rev.*, **12**, 265–299.

THOMPSON, W.J., TERASAKI, W., EPSTEIN, P. & STRADA, S.J. (1979). Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. *Adv. Cyclic Nucleotide Res.*, **10**, 69–92.

TORPHY, T.J. & CIESLINSKI, L.B. (1990). Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isoenzymes in canine tracheal smooth muscle. *Mol. Pharmacol.*, **37**, 206–214.

TORPHY, T.J., RINARD, G.A., RIETOW, M.G. & MAYER, S.E. (1983). Functional antagonism in canine tracheal smooth muscle by methacholine of the mechanical and biochemical responses to isoproterenol. *J. Pharmacol. Exp. Ther.*, **227**, 694–699.

TORPHY, T.J. & UNDEM, B.J. (1991). Phosphodiesterase inhibitors: new opportunities for the treatment of asthma. *Thorax*, **46**, 512–523.

WEISHAAR, R.E., CAIN, M.H. & BRISTOL, J.A. (1985). A new generation of phosphodiesterase inhibitors: multiple molecular forms of phosphodiesterase and the potential for drug selectivity. *J. Med. Chem.*, **28**, 537–545.

WHITE, J.R., TORPHY, T.J., CHRISTENSEN, S.B., LEE, J.A. & MONG, S. (1990). Partial purification of the rolipram sensitive phosphodiesterase from human monocytes. *FASEB J.*, **4**, A1987.

WIRTH, J.J. & KIERSZENBAUM, F. (1982). Inhibitory action of elevated levels of adenosine-3′:5′ cyclic monophosphate on phagocytosis effects of macrophage-*Trypanosoma cruzi* interactions. *J. Immunol.*, **129**, 2759–2762.

WRIGHT, C.D., KNIPERS, P.J., KOBYLARZ-SINGER, D., DEVALL, C.J., KLINKENFUS, B.A. & WEISHAAR, R.E. (1990). Differential inhibition of human neutrophil functions. Role of cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase. *Biochem. Pharmacol.*, **40,** 699–707.

YUKAWA, T., KROEGEL, C., CHANEZ, P., DENT, G., UKENA, D., CHUNG, K.F. & BARNES, P.J. (1989). Effects of theophylline and adenosine on eosinophil function. *Am. Rev. Respir. Dis.*, **140,** 327–333.

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Evidence for an external location of the dihydropyridine agonist receptor site on smooth muscle and skeletal muscle calcium channels

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1 The location of the binding domain for agonist dihydropyridines (DHP) has been studied by comparing the action of (+)-202 791 and (−)-Bay K 8644 on Ba^{2+} currents (I_{Ba}) in whole cell patch clamp experiments. Drug effects were examined upon internal and external (extracellular) application in A7r5 smooth muscle cells and BC_3H1 cells, a cell line expressing Ca channels of the skeletal muscle type.

2 Efficiency of internal drug application in the whole cell studies was demonstrated by inhibition of potassium currents and barium currents (I_{Ba}) upon internal perfusion with tetraethylammonium (TEA^+) (10 mM) and the permanently charged phenylalkylamine, D 890 (100 μM) respectively. The uncharged DHP, (−)-STBODIPY-DHP (2 μM) was used to estimate the time course of internal perfusion by monitoring its fluorescence.

3 Intracellular application of (+)-202 791 and (−)-Bay K 8644 (5 μM) in patch clamp experiments was ineffective in stimulating Ca^{2+} channel currents in both cell lines. In contrast a 50 fold lower agonist concentration (0.1 μM (−)-Bay K 8644) applied to the external face of the membrane induced typical changes in tail currents and a current increase under conditions when up to 10 μM of the agonist was present in the intracellular perfusion solution.

4 In cell-attached patches in A7r5 cells, (−)-Bay K 8644 increased and (+)-PN 200 110 inhibited single channel activity when applied via the bath solution. This suggests partitioning and lateral diffusion of the DHPs in the lipid of the plasma membrane.

5 We conclude that the binding site for agonist DHPs on Ca^{2+} channels in A7r5 and BC_3H1 cells is located close to the external surface of the membrane. The DHP binding domain can be reached by agonists and antagonists from the extracellular but not from the intracellular face of the membrane.

Keywords: Ca^{2+} channel; smooth muscle cells; skeletal muscle cells; dihydropyridines; phenylalkylamines

Introduction

Different classes of drugs (dihydropyridines, phenylalkylamines, benzothiazepines) bind to the α_1-subunit of the voltage-dependent L-type Ca^{2+} channel and modulate their physiological properties (Janis *et al.*, 1987; Triggle & Janis, 1987; Glossmann & Striessnig, 1990).

Recently the phenylalkylamine binding domain was mapped by means of photoaffinity labelling and peptide-specific antibodies to a 42-residue segment extending from Glu 1349 to Trp 1391 of the α_1-subunit from rabbit skeletal muscle (Catterall & Striessnig, 1992). It is, therefore likely that phenylalkylamines bind to the intracellular segment IV S6 which it is suggested, participates in the formation of the intracellular mouth of the ionic pore (Catterall & Striessnig, 1992).

In contrast to the phenylalkylamines, the situation with respect to DHPs is less clear. Regulla *et al.* (1991) found most [^{3}H]-azidopine photolabelled peptides at the intracellular C-terminal channel region (residues 1390–1399, 1410–1420, 1428–1437) but Nakayama *et al.* (1991) and Striessnig *et al.* (1991) mapped the DHP site close to the proposed outer mouth of the channel in the S5/S6 loop and the transmembrane segment S6 of the channel domain III.

Studies on isolated Ca^{2+} channels from rabbit skeletal muscle incorporated into planar lipid bilayers suggested binding of DHPs to external and internal binding domains, with a higher affinity for the external binding site (Valdivia & Coronado, 1990). In accord with an external site for the antagonists, permanently charged DHP-antagonists are ineffective when applied to the intracellular face of the membrane but are effective when applied from the external face (Kass *et al.*, 1991). Such information is not available for DHP agonists but would be of interest in light of other electrophysiological studies with the agonist and antagonist enantiomers of 202 791 which suggest the existence of separate binding sites for agonist and antagonist DHPs on L-type Ca^{2+} channels (Kamp *et al.*, 1989; Hughes *et al.*, 1990).

Therefore, we investigated the effects of intra- and extracellularly applied agonist enantiomers of Bay K 8644 and 202 791 on single cells from A7r5 and BC_3H1 cell lines to determine the localization of the DHP agonist binding site.

Methods

Cell culture

The experiments were performed on permanent cell lines A7r5 and BC_3H1 which were obtained from the American Type Culture Collection (ATCC, Rockville, U.S.A.). Both cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (FCS) (GIBCO, Paisley, U.K.), 2 mM L-glutamine, 63 μg ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Sigma, Deisenhofen, Germany). Before use in patch clamp experiments, A7r5 cells were maintained for 3 to 5 days in DMEM with 3% FCS. The smooth muscle-like brain tumour mouse cells (BC_3H1) are known to express dominantly slowly activating (skeletal muscle type) calcium channels when the serum content in the

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culture medium is reduced to 0.5% (Caffrey *et al.*, 1987; Biel *et al.*, 1991). In the present study, differentiation in BC_3H1 cells was induced at 30% cell confluency by stepwise reduction of FCS to 5% (for 2 days) and 0.5% (for 12 days).

Electrophysiology

Inward barium currents (I_{Ba}) were studied in single cells from the BC_3H1 and A7r5 cell lines by the patch-clamp technique in the whole-cell and cell-attached configuration (Hamill *et al.*, 1981). All experiments were carried out at 22–25°C and by means of a List EPC-7 patch clamp amplifier (List LM, Darmstadt, Germany). Patch pipettes were made from borosilicate glass (Clark Electromedical Instruments, Reading, U.K.) and had resistances of 2 to 4 MΩ when filled with normal intracellular solution. Data were filtered at 2 kHz (4 pole Bessel Filter) and sampled on line to the hard disk of an Olivetti M 380/T computer by means of a Labmaster interface TL-125 using pClamp 5.51 software (Axon Instruments, Foster City, U.S.A.). The leak currents were subtracted either digitally by use of average values of scaled leakage currents elicited by a 10 mV hyperpolarizing pulse or electronically by means of an analogue circuit. Off line data analysis was performed using pClamp 5.51 analysis programmes (Axon Instruments) and the Sigma Plot 4.0 software (Jandel Scientific, Corte Madera, U.S.A.). Results are indicated as mean values ± s.d.

Fluorescence measurements

The fluorescence measurements were done on a DIAPHOT-TMD microscope with G2-A filter (excitation wavelength 510–560 nm, barrier filter 580 nm) (Nikon, Tokyo, Japan). The microscope was equipped with an image acquisition system (Fairfield Imaging, Sussex, U.K.). Images were stored on computer hard disk and analysed with Image-Pro software (Media Cybernetics, Silver Spring, U.S.A.). The indicated fluorescence intensities express the mean intensity with respect to the whole surface of the analysed single cell.

Intracellular drug application

For intracellular application the tested drugs were diluted in the respective internal (pipette) solution and included either directly into the patch pipette (internal dialysis) or applied via an internal perfusion system (internal perfusion). Our patch pipette perfusion system was similar to that described previously by Soejima & Noma (1984), Kameyama *et al.* (1985) and Tang *et al.* (1990). A thin heat-pulled glass capillary (tip diameter 5–20 μm) was inserted into the patch pipette as close as 50–100 μm to the tip. A polyethylene tube was connected to the end of the glass capillary and filled with test solution. Internal drug application was initiated by application of positive hydrostatic pressure to the perfusion capillary in the patch pipette. To prevent early diffusion of drug out of the capillary the tip of the perfusion capillary was filled with normal pipette solution. Re-perfusion with control solution was achieved by application of suction to the perfusion capillary.

Solutions and drugs

The normal pipette solution for most whole-cell patch clamp measurements contained (mM): NaCl 126, $MgSO_4$ 3.2, NaH_2PO_4 1, EGTA 2, glucose 11.5, ATP 2, TEA-Cl 10, and HEPES 5, buffered to pH 7.2 with NaOH.

Barium currents were measured in high barium external solution containing (mM): $BaCl_2$ 20, NaCl 135 and HEPES 5, buffered to pH 7.4 with TEA-OH. There was no evidence for an overlap of slow inward barium currents by fast sodium inward or outward currents in the studied voltage range. The kinetics of the barium currents in BC_3H1 cells did not change on replacement of internal or external sodium by caesium.

Outward K^+ currents were recorded with a high potassium internal solution containing (mM): KCl 126, NaCl 6, $MgCl_2$ 1, EGTA 2, glucose 3 and HEPES 10, buffered to pH 7.2 with NaOH and a physiological salt solution as external solution (PSS) containing (mM): NaCl 130, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 5, HEPES 5, buffered to pH 7.4 with NaOH.

For cell-attached single channel recordings a high barium pipette solution containing (mM): $BaCl_2$ 110 and HEPES 5, buffered to pH 7.4 with TEA-OH and cells were bathed in an external solution containing (mM): K-aspartate 140, $MgCl_2$ 1, EGTA 10, HEPES 10, buffered to pH 7.4 with methansulphonic acid.

Chemicals were obtained from Sigma (Sigma Chemical, Deisenhoften, Germany). (−)-Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was from Bayer (Bayer, Leverkusen, Germany) and (+)-Sandoz 202 791 (isopropyl 4,2,1,3-benz-

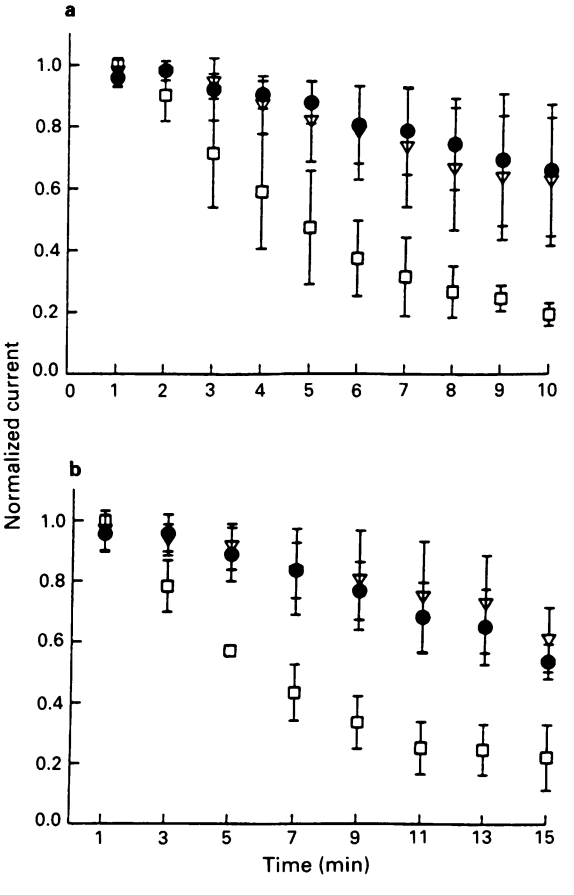


Figure 1 (a) Comparison of intracellular effects of D 890 (□, $n = 6$) and dihydropyridine (DHP)-Ca^{2+} channel agonists ((−)-Bay K 8644 and (+)-202 791) (△, $n = 4$) with I_{Ba} control 'run down' (●, $n = 6$) in A7r5 cells. D 890 (100 μM) and DHP-agonists (5 μM of (+)-202 791 or (−)-Bay K 8644) were included in the pipette solution. Data points are given as means ± s.d. (vertical bars). Inward I_{Ba} were elicited with 0.1 Hz by depolarizing voltage pulses of 50 ms from −70 to 0 mV. Peak inward current amplitudes were measured and normalized to the first peak current amplitude in each experiment (every 6th value is shown). (b) Comparison of intracellular effects of D890 (□, $n = 3$) and DHP-Ca^{2+} channel agonists ((−)-Bay K 8644 and (+)-202 791) (△, $n = 4$) with I_{Ba} control 'run down' (●, $n = 5$) in BC_3H1 cells. D 890 (100 μM) and DHP-agonists (5 μM of (+)-202 791 or (−)-Bay K 8644) were included in the pipette solution. Data points are given as means ± s.d. (vertical bars). I_{Ba} were elicited with 0.1 Hz by 150 ms voltage pulses from −70 to 0 mV. Peak inward current amplitudes were measured and normalized to the first peak current amplitude for each experiment (every 12th value is shown).

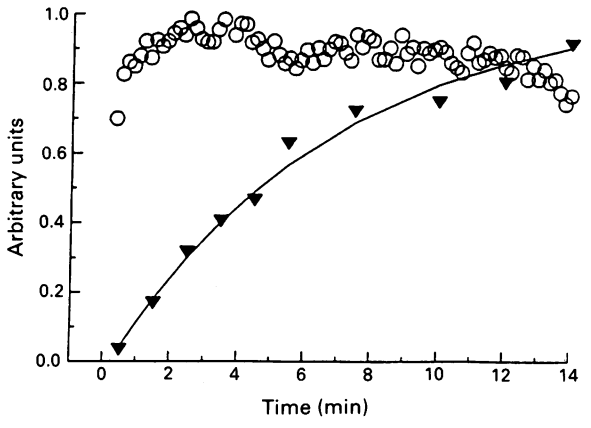


Figure 2 Simultaneous recordings of fluorescence and inward Ba^{2+} current of an A7r5 cell during intracellular dialysis with 2 μM (−)-STBODIPY-DHP included in the pipette solution. The bath contained standard Ba^{2+} solution. I_{Ba} (○) were elicited with 0.1 Hz by 50 ms voltage pulses from −70 to 0 mV. Peak inward current amplitudes were normalized to the maximal amplitude (−210 pA). Fluorescence intensities (▲) were measured at the denoted times and after 20 min (steady state). The intensities were normalized to the steady state fluorescence intensity and the time course was fitted to a single exponential function with $\tau = 6.75$ min (solid line).

oxadiazol-4-yl) 1,4-dihydrocarboxylate) referred to as (+)-202 791 throughout the text was from Sandoz (Sandoz, Basle, Switzerland). Stock solutions (10 mM) of both Ca^{2+} channel agonists were made up in 100% dimethylsulphoxide (DMSO). D 890 (N,N-dimethyl-N-[4-cyano-4-(3,4,5-trimethoxyphenyl)-5-methylhexyl]-N-(β-3,4-dimethoxyphenylethyl)-ammoniumchloride-monohydrate (from Knoll, Ludwigshafen, Germany) was dissolved (10 mM) in water.

(−)-STBODIPY-DHP (1,4-dihydro-2,6-dimethyl-4-(2-trifluromethylphenyl)-3,5-pyridinecarboxylic acid 2-(aminoethyl) ethyl ester coupled to the fluorescent moieties (−)-STBODIPY) was synthesized as described (Knaus *et al.*, 1992) and stored as a stock solution (1 mM) in 100% DMSO.

All drugs were diluted to the indicated concentrations in the respective solution immediately before the experiments.

Results

The mechanism of action of DHP-agonists has been extensively studied in single cells under various conditions in many tissues by means of the patch clamp technique (Hess *et al.*, 1984; Cognard *et al.*, 1986; Droogmans & Callewaert, 1988; Hering *et al.*, 1989; Lacerda & Brown, 1989), but little is known about their action from different sides of the plasma membrane.

Control experiments for diffusional exchange between patch pipettes and A7r5 or BC_3H1 cells

In a first series of experiments A7r5 and BC_3H1 cells were internally dialysed with the quaternary phenylalkylamine D 890 which blocks calcium channel currents when applied to the cytosolic but not to the external side of the membrane (Hescheler *et al.*, 1982; Leblanc & Hume, 1989). D 890 (100 μM) was included in the pipette solution and the peak I_{Ba} values were monitored over a period of 10 (A7r5 cells) or 15 min (BC_3H1 cells). As shown in Figure 1 (a,b) I_{Ba} exhibited a time-dependent 'run down' in all control experiments. In A7r5 control cells during 10 min I_{Ba} decreased to 66 ± 21% ($n = 6$) of the initial value (Figure 1a). However, a significantly faster decay in peak current amplitude was observed with 100 μM D 890 in the pipette. Within 10 min of internal dialysis I_{Ba} decreased to 19 ± 3.7% ($n = 6$). Similar results were obtained with BC_3H1 cells: within 15 min I_{Ba} decreased to 54 ± 6% ($n = 4$) in control cells and to 22 ± 10% ($n = 3$) during internal dialysis with D 890 (100 μM) (Figure 1b).

Alternatively, internal drug application was achieved by pipette perfusion via a perfusion capillary. The time for internal drug application with this system was tested by inhibition of potassium outward currents in BC_3H1 cells by internal perfusion with Cs^+ (135 mM) or block of I_K by TEA^+ (10 mM). A rapid inhibition of I_K (with time constants for I_K-inhibition ranging from 1 to 3 min) was achieved in both cases. Internal reperfusion of the cells with potassium resulted in partial recovery from block.

Due to 'run down' of I_{Ba} the time for observation of drug effects on internal application is limited. D 890, TEA^+ and Cs^+ are hydrophilic, charged molecules and ions, whereas the DHPs employed here are uncharged lipophilic compounds. According to Pusch & Neher (1988) diffusion of drugs applied via patch pipettes may be retarded due to drug binding and geometrical factors. We were concerned that uncharged DHPs could exhibit a retarded diffusion or alternatively diffuse rapidly via the membrane into the bath solution without achieving an effective steady concentration in the cell membrane. Therefore we have employed (−)-STBODIPY-DHP, a fluorescent high affinity L-type Ca^{2+} channel blocker (Knaus *et al.*, 1992) to measure the diffusion time constants for uncharged DHPs (Figures 2 and 3). (−)-STBODIPY-DHP (2 μM) was included in the pipette solution and fluorescence and I_{Ba} were measured simultaneously. The time constant for reaching a steady state fluorescence was 6.75 min and within 14 min fluorescence reached about 90% of the steady state value (Figure 2). There was only moderate I_{Ba} 'run down' in this experiment (compare with Figures 1a,b).

In contrast, externally applied (−)-STBODIPY-DHP (0.5 μM) caused an inhibition of I_{Ba} with an immediate onset. The I_{Ba} and corresponding fluorescence intensities (as pseudo colour coded images) after 6 min of external and internal application are shown in Figure 3. After 6 min the relative fluorescence intensity both after intra- and extracellular (−)-STBODIPY-DHP application reached the same level whereas inward Ba^{2+} current was 95% of control current with intracellularly and only 16% of control with extracellularly applied (−)-STBODIPY-DHP.

Internal and external application of agonist DHPs in A7r5 and BC_3H1 cells

In the next series of experiments we studied intracellular and extracellular effects of DHP-agonists on I_{Ba} in A7r5 and BC_3H1 cells during internal dialysis with (−)-Bay K 8644 or (+)-202 791 respectively. (−)-Bay K 8644 (5 μM) or (+)-202 791 (5 μM) were included in the normal pipette solution. The cells were dialyzed with internal solution after establishing the whole cell configuration. The I_{Ba} was recorded every 10th second by applying a voltage step to 0 mV from a holding potential of −70 mV (Figures 4 and 5). As shown by others (Marks & Jones, 1992) most A7r5 cells express exclusively the high threshold DHP-sensitive Ca^{2+} channel. No evidence for low threshold I_{Ba} was found in the voltage range studied in A7r5 and BC_3H1 cells (see Figure 6 and also Figures 3 and 10).

Figures 4 and 5 show the time-course of whole cell peak I_{Ba} in A7r5 and BC_3H1 cells when (−)-Bay K 8644 or (+)-202 791 (5 μM) were present in the patch pipette. Zero time (Figures 4 and 5) corresponds to the first current recording after establishing the whole cell configuration (usually after 10 to 20 s). The 'run down' with either 5 μM (+)-202 791 or 5 μM (−)-Bay K 8644 included in the patch pipette did not

significantly differ from the 'run down' of current in the absence of drug (compare with Figures 1a,b). No increase in I_{Ba} amplitude or changes in tail current kinetics could be detected in BC_3H1 or in A7r5 cells upon internal dialysis with (−)-Bay K 8644 or (+)-202 791. We applied a 5 to 50 fold lower agonist concentration to the external side of the membrane after internal perfusion. I_{Ba} was stimulated in an A7r5 cell by extracellular application of 0.1 μM (−)-Bay K 8644 after 23 min of internal dialysis with 5 μM (−)-Bay K 8644 (Figure 4).

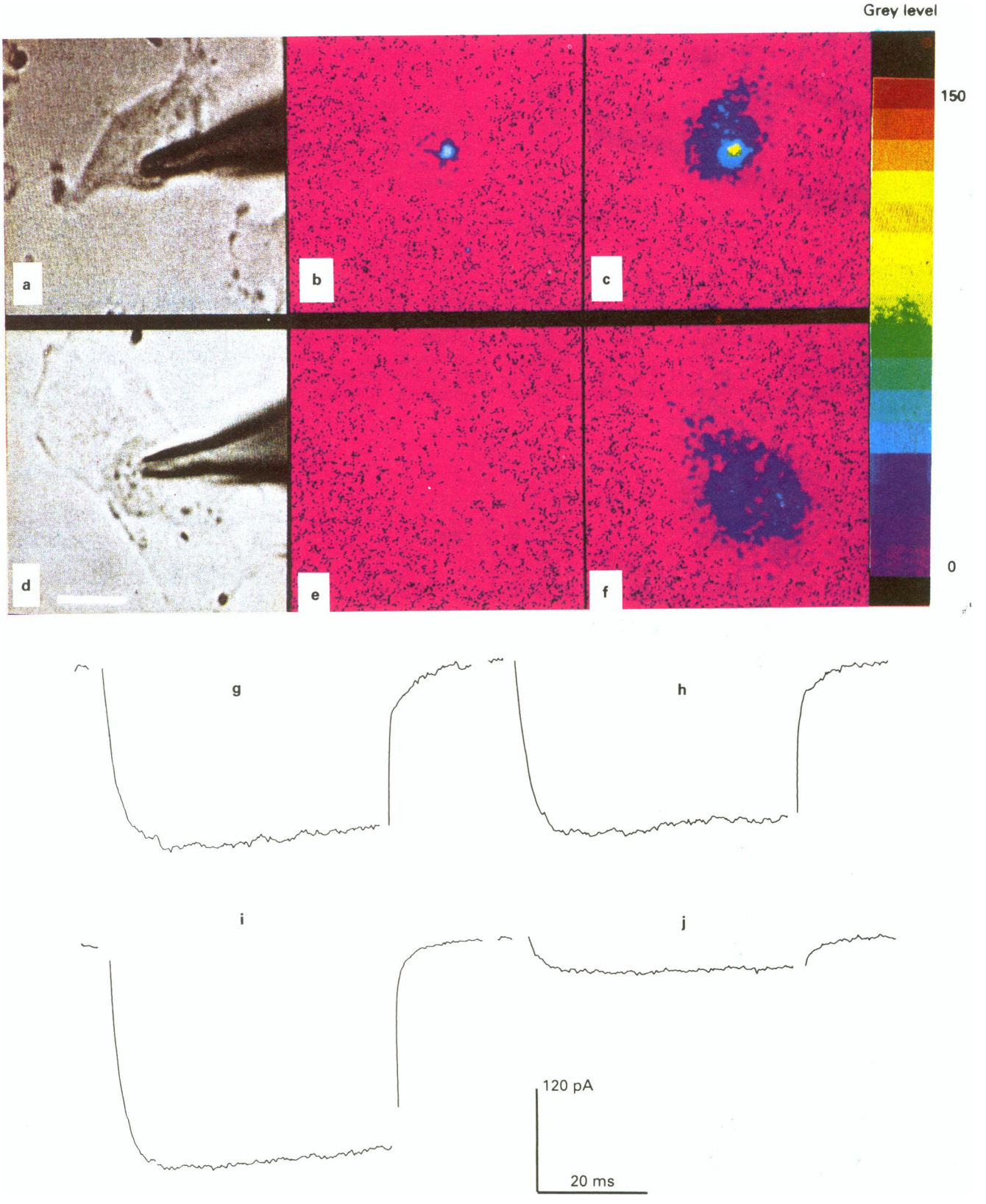


Figure 3 Fluorescence (b,c,e,f) and I_{Ba} (g–j) of A7r5 cells (a,d) during intracellular (2 μM, upper records) and extracellular (0.5 μM, lower records) application of (−)-STBODIPY-DHP. The pulse protocol for I_{Ba} measurements was similar to that in Figure 1. The records were taken immediately (b,e,g,i) and 6 min after drug application (c,f,h,j). Fluorescence intensities are coded as pseudo colours. Bar in (d) = 20 μm.

Therefore, in contrast to the internal application, external application of the agonists induced a 2 to 4 fold increase in I_{Ba} when 1 μM agonist was applied ($n = 5$) and typical changes in tail currents in all cells studied (Figure 6).

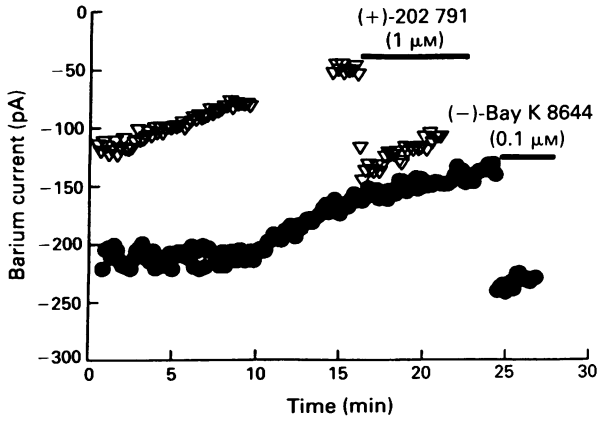


Figure 4 Peak I_{Ba} in A7r5 cells as function of time during internal dialysis with (+)-202 791 (5 μM) (Δ, between min 10 and 14 the cell was not depolarized) and (−)-Bay K 8644 (5 μM) (●), pulse protocol as in Figure 1. I_{Ba} was stimulated by external agonist application (1 μM or 0.1 μM, horizontal bars).

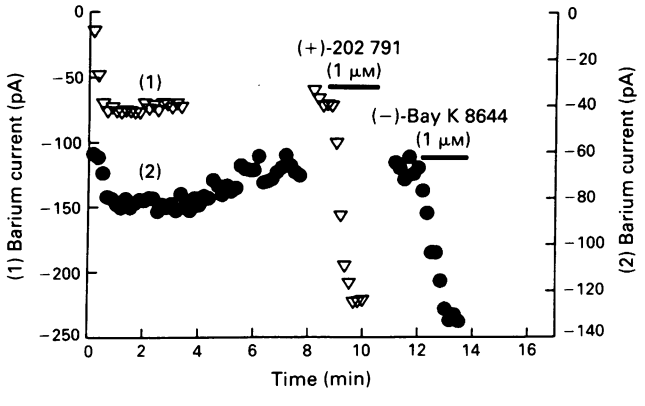


Figure 5 Peak I_{Ba} in BC_3H1 cells as function of time during internal dialysis with (+)-202 791 (5 μM) (Δ) and (−)-Bay K 8644 (5 μM) (●), pulse protocol as in Figure 2. The horizontal bars indicate the period of external agonist (1 μM) application.

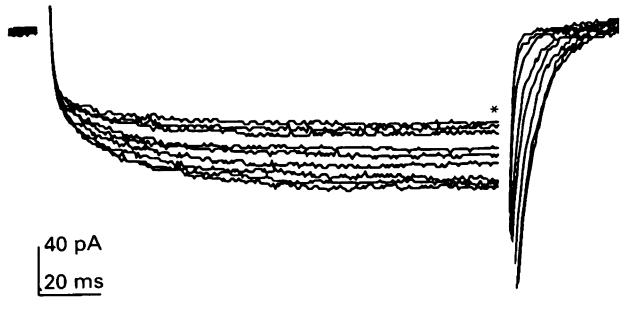


Figure 6 Effects on peak I_{Ba} and tail current amplitude in BC_3H1 cells during extracellular application of (−)-Bay K 8644 (1 μM). (−)-Bay K 8644 (5 μM) had been infused intracellularly for 12 min before extracellular application (same cell as in Figure 5). Voltage protocol as described in Figure 2. Low amplitude trace with fast tail current just before external agonist application (*) and 7 consecutive traces during external agonist application are shown.

An increase in I_{Ba} during the first 20–40 s of the experiments was observed under control conditions as well as with agonist-DHP included in the pipette solution (Figures 4 and 5). This 'run up' of current correlated with the washout of K^+ from the cytoplasm (see Figure 7) and was less prominent in A7r5 cells (Figure 4), presumably because of smaller cell size. To prevent an underestimation of fast agonist effects during the K^+-wash period, drugs were applied after a defined control period under steady state conditions by means of a pipette perfusion system (see Methods). Internal perfusion of A7r5 cells with 10 μM (+)-202 791 (Figure 8) or BC_3H1 cells with 5 μM (−)-Bay K 8644 (Figure 9) was started after 4 or 3 min perfusion with normal internal solution respectively. As observed previously internal application of agonists did not affect peak current values or current kinetics of the recorded Ba^{2+} currents. Again, externally applied agonist stimulated the I_{Ba} (Figures 8 and 9).

Action of (−)-Bay K 8644 on single L-type Ca^{2+} channels

To answer the question whether the DHP-agonist binding site is accessible only from the aqueous phase or also after partitioning and lateral diffusion of the DHP-agonist in the plasma membrane, we performed single channel recordings on L-type Ca^{2+} channels in A7r5 cells. The cells were bathed in a 140 mM potassium aspartate solution to zero the resting membrane potential. The patch pipette was filled with high

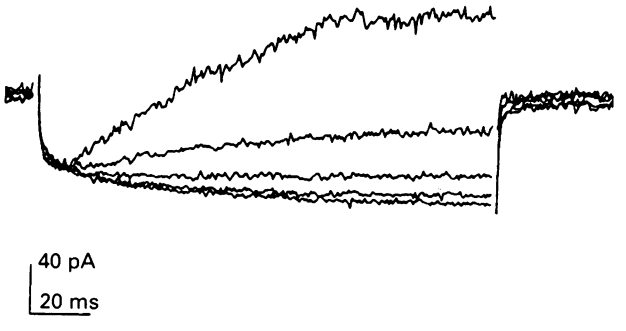


Figure 7 Block of outward K^+ current in BC_3H1 cells starting 10–20 s after establishing the whole cell configuration. The holding potential was −70 mV and voltage pulses (150 ms) to 0 mV were applied to 0.1 Hz. The pipette contained normal internal solution and the bath high barium solution. The experiment illustrates the speed of K^+-wash out or replacement by Na^+ respectively in BC_3H1 cells.

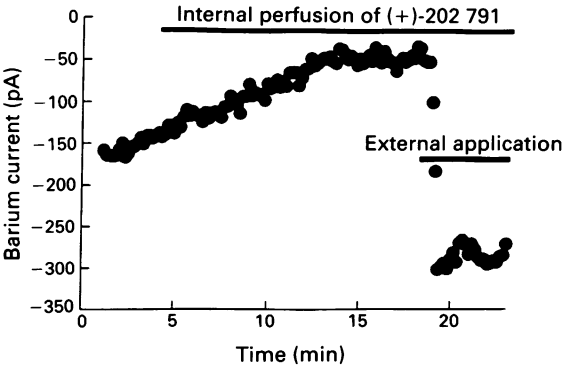


Figure 8 Time course of peak I_{Ba} of an A7r5 cell during intracellular perfusion of (+)-202 791 (10 μM) and following external application of 1 μM (+)-202 791. The horizontal bars indicate the start of pipette perfusion with agonist or extracellular application drug. Same pulse protocol as in Figure 1.

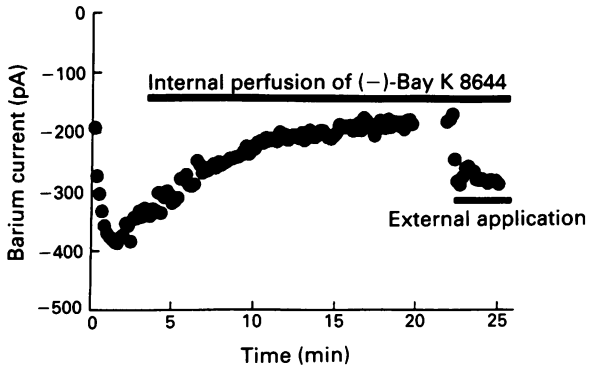


Figure 9 Time course of peak I_{Ba} of a BC_3H1 cell during intracellular perfusion of (−)-Bay K 8644 (5 μM) and following external application of 0.1 μM (−)-Bay K 8644. The horizontal bars indicate the start of pipette and extracellular perfusion with the agonist. The pulse protocol for BC_3H1 cells (as in Figure 2) was used.

barium solution (110 mM) and single channel currents were recorded in the cell-attached mode. Inward Ba^{2+} currents through L-type Ca^{2+} channels were evoked every 4th second by voltage steps to 0 mV from a holding potential of − 70 mV. Under control conditions only rare openings of L-type Ca^{2+} channels were observed. Figure 10a shows 8 representative control current sweeps and the ensemble average current of 100 consecutive sweeps (Figure 10).

After addition of 5 μM (−)-Bay K 8644 to the bath the Ca^{2+} channel open-probability and the related ensemble average current strongly increased (see Figure 10 lower traces) and typical agonist-induced channel gating with long openings (Hess *et al.*, 1984) was observed (Figure 10b). The onset of drug action occurred within 1 to 2 min. The antagonist (+)-PN 200 110 (5 μM) added to the bath inhibited the calcium channel activity in the presence of (−)-Bay K 8644 (5 μM) (Figure 10c).

Our experiments suggest that the DHP-agonists (−)-Bay K 8644 and (+)-202 791 cannot stimulate I_{Ba} when applied to the intracellular side of the membrane but nevertheless could reach their receptor sites via the membrane within less than 2 min when applied to the external solution during a tight-seal cell-attached recording.

Discussion

Our results suggest the DHP-agonist binding site is located close to the extracellular side of the plasma membrane in smooth and skeletal muscle DHP-sensitive Ca^{2+} channels. As the intracellular application of substances in the whole cell patch clamp configuration is limited by the speed of diffusion of substances from the patch pipette into the cell, control experiments on the efficiency of internal drug application were essential for studying internal effects of DHPs in A7r5 and BC_3H1 cells. The speed of diffusional exchange depends on cell size and shape, pipette resistance (access resistance), and molecular mass of the infused substances (Pusch & Neher, 1988). The difficulties in finding exact quantitative solutions for the intracellular perfusion process have been demonstrated by Oliva *et al.* (1988) and Mogul *et al.* (1989) who modelled the diffusion of substances from patch pipettes into cells with respect to geometric factors of the pipette and cell. However, I_{Ba} inhibition with the charged non-membrane permeable phenylalkylamine, D 890 and I_K inhibition by TEA^+ and/or Cs^+ in control experiments demonstrate that the time for internal perfusion of A7r5 and BC_3H1 cells with either (+)-202 791 or (−)-Bay K 8644 was sufficient to yield a concentration of agonist DHP within the cells which effectively stimulates I_{Ba} when applied extracellularly. Furthermore, the speed of internal perfusion with a DHP could be observed by simultaneous measurements of (−)-STBODIPY-DHP effects on I_{Ba} and fluorescence intensity in A7r5 cells upon internal application of drug (Figures 2 and 3). An even faster diffusion can be expected for the studied DHP-agonists as those drugs have a lower molecular weight (≈350 D) than the studied (−)-STBODIPY-DHP (≈750 D). Calcium channel blocking properties of (−)-STBODIPY-DHP have been shown previously in calcium uptake measurements by Knaus *et al.* (1992). In the present paper we report the first patch clamp studies on the effects of (−)-STBODIPY-DHP on I_{Ba} . Our experiments confirm that (−)-STBODIPY-DHP is an efficient blocker of calcium channels when applied to the external side of the membrane (Figure 3i,j). However, there

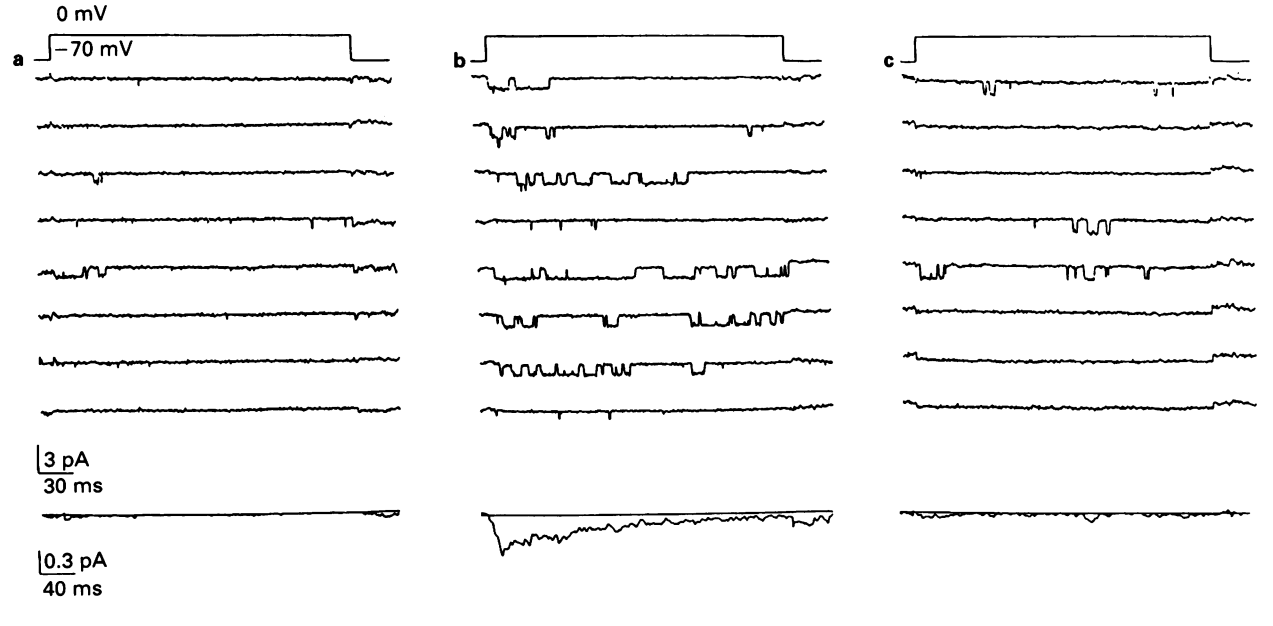


Figure 10 Single channel recordings of L-type Ca^{2+} channels in an A7r5 cell. The pipette was filled with high Ba^{2+} solution and cells were bathed in a 140 mM K^+-aspartate solution. Control currents are shown (a) as 8 consecutive sweeps (upper traces) and the respective ensemble average current (lower trace). Channel open probability increased after addition of 5 μM (−)-Bay K 8644 to the outer bath (b) and decreased in the presence of (−)-Bay K 8644 by 5 μM (+)-PN 200 110 (c).

was no significant inhibition of I_{Ba} by 2 μM during 14 min of internal perfusion (Figures 2 and 3). This supports findings by Kass *et al.* (1991) showing the absence of DHP calcium antagonist effects when applied intracellularly.

Accessibility of the DHP-agonist binding site in A7r5 and BC_3H1 cells

The observed side-dependent effects of (−)-Bay K 8644 and (+)-202 791 in whole cell experiments are surprising (see Figures 4, 5, 8 and 9). Although these compounds are highly lipophilic our results demonstrate that they have access to their receptor domain only from the outer leaf of the plasma membrane (Figure 10). Agonist effects of (+)-202 791 in A7r5 cells have been demonstrated on application of drug concentrations as low as 10 nM in patch clamp studies (Marks & Jones, 1992). This concentration is three orders of magnitude lower than the highest agonist concentration (10 μM (+)-202 791, see Figure 8) used for internal drug application in our experiments which makes insufficient diffusion of the drug to an intracellular receptor domain very unlikely. However, the diffusion of intracellularly applied agonist DHPs to a receptor domain located at the external surface of the plasma membrane may be restricted or delayed (not observable during the time available for I_{Ba} whole cell recordings) due to specific partitioning and enrichment of DHPs within the plasma membrane (Mason *et al.*, 1989).

Valdivia & Coronado (1990) found opposite effects of (−)-Bay K 8644 when added to intra- (*cis*) and extracellular (*trans*) side in studies of Ca^{2+} channels from skeletal muscle T-tubules incorporated into planar lipid bilayers. At low (nanomolar) concentrations (−)-Bay K 8644 decreased channel life time when applied to the *cis* side of the bilayer; however, the drug acted as agonist when applied to the *trans* side. Higher (−)-Bay K 8644 concentrations applied to the *cis* side induced agonist effects (Valdivia & Coronado, 1990).

Although the experimental conditions in our studies differ significantly from bilayer studies, these discrepancies cannot be attributed to species differences as BC_3H1 cells are known to express Ca^{2+} channels with a high degree of sequence similarity to the rabbit skeletal muscle α_1-subunit (Perez-Reyes *et al.*, 1990). In contrast to Valdivia & Coronado (1990) we have no evidence for functionally different agonist DHP binding sites located on the cytosolic and extracellular side of the α_1-subunit as postulated from their bilayer experiments. Our results indicate, that DHP-agonists like DHP-antagonists bind to a site located on the extracellular channel surface which can be reached via the membrane bilayer.

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References

BIEL, M., HULLIN, R., FREUDNER, S., SINGER, D., DASCAL, N., FLOCKERZI, V. & HOFMANN, F. (1991). Tissue-specific expression of high-voltage-activated dihydropyridine-sensitive L-type calcium channels. *Eur. J. Biochem.*, **200**, 81–88.

CAFFREY, J.M., BROWN, A.M. & SCHNEIDER, M.H. (1987). Mitogens and oncogenes can block the induction of specific voltage-gated ion channels. *Science*, **236**, 570–573.

CATTERALL, W.A. & STRIESSNIG, J. (1992). Receptor sites for Ca^{2+} channel antagonists. *Trends Pharmacol. Sci.*, **13**, 256–262.

COGNARD, C., ROMEY, G., GALIZZI, J.-P., FOSSET, M. & LAZDUNSKI, M. (1986). Dihydropyridine-sensitive Ca^{2+} channels in mammalian skeletal muscle cells in culture: electrophysiological properties and interactions with Ca^{2+} channel activator (Bay K8644) and inhibitor (PN 200-110). *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 1518–1522.

DROOGMANS, G. & CALLEWAERT, G. (1988). Ca^{2+}-channel current and its modification by the dihydropyridine agonist Bay K 8644 in isolated smooth muscle cells. *Pflügers Arch.*, **406**, 259–265.

GLOSSMANN, H. & STRIESSNIG, J. (1990). Molecular properties of calcium channels. *Rev. Physiol. Biochem. Pharmacol.*, **114**, 1–105.

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.

HERING, S., KLEPPISCH, T., TIMIN, E.N. & BODEWEI, R. (1989). Characterisation of the calcium channel state transitions induced by the enantiomers of the 1,4-dihydropyridine Sandoz 202 791 in neonatal rat heart cells: a nonmodulated receptor model. *Pflügers Arch.*, **414**, 690–700.

HESCHELER, J., PELZER, D., TRUBE, G. & TRAUTWEIN, W. (1982). Does the organic calcium channel blocker D 600 act from inside or outside on the cardiac cell membrane? *Pflügers Arch.*, **393**, 287–291.

HESS, P., LANSMAN, J.B. & TSIEN, R.W. (1984). Different modes of Ca^{2+} channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature*, **311**, 538–544.

HUGHES, A.D., HERING, S. & BOLTON, T.B. (1990). Evidence that agonist and antagonist enantiomers of the dihydropyridine PN 202-791 act at different sites on the voltage-dependent calcium channel of vascular muscle. *Br. J. Pharmacol.*, **101**, 3–5.

JANIS, R.A., SILVER, P.J. & TRIGGLE, D.J. (1987). Drug action and cellular calcium regulation. *Adv. Drug. Res.*, **16**, 309–591.

KAMEYAMA, M., HOFMANN, F. & TRAUTWEIN, W. (1985). On the mechanism of β-adrenergic regulation of the Ca channel in the guinea-pig heart. *Pflügers Arch.*, **405**, 285–293.

KAMP, T.J., SANGUINETTI, M.C. & MILLER, R.J. (1989). Voltage- and use-dependent modulation of cardiac calcium channels by the dihydropyridine (+)-202-791. *Circ. Res.*, **64**, 338–351.

KASS, R.S., ARENA, J.P. & CHIN, S. (1991). Block of L-type calcium channels by charged dihydropyridines. *J. Gen. Physiol.*, **98**, 63–75.

KNAUS, H.-G., MOSHAMMER, T., FRIEDRICH, K., KANG, H.C., HAUGLAND, R.P. & GLOSSMANN, H. (1992). In vivo labeling of L-type Ca^{2+} channels by fluorescent dihydropyridines: evidence for a functional, extracellular heparin-binding site. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3586–3590.

LACERDA, A.E. & BROWN, A.M. (1989). Nonmodal gating of cardiac calcium channels as revealed by dihydropyridines. *J. Gen. Physiol.*, **93**, 1243–1273.

LEBLANC, N. & HUME, J.R. (1989). D 600 block of L-type Ca^{2+} channel in vascular smooth muscle cells: comparison with permanently charged derivate, D 890. *Am. J. Physiol.*, **257**, 689–695.

MARKS, T.N. & JONES, S.W. (1992). Calcium currents in the A7r5 smooth muscle-derived cell line. *J. Gen. Physiol.*, **99**, 367–390.

MASON, R.P., GONYE, G.E., CHESTER, D.W. & HERBETTE, L.G. (1989). Partitioning and location of Bay K 8644, a 1,4-dihydropyridine calcium channel agonist, in model and biological membranes. *Biophys. J.*, **55**, 769–778.

MOGUL, D.J., SINGER, D.H. & TEN EICK, R.E. (1989). Ionic diffusion in voltage clamped isolated cardiac myocytes. *Biophys. J.*, **56**, 565–577.

NAKAYAMA, H., TAKI, M., STRIESSNIG, J., GLOSSMANN, H., CATTERALL, W.A. & KANAOKA, Y. (1991). Identification of 1,4-dihydropyridine binding regions within the α_1 subunit of skeletal muscle Ca^{2+} by photoaffinity labeling with diazipine. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 9203–9207.

OLIVA, C., COHEN, I.S. & MATTHIAS, R.T. (1988). Calculation of time constants for intracellular diffusion in whole cell patch clamp configuration. *Biophys. J.*, **54**, 791–799.

PEREZ-REYES, E., WEI, X., CASTELLANO, A. & BIRNBAUMER, L. (1990). Molecular diversity of L-type calcium channels. Evidence for alternative splicing of the transcripts of three non-allelic genes. *J. Biol. Chem.*, **265**, 20430–20436.

PUSCH, M. & NEHER, E. (1988). Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflügers Arch.*, **411**, 204–211.

REGULLA, S., SCHNEIDER, T., NASTAINCZYK, W., MEYER, H.E. & HOFMANN, F. (1991). Identification of the site of interaction of the dihydropyridine channel blockers nitrendipine and a azidopine with the calcium-channel α_1 subunit. *EMBO J.*, **10**, 45–49.

SOEJIMA, M. & NOMA, A. (1984). Mode of regulation of the Ach-sensitive K-channel by muscarinic receptor in rabbit atrial cells. *Pflügers Arch.*, **400**, 424–431.

STRIESSNIG, J., MURPHY, B.J. & CATTERALL, W.A. (1991). Dihydropyridine receptor of L-type Ca^{2+} channels: identification of binding domains for [^{3}H](+)-PN 200-110 and [^{3}H]-azidopine within the α_1 subunit. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10769–10773.

TANG, J.M., WANG, J., QUANDT, F.N. & EISENBERG, R.S. (1990). Perfusing pipettes. *Pflügers Arch.*, **416**, 347–350.

TRIGGLE, D.J. & JANIS, R.A. (1987). Calcium channel ligands. *Annu. Rev. Pharmacol. Toxicol.*, **27**, 347–369.

VALDIVIA, H.H. & CORONADO, R. (1990). Internal and external effects of dihydropyridines in the calcium channel of skeletal muscle. *J. Gen. Physiol.*, **95**, 1–27.

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Effects of the immunosuppressants cyclosporin A and FK 506 on exocytosis in the rat exocrine pancreas *in vitro*

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1 We have examined the effects of the immunosuppressive drugs cyclosporin A (CsA) and FK 506 on exocytosis in two *in vitro* preparations of the exocrine pancreas–lobules and dispersed acini.

2 In lobules taken from starved rats and stimulated with the secretagogue caerulein, both CsA and FK 506, given shortly before stimulation, caused a dose-dependent inhibition of amylase secretion. In lobules from rats that had been pretreated *in vivo* with the protease inhibitor FOY-305 to stimulate secretion maximally, both CsA and FK 506 inhibited secretion of newly synthesized proteins, whereas only FK 506 inhibited caerulein-stimulated amylase release.

3 These different effects of the immunosuppressants on amylase release were reflected in their effects on degranulation, as revealed by electron microscopy. Control acinar cells in lobules from FOY-305-treated rats were almost completely degranulated, whereas treatment with FK 506, but not CsA, caused the accumulation of zymogen granules close to the apical plasma membrane.

4 In dispersed acini, stimulated with the cholinomimetic secretagogue bethanechol, both CsA and FK 506 reduced the secretory response, to about 45% of control; IC_{50} values were 50 nM and 3 nM, respectively. A similar partial inhibition of exocytosis was seen in acini permeabilized with the bacterial toxin streptolysin O and stimulated with 10 μM Ca^{2+}.

5 These results demonstrate that the immunosuppressants cause an inhibition of exocytosis in the exocrine pancreas that is both rapid in onset and potent. The loss of the inhibitory effect of CsA on amylase release in lobules taken from FOY-305-treated rats may reveal a change in the characteristics of exocytosis as a consequence of the high level of stimulation, and also indicates that CsA and FK 506 have subtly different effects on secretion. We suggest that these drugs might be useful tools in the dissection of the molecular mechanisms of exocytosis.

Keywords: Cyclosporin A; FK 506; exocytosis; amylase release; exocrine pancreas

Introduction

Cyclosporin A (CsA) and FK 506 are powerful immunosuppressive agents that are used clinically to prevent rejection of transplanted tissues and organs (Schreiber, 1991). Both CsA and FK 506 produce significant side-effects, including nephro- and hepatotoxicity and impairment of the function of both the endocrine and exocrine pancreas (Müller *et al.*, 1988; Carroll *et al.*, 1991; Doi *et al.*, 1992; Hirano *et al.*, 1992). Over the past few years, several long-term *in vivo* studies in animals (usually rats) have been carried out to examine the effects of CsA and FK 506 on pancreatic function. CsA has been found to cause lesions in pancreatic β-cells and a corresponding reduction in insulin secretion and glucose tolerance, resulting in the development of hyperglycaemia (Helmchen *et al.*, 1984; Fehmann *et al.*, 1987; Müller *et al.*, 1988; Suzuki & Oka, 1989). Structural damage to the acinar cells of the exocrine pancreas has also been observed, along with reductions in plasma amylase levels and in cholecystokinin (CCK)-stimulated output of amylase, lipase and trypsin (Stöckmann *et al.*, 1985; Fernandez-Cruz *et al.*, 1988; Müller *et al.*, 1988; Suzuki & Oka, 1989; Bani *et al.*, 1990; Lopez-Miranda *et al.*, 1991; Hirano *et al.*, 1992). There is some debate about the doses of CsA required to produce these effects, and in particular about whether they occur at therapeutic (10 mg kg^{-1} day^{-1}) or only at toxic (50 mg kg^{-1} day^{-1}) levels. The consensus seems to be that even therapeutic doses of CsA cause significant adverse effects on the pancreas. Similar effects on pancreatic structure and function have been found following treatment with FK 506 *in vivo*, although this drug is 10–100 times more potent than CsA (Ricordi *et al.*, 1991; Doi *et al.*, 1992; Strasser *et al.*, 1992). For example, isolated pancreatic acini from rats receiving FK 506 over two weeks showed a reduction in CCK-stimulated amylase release (Doi *et al.*, 1992). Histological examination revealed that FK 506 treatment at 0.5 mg kg^{-1} day^{-1} caused the appearance of a small number of vacuoles in the cytoplasm of the acinar cells, while at ten times this dose numerous vacuoles and pyknotic nuclei were seen. A small number of studies on the effects of the immunosuppressants on the endocrine pancreas *in vitro* have also been carried out (Andersson *et al.*, 1984; Martin & Bedoya, 1990; Carroll *et al.*, 1991); inhibitory effects on β-cell function were observed, but no changes in cell structure were reported.

CsA and FK 506 exert their major therapeutic effect by inhibiting T-cell activation (Schreiber, 1991; Schreiber & Crabtree, 1992). They bind to different sets of endogenous intracellular receptors, called immunophilins (Handschumacher *et al.*, 1984; Harding *et al.*, 1989; Siekierka *et al.*, 1989; Harrison & Stein, 1990), and the immunophilin-ligand complexes in turn bind to and inhibit the calcium/calmodulin-dependent serine/threonine protein phosphatase calcineurin (Liu *et al.*, 1991). Our interest in these agents was first aroused by a report that they were also able to inhibit IgE receptor-mediated degranulation in the mast cell line RBL-2H3 (Hultsch *et al.*, 1991), with IC_{50} values close to those for inhibition of T-cell receptor-mediated interleukin-2 transcription. This finding raises the possibility that they may be useful tools in the dissection of the molecular mechanisms of exocytosis.

The acinar cells of the exocrine pancreas synthesize and secrete a complement of 15–20 digestive enzymes and proenzymes (zymogens). The intracellular transport and discharge of these proteins involves a series of interrelated

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events, which have been described in the classic work of Palade (1975). After synthesis and translocation into the lumen of the rough ER, the proteins are transported vectorially through the Golgi complex, In the *trans* Golgi network, condensing vacuoles pinch off into the cytosol. These condensing vacuoles mature, with further condensation of their contents, to form zymogen granules. In the fasted state, the zymogen granules accumulate at the apical pole of the cell. Stimulation of pancreatic secretion is controlled by both neural and hormonal factors, such as acetylcholine and CCK. These secretagogues induce a rapid, oscillatory release of Ca^{2+} from intracellular stores (Tsunoda *et al.*, 1990), that results in the fusion of the zymogen granules with the apical domain of the plasma membrane. While the regulation of the synthesis and intracellular transport of the individual enzymes has been extensively studied (Vasiloudes *et al.*, 1991), little is known about the molecular details of exocytosis. It was of interest to us, therefore, to know whether the immunosuppressants CsA and FK 506 might shed light on this process in the pancreatic acinar cell. In this study, we have examined the effects of CsA and FK 506 on two *in vitro* preparations of the rat exocrine pancreas–lobules and isolated dispersed acini. Our aims were to establish precisely which cellular functions are affected by these agents (and in particular whether exocytosis is specifically inhibited), and also to determine the potency of the drugs and the rapidity of their effect. A preliminary account of this work has been presented to the British Pharmacological Society (Waschulewski *et al.*, 1992).

Methods

Preparation of pancreatic lobules

Pancreatic lobules were prepared from male Wistar rats (200–300 g; Zentralinstitut für Versuchstierkunde GmbH, Hannover, Germany) in which pancreatic exocytosis had either been inhibited by starvation for 18 h, or maximally stimulated by feeding with the protease inhibitor FOY-305. FOY-305 produces this effect by inhibiting the proteolytic activity of secreted trypsin and thereby removing the normal feedback inhibition of CCK release, so that CCK levels remain elevated for several hours (Rausch *et al.*, 1987). Animals received the FOY-305 at a dose of 50 mg kg^{-1} body weight in 1 ml tap water via an orogastric tube 6–8 h before they were killed. Rats were killed by exsanguination under light ether anaesthesia, and the pancreas was quickly removed and placed in Krebs-Ringer-HEPES (KRH) buffer, pH 7.4, containing physiological concentrations of amino acids. Pancreatic lobules were then prepared as described by Scheele & Palade (1975). For the preparation of lobules from FOY-305-treated rats, all buffers contained the CCK analogue caerulein (1 ng ml^{-1}) to maintain the exocytotic stimulus.

Exocytosis in lobules

Lobules were prepared either from starved rats or from FOY-305-treated rats. Batches of about 15–20 lobules were pre-incubated at 37°C for 20 min, with shaking, in 5 ml KRH buffer containing either CsA, FK 506 or vehicle alone (1% (v/v) final concentration). After pre-incubation, the lobules were washed with KRH buffer in a Buchner funnel and transferred to flasks containing 5 ml buffer together with appropriate drug concentrations and caerulein (1 ng ml^{-1}). The lobules were then incubated at 37°C for 90 min, with shaking, and a 1 ml sample was taken from the medium at 15, 30, 60 and 90 min and replaced with fresh medium. Oxygenation was carried out every 15 min. At the end of the incubation the lobules were homogenized in 5 ml of 0.05 M Tris-HCl, pH 8.8 containing 0.1% (v/v) Triton X-100.

Amylase in medium and homogenates was assayed by the method of Bernfeld (1955). Secretion was expressed as a percentage of total amylase content. To assess the degree of cell damage, the release of the cytosolic marker enzyme lactate dehydrogenase (LDH) was also measured, by the method of Bergmeyer (1963). Again, release was expressed as a percentage of total LDH content.

Release of metabolically-labelled secretory proteins from lobules

Lobules prepared from FOY-305-treated rats were pre-incubated with immunosuppressant or vehicle as described above, and then pulse-labelled at 37°C for 8 min in methionine-free KRH buffer containing 100 μCi [^{35}S]-methionine in the continuous presence of caerulein (1 ng ml^{-1}). Lobules were transferred to Buchner funnels and washed with warm buffer containing a 100 fold excess of unlabelled methionine for 1–2 min. They were then incubated at 37°C, with shaking, in the same buffer. The incubation medium was sampled as described above for the measurement of amylase release. [^{35}S]-methionine-labelled proteins were quantitated by measuring trichloroacetic acid-insoluble radioactivity in medium and homogenates by the method of Mans & Novelli (1961). Secretion of labelled proteins was expressed as a percentage of the total contained in homogenized tissue samples.

Morphology of lobules

Lobules prepared from FOY-305-treated rats were pre-incubated with immunosuppressant or vehicle as described above. After incubation with caerulein (1 ng ml^{-1}) for 30, 60 or 90 min, lobules were fixed at 4°C for 2 h in a solution containing 2.5% (v/v) glutaraldehyde, 2.5% (w/v) paraformaldehyde, 0.05% (v/v) picric acid and 0.1% (w/v) sodium cacodylate, pH 7.4. Following post-fixation in reduced 1% (w/v) osmium tetroxide, tissues were stained in 0.5% (w/v) uranyl acetate in 50 mM malate buffer. Tissues were then dehydrated in a graded series of ethanol and embedded in Epon. Thin sections were cut with an Ultracut E microtome (Leica, Germany), stained in lead citrate solution and examined using a Zeiss EM 9S electron microscope.

Preparation of pancreatic acini

Pancreatic acini were prepared from male Sprague-Dawley rats (200–250 g), that had been starved overnight. Rats were killed by cervical dislocation and acini were prepared essentially as described by Rogers *et al.* (1988). A 5 ml volume of oxygenated, modified Krebs-Henseleit solution (isolation buffer, with the following composition, mM: NaCl 103, KCl 4.8, $MgCl_2$ 1.2, $CaCl_2$ 2.0, Na_2HPO_4 1.2, $NaHCO_3$ 2.5, glucose 14, HEPES 25, glutamine 2, basal Eagle's medium amino acid supplement, bovine serum albumin (BSA) 2 mg ml^{-1}, soybean trypsin inhibitor 0.1 mg ml^{-1}, pH 7.2), containing collagenase (800 units ml^{-1}) was injected into the pancreas. The tissue was then incubated at 37°C for 10 min with shaking (60 cycles min^{-1}). The collagenase-containing buffer was removed and the tissue washed twice in fresh isolation buffer. It was then incubated for 10 min in isolation buffer and for two further 10 min periods in collagenase-containing buffer, separated by a 10 min wash in isolation buffer. The tissue was then dispersed by repetitive pipetting with a plastic Pasteur pipette and filtered through 200 μm mesh nylon gauze. The acinar cell suspension was layered onto 2 × 5 ml isolation buffer containing 4% (w/v) BSA and centrifuged at 500 r.p.m. in a bench centrifuge for 3 min. The pellets were washed twice in the same buffer and the cells were resuspended in 20 ml of isolation buffer. The cells were incubated in this buffer at 37°C for 30 min with shaking and oxygenation, and finally washed three times before use.

Exocytosis in intact acini

Acinar cell suspensions, in isolation buffer, were distributed into microfuge tubes (final volume 1 ml) and incubated at 37°C for 10 min with either dimethylsulphoxide (DMSO, final concentration 0.1% (v/v), which by itself had no effect on exocytosis) or DMSO containing CsA or FK 506. An appropriate concentration of the cholinomimetic secretagogue bethanechol was then added and the acini were incubated at 37°C for a further 15 min, to allow exocytosis to proceed to completion (Edwardson *et al.*, 1990). At the end of the incubation, the tubes were centrifuged at 500 r.p.m. in a bench centrifuge to pellet the acini and a sample of the supernatant was taken for amylase determination.

Amylase was assayed by the method of Rinderknecht *et al.* (1967), using amylose azure as substrate, and porcine amylase as standard. All determinations were made in triplicate. Amylase release was expressed as a percentage of the total amylase content of the acini, determined by treating samples with 0.2% (v/v) Triton X-100. Results from several separate experiments were combined by use of a weighted means computer programme.

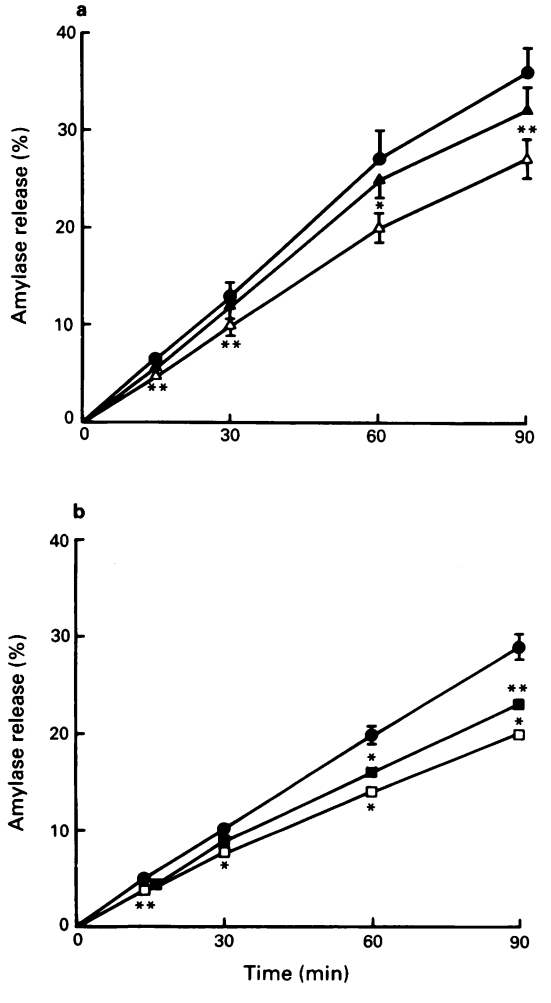


Figure 1 Inhibition by cyclosporin A (CsA, a) and FK 506 (b) of caerulein-stimulated amylase release from pancreatic lobules taken from starved rats. Lobules were pre-incubated for 20 min with vehicle (●), CsA at 10^{-6} M (▲) or 10^{-4} M (△), or FK 506 at 10^{-7} M (■) or 10^{-5} M (□), before stimulation with caerulein (1 ng ml^{-1}). Values are means ± s.e.mean from 3 (a) and 4 (b) separate experiments. Error bars are not shown where they lie within the dimensions of the symbols. $*P < 0.05$ and $**P < 0.01$, when compared with control values.

Exocytosis in permeabilized acini

Acini were cooled on ice and washed twice in ice-cold buffer solution containing KCl (140 mM), $MgCl_2$ (1 mM), EGTA (1 mM) and HEPES (20 mM), pH 6.5. They were then incubated at 37°C for 10 min in the same buffer with either DMSO (0.1% v/v) or DMSO containing CsA or FK 506. The tubes were returned to ice and streptolysin O (0.2 u ml^{-1}) and either water or $CaCl_2$ (to give a final free Ca^{2+} concentration of 10 μM (Fabiato & Fabiato, 1979)) were added. The tubes were then incubated at 37°C for 15 min. At the end of the incubation, the tubes were cooled on ice, centrifuged to pellet the acini and the amylase in the supernatants was assayed as above. Results are in the form of Ca^{2+}-dependent amylase release, expressed as a percentage of total amylase content.

Drugs and reagents

CsA was kindly provided by Sandoz Pharmaceuticals, Basel, Switzerland, and was dissolved in ethanol:water:Tween-80, 50:48:2 by volume. FK 506 was kindly provided by Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan, and was dissolved in ethanol. FOY-305 (N,N-dimethylcarbamylmethyl-*p*-(*p*-guanidinobenzoyloxy) phenylacetate-methansulphonate) was from Sanol-Schwartz, Monheim, Germany. Caerulein (Takus) was from Farmitalia, Freiburg, Germany. [^{35}S]-methionine was from Amersham Buchler, Braunschweig, Germany. Bovine serum albumin (BSA Type V), soybean trypsin inhibitor, collagenase (Type XI), bethanechol and porcine amylase (Type VI-B) were from Sigma, Poole, U.K. Streptolysin O was from Wellcome Diagnostics, Dartford, U.K.

Statistical evaluation of data

The statistical significance of differences between groups of data was assessed by Student's *t* test (paired for results with lobules and unpaired for results with acini). A *P* value of <0.05 was considered to be significant. All values are expressed as mean ± s.e.mean.

Results

Amylase release in lobules

Lobules were taken either from starved rats, in which exocytosis would have been inhibited, or from rats that had been treated with the low molecular weight protease inhibitor FOY-305 to stimulate exocytosis maximally (Rausch *et al.*, 1987). In both cases, approximately 40% of the total amylase initially present in the lobules was released into the bathing medium over the 90 min incubation period used in these experiments (Figures 1 and 2). A 20 min pretreatment with either CsA or FK 506 inhibited amylase release from lobules taken from starved rats (Figure 1). The inhibition produced was dose-dependent, with FK 506 being about ten times more potent than CsA. For example, at the highest concentrations of the drugs used (10^{-4} M CsA and 10^{-5} M FK 506), amylase release over 90 min was reduced to 75 ± 7% and 69 ± 3% of control, respectively. A different picture emerged when lobules were taken from FOY-305-stimulated rats (Figure 2). In this case CsA (10^{-4} M) did not significantly inhibit amylase release, whereas FK 506 (10^{-5} M), although now less effective, still reduced release over 90 min, to 80 ± 9% of control.

The release of LDH from the lobules over the 90 min incubation period was always less than 5% of the total content, which indicates that the vast majority of the acinar cells maintained their integrity during the experiments. LDH release was not affected by treatment of the lobules with either CsA or FK 506. Hence, there was no evidence that the immunosuppressants caused any gross damage to the cells.

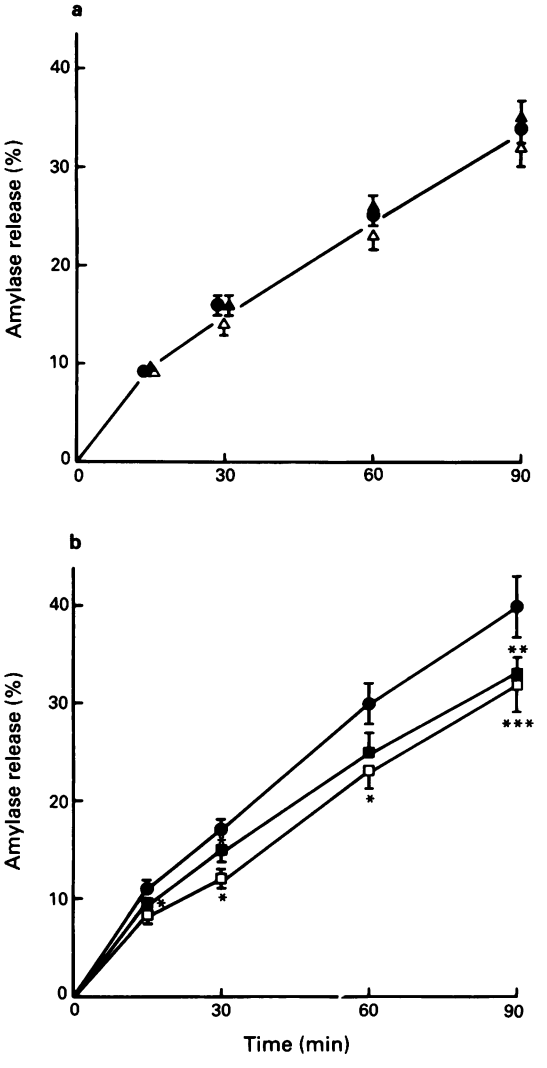


Figure 2 Effects of cyclosporin A (CsA, a) and FK 506 (b) on caerulein-stimulated amylase release from pancreatic lobules taken from FOY-305-treated rats. Experimental procedure and symbols are as for Figure 1. Values are means ± s.e.mean from 6 (a) and 4 (b) separate experiments. $*P<0.05$, $**P<0.01$ and $***P<0.001$, when compared with control values.

Protein secretion in lobules

Secretion of proteins that had been pulse-labelled with [^{35}S]-methionine from lobules taken from FOY-305-treated rats was detected after a lag period of about 15 min, and 60% of the total labelled protein was released into the medium over 90 min (Figure 3). Secretion was inhibited by both CsA and FK 506; for example, secretion over 90 min was reduced to 75 ± 3% of control by CsA (10^{-4} M) and to 72 ± 3% of control by FK 506 (10^{-5} M).

Morphology of lobules

To determine whether the inhibitory effect of the immunosuppressants on exocytosis had any morphological correlate, lobules from FOY-305-treated rats were examined at the ultrastructural level. Figure 4a shows the appearance of control acinar cells after a 90 min incubation with vehicle alone. It is apparent that treatment of the rats with FOY-305 caused an extensive degranulation of the acinar cells; only a few electron-translucent secretory granules are found in the vicinity of the Golgi complex or near the acinar lumen. This degranulation was not affected by incubation of the lobules with CsA (10^{-4} M; Figure 4b). In contrast, acinar cells in lobules that had been incubated with FK 506 (10^{-6} M) contained many zymogen granules, located primarily at the apical pole, indicating an inhibition of degranulation (Figure 4c). These granules, although of smaller size than those seen in control animals, revealed an electron-dense granule content, indicating maturation during storage (Figure 4d). Finally, neither FK 506 (Figure 5) nor CsA (not shown) had any effect on the morphology of the Golgi complex, or indeed on the structure of any other cellular organelle.

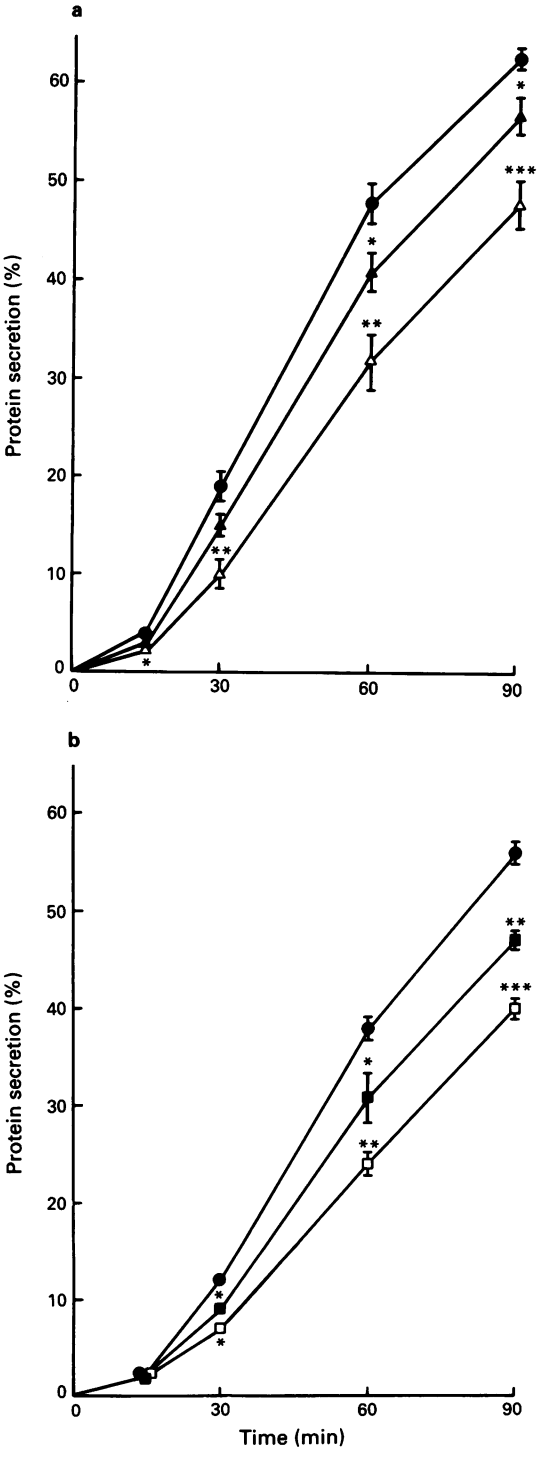


Figure 3 Inhibition by cyclosporin A (CsA, a) and FK 506 (b) of caerulein-stimulated release of pulse-labelled secretory proteins from pancreatic lobules taken from FOY-305-treated rats. Pre-incubation with drugs was for 20 min; symbols are as for Figure 1. Values are means ± s.e.mean from 6 (a) and 5 (b) separate experiments. $*P<0.05$, $**P<0.01$ and $***P<0.001$, when compared with control values.

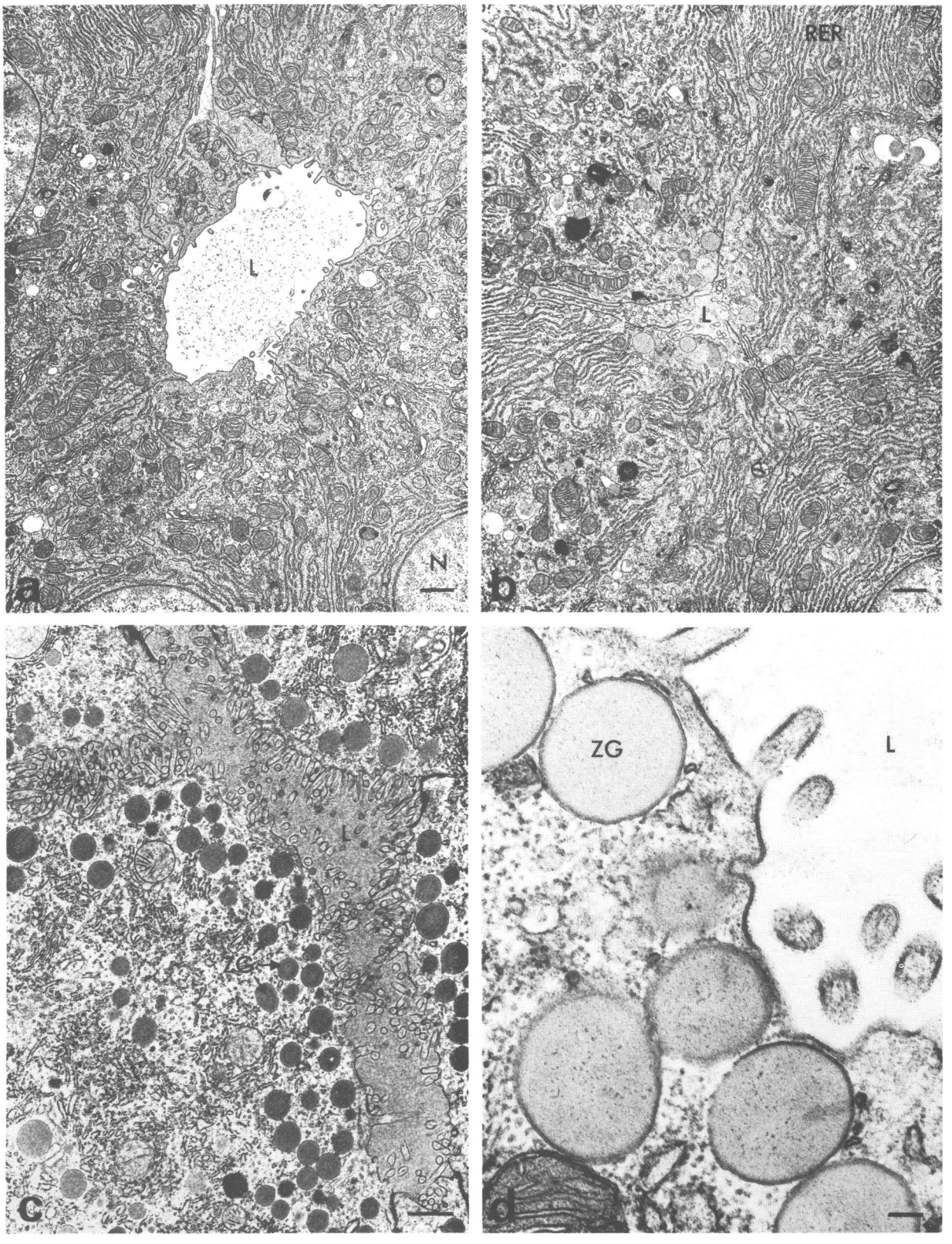


Figure 4 Effects of cyclosporin A (CsA) and FK 506 on caerulein-stimulated degranulation of acinar cells in lobules taken from FOY-305-treated rats. Lobules were pre-incubated for 20 min with vehicle (a), CsA at 10^{-4} M (b) or FK 506 at 10^{-6} M (c) or 10^{-5} M (d), and then incubated for 90 min in the presence of caerulein (1 ng ml^{-1}), before fixation and processing. RER, rough endoplasmic reticulum; L, acinar lumen; N, nucleus; ZG, zymogen granule. Bar 1 μm (a–c), 100 nm (d).

Exocytosis in intact acini

Bethanechol caused a stimulation of amylase release from intact acini of approximately 4% over a 15 min period, with half-maximal stimulation occurring at a concentration of 10^{-5} M. The percentage of the total amylase released from the acini is similar to the extent of release from lobules over the same period (see Figure 1). The difference between the two preparations is that release from acini is complete after 15 min (Edwardson *et al.*, 1990), whereas release from

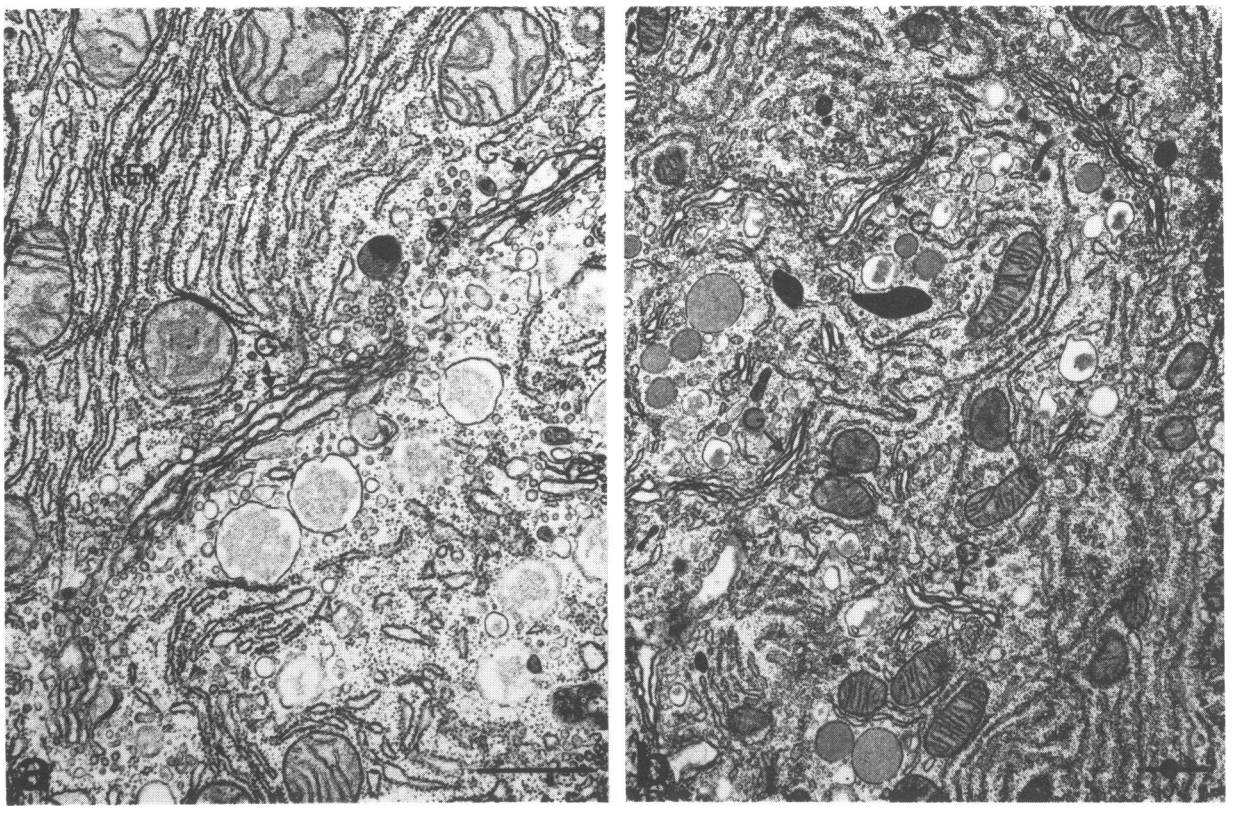


Figure 5 Effect of FK 506 on ultrastructure of acinar cells in lobules taken from FOY-305-treated rats. Lobules were pre-incubated for 20 min with vehicle (a) or FK 506 at 10^{-5} M (b), and then incubated with caerulein (1 ng ml^{-1}) for 30 min. RER, rough endoplasmic reticulum; G, Golgi complex. Bar 1 μm.

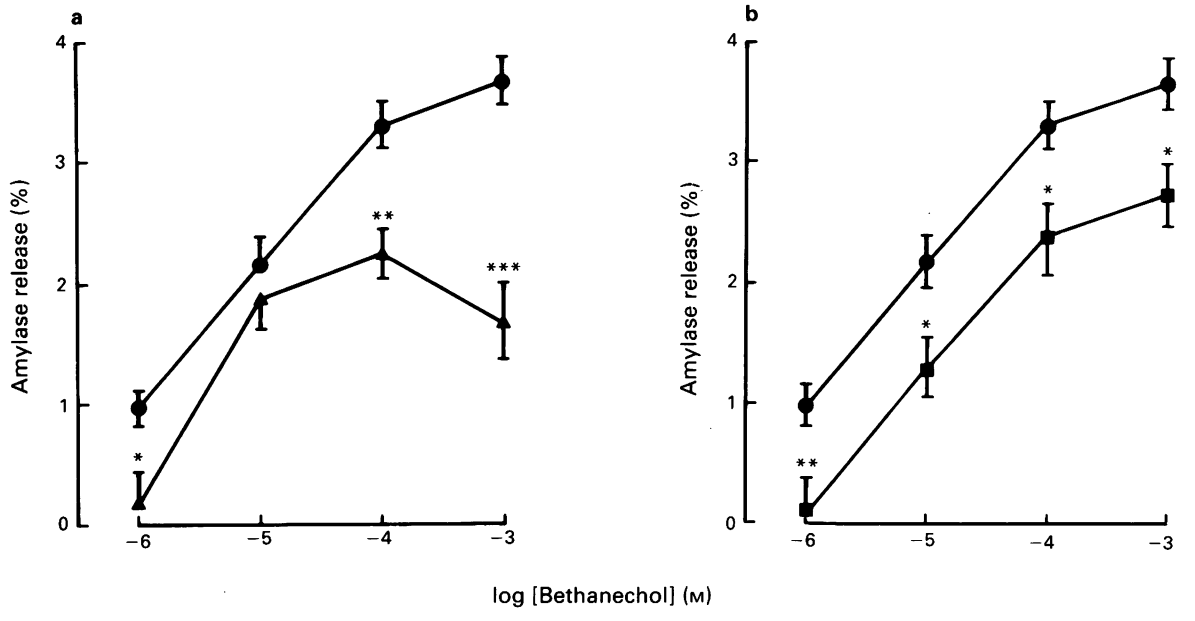


Figure 6 Inhibition by cyclosporin A (CsA, a) and FK 506 (b) of bethanechol-stimulated amylase release from dispersed intact pancreatic acini taken from starved rats. Acini were pre-incubated for 10 min with dimethylsulphoxide vehicle (●), or with CsA at 10^{-6} M (▲) or FK 506 at 10^{-7} M (■), before stimulation with various concentrations of bethanechol for 15 min. Values are means ± s.e.mean from 8 (a) and 10 (b) separate experiments. $*P<0.05$, $**P<0.01$ and $***P<0.001$, when compared with control values.

lobules continues beyond 90 min. A 10 min pretreatment of the acini with either CsA (10^{-6} M) or FK 506 (10^{-7} M) caused a reduction in this bethanechol-dependent amylase release (Figure 6). CsA did not reduce basal amylase release, whereas FK 506 did cause a small reduction. The concentration-dependencies of the effects of the immunosuppressants on exocytosis are shown in Figure 7. Neither drug completely abolished exocytosis, even at high concentrations. Exocytosis was reduced to 43 ± 8% (CsA) and 45 ± 5% (FK 506) of control, with IC_{50} values of 50 nM (CsA) and 3 nM (FK 506).

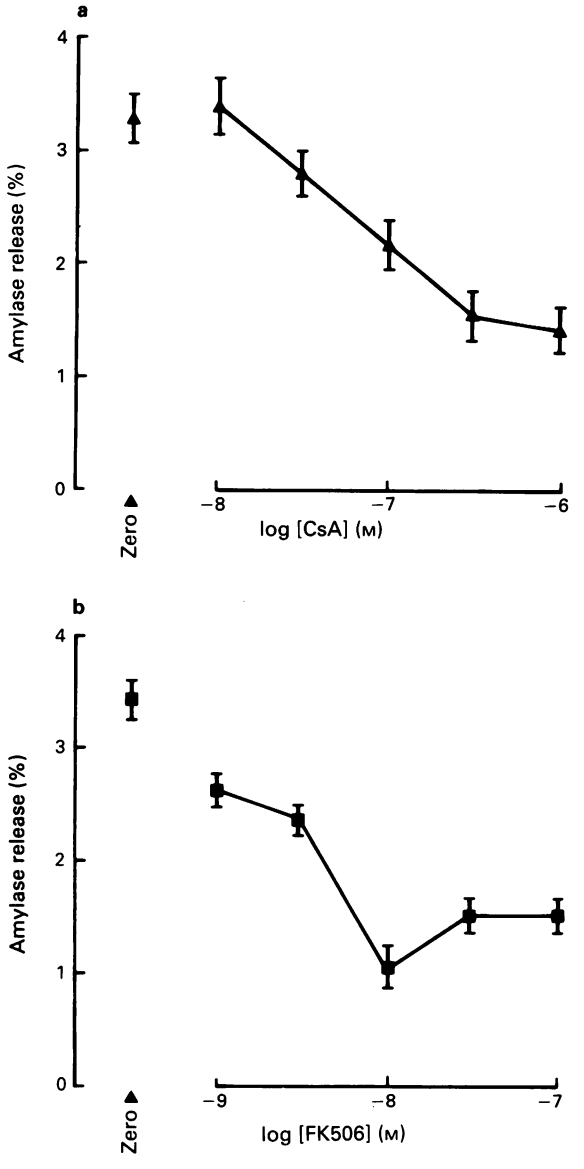


Figure 7 Concentration-dependence of the inhibitory effects of cyclosporin A (CsA, a) and FK 506 (b) on bethanechol-stimulated amylase release from intact pancreatic acini taken from starved rats. Experimental procedure was as for Figure 6, except that the bethanechol concentration was kept constant at 10^{-4} M and the concentration of immunosuppressant was varied. Values are means ± s.e.mean from 10 (a) and 17 (b) separate experiments.

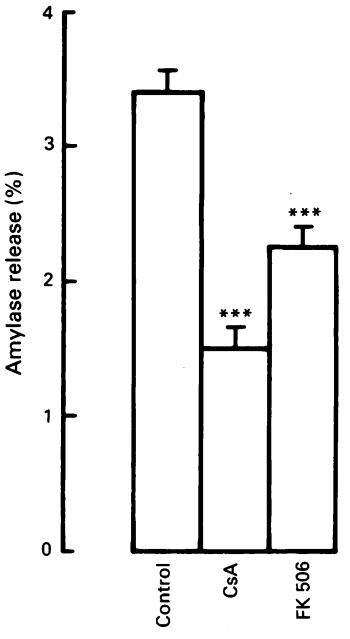


Figure 8 Inhibition by cyclosporin A (CsA, 10^{-6} M) and FK 506 (10^{-7} M) of Ca^{2+}-stimulated release of amylase from streptolysin O-permeabilized pancreatic acini taken from starved rats. Amylase release was allowed to proceed to completion (15 min). Values are means ± s.e.mean from 19 (control), 14 (CsA) and 18 (FK 506) separate experiments. ***$P < 0.001$, when compared with control values.

Exocytosis in permeabilized acini

In acini permeabilized with the bacterial toxin, streptolysin O, exocytosis can be triggered by raising the free Ca^{2+} concentration (Edwardson *et al.*, 1990). In this study, the mean Ca^{2+}-dependent exocytosis was 3.4% of total cellular amylase over a 15 min period. Pretreatment of the acini with the immunosuppressive drugs, at concentrations that were maximally effective in intact acini, 1 μM CsA and 100 nM FK 506, caused a significant reduction in the extent of exocytosis observed, to 44 ± 6% of control with CsA and 66 ± 6% of control with FK 506 (Figure 8).

Discussion

In this study, we have examined the effects of CsA and FK 506 on secretion from lobules taken either from starved rats or from rats treated with the low molecular weight protease inhibitor FOY-305. Caerulein-stimulated secretion of newly-synthesized proteins from lobules taken from starved rats has previously been shown to occur at a low rate (20% of pulse-labelled protein released over 90 min (Vasiloudes *et al.*, 1991)). Hence, the amylase release occurring in these experiments (40% of total cell content over 90 min) must largely represent release of pre-formed amylase that had been stored in zymogen granules. The inhibition by CsA and FK 506 of this release, therefore, indicates a direct effect of these drugs on the final stage in the exocytotic pathway, that is, the fusion of the granules with the plasma membrane. Treatment of rats with FOY-305 results in the release of endogenous CCK, which in turn causes persistent stimulation of secretion in the exocrine pancreas (Rausch *et al.*, 1987). It has also been shown (Vasiloudes *et al.*, 1991) that pre-stimulation *in vivo* with caerulein for 24 h results in a five fold increase in the rate of secretion of pulse-labelled proteins in response to caerulein *in vitro*, but that the proteins still pass through zymogen granules en route to the plasma membrane. As expected from these previous results, it was found in the present study that about 60% of pulse-labelled protein was released over 90 min from lobules taken from FOY-305-treated rats. In these lobules, the storage of amylase in zymogen granules will be greatly reduced and the amylase released will largely be newly-synthesized. Inhibition of amylase release, therefore, could result from an effect at any point on the exocytotic pathway. It was found that CsA did not significantly inhibit amylase release from lobules taken from FOY-305-treated rats, but that FK 506, although now less effective than in lobules taken from starved rats, still had an inhibitory effect. This picture was corroborated by morphological experiments conducted in parallel, which showed that FK 506, but not CsA, inhibited the degranulation of acinar cells produced by caerulein. These results indicate that in lobules taken from FOY-305-treated rats, only FK 506, and not CsA, has an effect on the fusion of zymogen granules with the plasma membrane. Hence, the two drugs have subtly different actions in the acinar cell.

Since both drugs inhibit the release of pulse-labelled proteins, and the inhibition caused by FK 506 is greater than its effect on amylase release, it would appear that both drugs have an additional effect at a point earlier in the exocytotic pathway, although there was no indication of any ultrastructural changes in cytoplasmic organelles, such as the rough ER or the Golgi complex, occurring in their presence. At present, it is not clear why the inhibitory effect of CsA on amylase release should be lost in lobules taken from FOY-305-treated rats, or how in these lobules CsA can inhibit the release of newly synthesized proteins without affecting amylase release. These results do suggest, however, that the properties of the exocytotic event may be changed as a consequence of persistent stimulation.

It is difficult to relate the concentrations that are effective here to the doses required to affect pancreatic function *in vivo*, because of pharmacokinetic complications. It is clear, however, that effects on acinar cell function occur at concentrations that do not cause any detectable adverse effects on cell integrity or morphology. Although the concentrations of the immunosuppressants required to produce maximal inhibition of secretion in lobules have not been determined here, our experiments show that dispersed acinar cell suspensions are considerably more sensitive than lobules to the effects of the immunosuppressants. For example, the inhibitory effect of CsA on exocytosis in acini is maximal at 10^{-6} M, a concentration which in lobules from starved rats produces only about 50% of the inhibition of amylase release caused by CsA at 10^{-4} M. The reasons for this difference in potency are unclear, although access of the drug to the cells may be a problem in the lobules. Despite these complications, the potency of FK 506 in both lobules and acini is of the order of ten times that of CsA, as reported in other systems.

Recent interest has focused on the potential utility of these immunosuppressive drugs in probing intracellular communication pathways (McKeon, 1991). For example, the inhibition by CsA and FK 506 of exocytosis in RBL-2H3 cells occurs at concentrations similar to those required to inhibit T-cell receptor-mediated interleukin-2 transcription (Hultsch *et al.*, 1991). Both drugs appear to act at a step distal to the early events at the plasma membrane, such as IgE receptor aggregation, phosphatidyl inositol bis-phosphate hydrolysis and elevation of intracellular Ca^{2+} concentration, but proximal to late events, such as exocytotic membrane fusion. The results of the present study, and in particular the close correspondence between the IC_{50} values for the drugs in the two systems, indicate a similar type of effect in pancreatic acinar cells. The inhibition of exocytosis by CsA and FK 506 is seen both in lobules stimulated by the CCK analogue caerulein and in acini stimulated by the cholinomimetic secretagogue bethanechol. The immunosuppressants also inhibit exocytosis in permeabilized acini triggered directly by Ca^{2+}. These results provide clear evidence for a site of action downstream of receptor occupation and second messenger production. We concur, therefore, with the idea that these drugs may prove to be important probes of the mechanisms of intracellular membrane transport in general and of regulated exocytosis in particular.

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References

ANDERSSON, A., BORG, H., HALLBERG, A., HELLERSTROM, C., SANDLER, S. & SCHNELL, A. (1984). Long-term effects of cyclosporin A on cultured mouse pancreatic islets. *Diabetologia*, **27**, 66–69.

BANI, D., FILIPPONI, F., MAGNANI, L., HOUSSIN, D. & ROMAGNOLZ, P. (1990). Morphological changes in rat pancreatic acinar cells induced by long-term treatment with cyclosporine and their reversal after withdrawal. *Transplantation*, **50**, 830–834.

BERGMEYER, H.U. (1963). Determination of lactate dehydrogenase. In *Methods of Enzymatic Analysis*, pp. 736–743. New York: Academic Press.

BERNFELD, P. (1955). Amylases α and β. *Meth. Enzymol.*, **1**, 149–158.

CARROLL, P.B., BOSCHERO, A.C., LI, M.-Y., TZAKIS, A.G., STARZL, T.E. & ATWATER, I. (1991). Effect of the immunosuppressant FK 506 on glucose-induced insulin secretion from adult rat islets of Langerhans. *Transplantation*, **51**, 275–278.

DOI, R., TANGOKU, A., INOUE, K., CHOWDHURY, P. & RAYFORD, P.L. (1992). Effects of FK 506 on exocrine pancreas in rats. *Pancreas*, **7**, 197–204.

EDWARDSON, J.M., VICKERY, C. & CHRISTY, L.J. (1990). Rat pancreatic acini permeabilised with streptolysin O secrete amylase at Ca^{2+} concentrations in the micromolar range, when provided with ATP and GTPγS. *Biochim. Biophys. Acta*, **1053**, 32–36.

FABIATO, A. & FABIATO, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)*, **75**, 463–505.

FEHMANN, H.C., HAVERICH, R., STÖCKMANN, F. & CREUTZFELDT, W. (1987). Cyclosporine A in low doses induces functional and morphologic changes in rat pancreatic B cells. *Transpl. Proc.*, **19**, 4015–4016.

FERNANDEZ-CRUZ, L., TARGARONA, E.M., RIBERA, M.T., COLOMER, J., PI, F., SAENZ, A., CASAS, A. & HOTTER, G. (1988). The effect of cyclosporine and standard immunosuppressive drugs on the exocrine rat pancreas. *Transpl. Proc.*, **20**, 453–456.

HANDSCHUMACHER, R.E., HARDING, M.W., RICE, J., DRUGGE, R. & SPEICHER, D.W. (1984). Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science*, **226**, 544–547.

HARDING, M.W., GALAT, A., UEHLING, D.E. & SCHREIBER, S.L. (1989). A receptor for the immunosuppressant FK 506 is a *cis-trans* peptidyl-prolyl isomerase. *Nature*, **341**, 758–760.

HARRISON, R.K. & STEIN, R.L. (1990). Substrate specificities of the peptidyl-prolyl cis-trans isomerase activities of cyclophilin and FK 506 binding protein: evidence for the existence of a family of distinct enzymes. *Biochemistry*, **29**, 3813–3816.

HELMCHEN, U., SCHMIDT, W.E., SIEGEL, E.G. & CREUTZFELDT, W. (1984). Morphological and functional changes of pancreatic B cells in cyclosporin A-treated rats. *Diabetologia*, **27**, 416–418.

HIRANO, T., MANABE, T., ANDO, K. & TOBE, T. (1992). Acute cytotoxic effect of cyclosporin A on pancreatic acinar cells in rats. *Scand. J. Gastroenterol.*, **27**, 103–107.

HULTSCH, T., ALBERS, M.W., SCHREIBER, S.L. & HOHMAN, R.J. (1991). Immunophilin ligands demonstrate common features of signal transduction leading to exocytosis or transcription. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6229–6233.

LIU, J., FARMER, J.D., LANE, W.S., FRIEDMAN, J., WEISSMAN, I. & SCHREIBER, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK 506 complexes. *Cell*, **66**, 807–815.

LOPEZ-MIRANDA, J., BLANCO-MOLINA, A., LOPEZ-SEGURA, F., NICOLAS-PUIGGARI, M., TORRE-CISNEROS, J. & PEREZ-JIMENEZ, F. (1991). The effect of cyclosporine on exocrine function of the rat pancreas – an in vitro study. *Transplantation*, **51**, 562–565.

MCKEON, F. (1991). When worlds collide: immunosuppressants meet protein phosphatases. *Cell*, **66**, 823–826.

MANS, R.J. & NOVELLI, G.D. (1961). Measurement of the incorporation of radioactive amino acids into protein by a filter paper disc method. *Arch. Biochem. Biophys.*, **94**, 48–53.

MARTIN, F. & BEDOYA, F.J. (1990). Short-term effects of cyclosporine on secretagogue-induced insulin release by isolated islets. *Transplantation*, **50**, 551–553.

MÜLLER, M.K., BERGMANN, K., DEGENHARDT, H., KLOPPEL, G., LOHR, M., COONE, H.J. & GOEBELL, H. (1988). Differential sensitivity of rat exocrine and endocrine pancreas to cyclosporine. *Transplantation*, **45**, 698–700.

PALADE, G. (1975). Intracellular aspects of the process of protein secretion. *Science*, **189**, 347–358.

RAUSCH, U., ADLER, G., WEIDENBACH, H., WEIDENBACH, F., RUDOLFF, D., KOOP, I. & KERN, H.F. (1987). Stimulation of pancreatic secretory process in the rat by low-molecular weight proteinase inhibitor. *Cell Tissue Res.*, **247**, 187–193.

RICORDI, C., ZENG, Y., ALEJANDRO, R., TZAKIS, A., VENKATARAMANAN, R., FUNG, J., BEREITER, D., MINTZ, D.H. & STARZL, T.E. (1991). In vivo effect of FK 506 on human pancreatic islets. *Transplantation*, **52**, 519–522.

RINDERKNECHT, H., WILDING, P. & HAVERBACK, B.J. (1967). A new method for determination of α-amylase. *Experientia*, **23**, 805.

ROGERS, J., HUGHES, R.G. & MATTHEWS, E.K. (1988). Cyclic GMP inhibits protein kinase C-mediated secretion in rat pancreatic acini. *J. Biol. Chem.*, **263**, 3713–3719.

SCHEELE, G.A. & PALADE, G.E. (1975). Studies on the pancreas of the guinea pig. Parallel discharge of exocrine enzyme activities. *J. Biol. Chem.*, **250**, 2660–2670.

SCHREIBER, S.L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science*, **251**, 283–287.

SCHREIBER, S.L. & CRABTREE, G.R. (1992). The mechanism of action of cyclosporin A and FK 506. *Immunol. Today*, **13**, 136–142.

SIEKIERKA, J.J., HUNG, S.H.Y., POE, M., LIN, C.S. & SIGAL, N.H. (1989). A cytosolic binding protein for the immunosuppressant FK 506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature*, **341**, 755–757.

STÖCKMANN, F., GÖKE, B., OTTO, J. & CREUTZFELDT, W. (1985). Effect of cyclosporin A on rat exocrine pancreatic function. *Digestion*, **32**, 219.

STRASSER, S., ALEJANDRO, R., SHAPIRO, E.T., RICORDI, C., TODO, S. & MINTZ, D.H. (1992). Effect of FK 506 on insulin secretion in normal dogs. *Metabolism*, **41**, 64–67.

SUZUKI, S. & OKA, T. (1989). Inhibitory effects of cyclosporin on exocrine and endocrine functions of the rat pancreas. *Transpl. Proc.*, **21**, 913–914.

TSUNODA, Y., STUENKEL, E.L. & WILLIAMS, J.A. (1990). Oscillatory mode of calcium signalling in rat pancreatic acinar cells. *Am. J. Physiol.*, **258**, C147–C155.

VASILOUDES, P., PAUL, N., TOOZE, J. & KERN, H.F. (1991). Acceleration of asynchronous enzyme transport in the rat exocrine pancreas following hormonal stimulation in vivo. *Eur. J. Cell Biol.*, **54**, 27–37.

WASCHULEWSKI, I.H., HALL, D.V., KERN, H.F. & EDWARDSON, J.M. (1992). Effects of the immunosuppressants cyclosporin A and FK 506 on exocytosis in the rat exocrine pancreas in vitro. *Br. J. Pharmacol.*, **107**, 39P.

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Redirection of arachidonic acid metabolism by ICI D1542: effects on thrombus formation in the coronary artery of the anaesthetized dog

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1 The effects of simultaneous redirection of arachidonic acid metabolism, by inhibition of thromboxane A_2 (TXA_2) synthase and blockade of the platelet thromboxane A_2 receptor (TP-receptor), was examined on the rate of thrombus formation in a stenosed coronary artery with damaged endothelium in an anaesthetized dog.

2 Redirection of arachidonic acid metabolism was achieved by intravenous doses of ICI D1542, a selective and potent inhibitor of TXA_2 synthase and the TP-receptor.

3 Redirection of arachidonic acid metabolism was demonstrated in whole blood, stimulated *ex vivo* by collagen. The ED_{50} for inhibition of TXB_2 production was 7.1 $\mu g\,kg^{-1}$, i.v.; there were corresponding increases in the production of the eicosanoids prostaglandin D_2 (PGD_2), PGE_2 and $PGF_{2\alpha}$.

4 Thrombus formation was inhibited by D1542 (ED_{50} 0.55 $\mu g\,kg^{-1}$, i.v.), but could be restarted by an intravenous infusion of adrenaline (0.2–38 $\mu g\,kg^{-1}\,min^{-1}$, i.v.). In the presence of the maximum effective dose of D1542 (1 $mg\,kg^{-1}$, i.v.) a 190 fold increase in the infusion rate of adrenaline was required to restore thrombus formation.

5 In the presence of D1542, removal of endoperoxide metabolites by inhibition of cyclo-oxygenase with aspirin (5 $mg\,kg^{-1}$, i.v.) caused thrombus formation to restart, indicating the ability of the endoperoxide metabolites to inhibit thrombus formation *in vivo*.

6 These results indicate that, in the stenosed and damaged coronary artery of the dog, redirection of arachidonic acid metabolism by D1542 is more effective at preventing thrombus formation than inhibition of cyclo-oxygenase by aspirin.

Keywords: Platelet thrombus; coronary artery; thromboxane A_2; ICI D1542

Introduction

Activation and consequent aggregation of platelets by shear stresses and exposure to thrombogenic surfaces results in the synthesis of thromboxane (TXA_2) from arachidonic acid, which, along with the intermediate prostaglandin endoperoxide (PGH_2), promotes further aggregation and vasoconstriction. Clinical evidence of the importance of arachidonic acid products in coronary artery disease has been demonstrated by the efficacy of aspirin (Antiplatelet Trialists' Collaboration, 1988) and in the treatment of unstable angina (The RISC Group, 1990). However, inhibition of cyclo-oxygenase by aspirin, removes all PGH_2 metabolites and their potential benefits as inhibitors of platelet aggregation and smooth muscle cell proliferation.

Redirection of arachidonic acid metabolism, by inhibition of thromboxane synthase, coupled with simultaneous blockade of TP-receptor, provides a method of removing the effects of TXA_2 and PGH_2 and increasing the production of PGD_2 and PGI_2. In human platelets, aggregated *ex vivo* with collagen, Watts *et al.* (1991) showed that combined TXA_2 synthase inhibition and receptor blockade (dazoxiben and G332191, a TP-receptor antagonist) was more effective than aspirin in preventing aggregation. We obtained similar results on thrombus formation in the dog coronary artery and were able to show that after sequential synthase inhibition and receptor blockade, removal of all PGH_2 metabolites by inhibition of cyclo-oxygenase with aspirin, resulted in an increase in the rate of thrombus formation (McAuliffe *et al.*, 1992).

The purpose of the present experiments was to examine the effects of simultaneous TXA_2 synthase inhibition and TP-receptor blockade by ICI D1542 (4(Z)-6-[(2S,4S,5R)-2-[(1-methyl-1-(2-nitro-4-tolyloxy)-4-(3-pyridyl)-1,3-dioxan-5-yl] hex-4-enoic acid) a potent and selective inhibitor of TXA_2 synthase and the TP-receptor (Brownrigg *et al.*, 1992), on the rate of thrombus formation in a stenosed coronary artery with damaged endothelium in the anaesthetized dog.

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Methods

General methods

Experiments were carried out in 13 beagle dogs, mean weight 16.7 kg (range 22.6–13.0). The dogs were sedated with morphine sulphate (10 mg, i.m.) and 30 min later were anaesthetized with pentobarbitone (induction 30 $mg\,kg^{-1}$, i.v., maintenance 3 $mg\,kg^{-1}$, i.v. every 30 min) through a cannula inserted into the long saphenous vein under local anaesthesia.

Ventilation was via a tracheotomy with a mixture of 40% oxygen in room air supplied with a Harvard animal ventilator. When the chest was opened a resistance to expiration was produced by placing the expiratory output of the pump under 3 cm of water. Samples of arterial blood were withdrawn at intervals throughout each experiment and pH, P_{O_2} and P_{CO_2} measured with a Corning EEL blood gas analyser. End-tidal P_{CO_2} was continuously measured by aspirating expired air from the trachea into an infra-red CO_2 analyser (P.K. Morgan Ltd.). The arterial P_{CO_2} was kept within the limits 34–42 mmHg and the arterial blood pH between 7.33 and 7.43 either by adjustment of respiration or the intravenous injection of 1.0 M $NaHCO_3$ solution. Oesophageal temperature was recorded and maintained at 37 ± 1°C (range) by heating lamps above and a thermostatically controlled

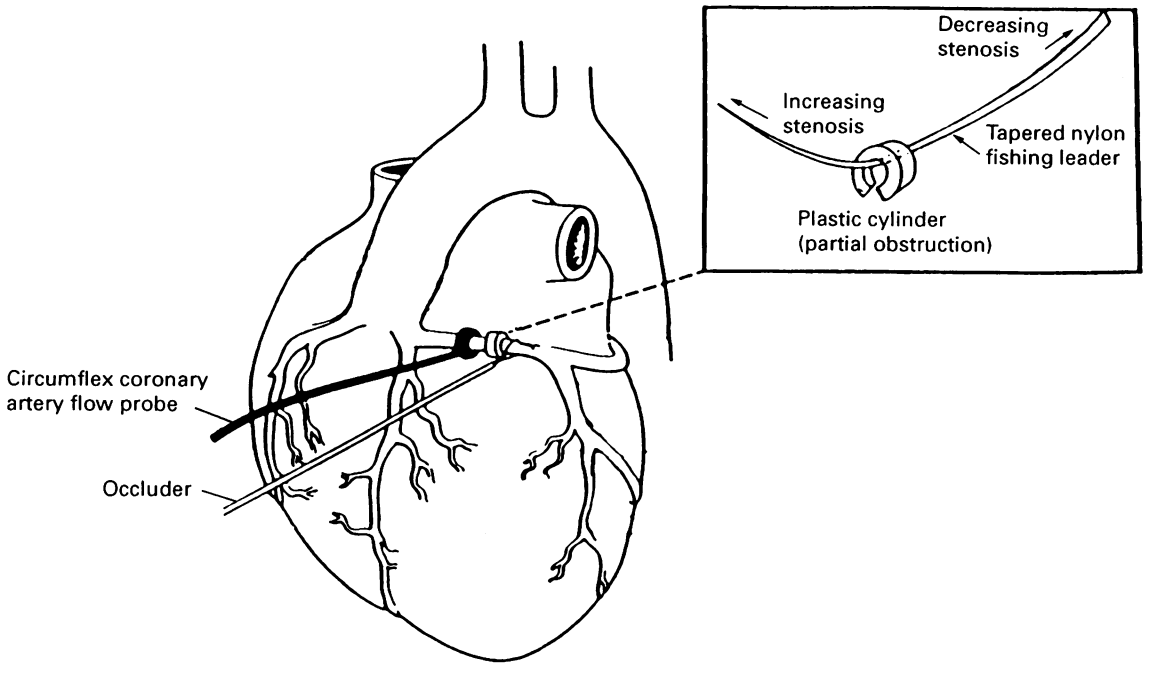


Figure 1 Diagram of preparation showing position of ultrasonic flow probe on the circumflex coronary artery, plastic cuff producing a stenosis over the damaged section of artery and snare occluder for producing periods of total obstruction as a test for reactive hyperaemia. The inset shows the tapered nylon fishing leader used to adjust the degree of stenosis until maximal vasodilatation is obtained in the distal vascular bed and reactive hyperaemia prevented.

heated blanket below the animal.

The ECG lead II was recorded and the signal used to drive a cardiotachometer. Systemic arterial pressure was recorded through a cannula inserted into the left femoral artery. All pressures were measured with strain gauge manometers (Hewlett Packard 4-442-001) attached to d.c. bridge amplifiers (Electromed). Blood flow in the left circumflex coronary artery was measured with either an electromagnetic flowmeter (Carolina Instruments Inc.) or an ultra-sound flowmeter (Transonic Inc.). The ECG, heart rate (HR), systemic arterial pressure and the mean and pulsatile coronary artery blood flow were recorded with a thermal array recorder (Gould TA4000).

Surgical procedures

The chest was opened in the 5th intercostal space, the left phrenic nerve divided and the pericardium opened to expose the left circumflex artery and vein. The circumflex coronary artery was damaged and stenosed as described by Folts *et al.* (1976); briefly, about 2 cm of the left circumflex coronary artery was dissected free of surrounding tissue and the endothelium of a 5 mm length of artery damaged by crushing with a pair of Spencer Wells forceps. The damaged section of the artery was stenosed by a stiff plastic cuff which occluded the artery by about 60%. A flow probe was placed proximal to and a snare distal to the stenosis. Fine adjustment of the

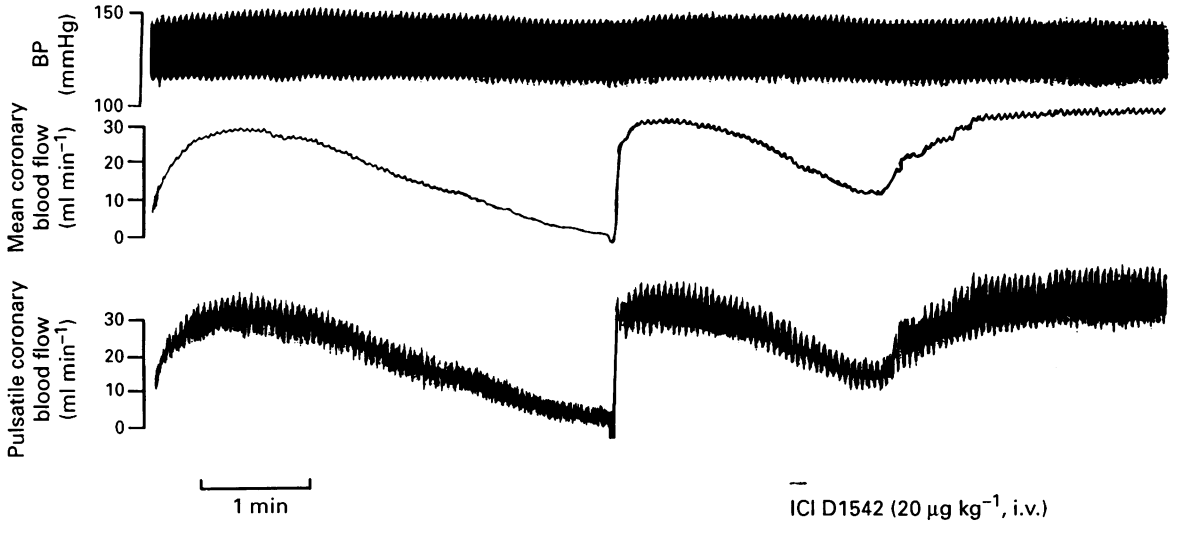


Figure 2 An example of records obtained in one dog of arterial blood pressure (BP), and mean and pulsatile coronary blood flow, with cyclical flow reductions (CFRs). Since the distal vascular bed is maximally dilated and arterial blood pressure constant, decreases in flow represent an increase in resistance at the site of stenosis as platelet aggregation occurs and a thrombus forms. Embolization produces an abrupt increase in flow. CFRs were abolished by D1542 (20 $\mu g\ kg^{-1}$, i.v.).

degree of stenosis was accomplished by passing a tapered nylon fishing line through the cuff (Figure 1). The degree of stenosis was adjusted until a 20 s occlusion with the distal snare did not produce a reactive hyperaemia, i.e. the distal vascular bed was fully dilated and therefore changes in blood flow (at constant heart rate and blood pressure) indicated changes in resistance at the stenosis.

In the above preparation the combination of stenosis and damaged endothelium induces platelet aggregation and the formation of a thrombus, increasing the resistance to blood flow. When blood flow approached zero, thrombi embolized either spontaneously or after gentle vibration of the plastic cuff; blood flow and thrombus formation then restarted and a pattern of cyclic flow reductions (CFRs) began, e.g. Figure 2 (Folts *et al.*, 1976; Folts, 1982).

Arachidonic acid metabolites

Samples of arterial blood (2 ml) were collected into citrated tubes. Equine collagen fibrils were added, and the whole incubated (2 h, 37°C) in a shaking water bath. Cellular debris was sedimented by centrifugation (25,000 *g*, 2 min), and the resultant platelet-poor plasma subjected to radioimmunoassay to determine the concentration of TXB_2 and PGD_2, PGE_2, $PGF_{2\alpha}$.

Experimental protocol

In 8 dogs, cumulative intravenous doses of D1542 (range 0.1–100 μg kg^{-1}) were given. Heart rate, arterial blood pressure and coronary blood flow were recorded throughout and measurements made 10 min after each dose.

In 5 dogs the effects of infusions of adrenaline (range 0.2–20 μg kg^{-1} min^{-1}, i.v.) on CFRs were obtained in the absence and presence of cumulative doses of D1542 (range 1–1000 μg kg^{-1}, i.v.), followed by aspirin 5 mg kg^{-1}, i.v.

Drugs

Adrenaline bitartrate (Sigma, U.K.); acetylsalicyclic acid (Sigma, U.K.) and D1542 (ICI Pharmaceuticals) were used. All drugs were dissolved in sterile pyrogen free 0.85% sodium chloride solution.

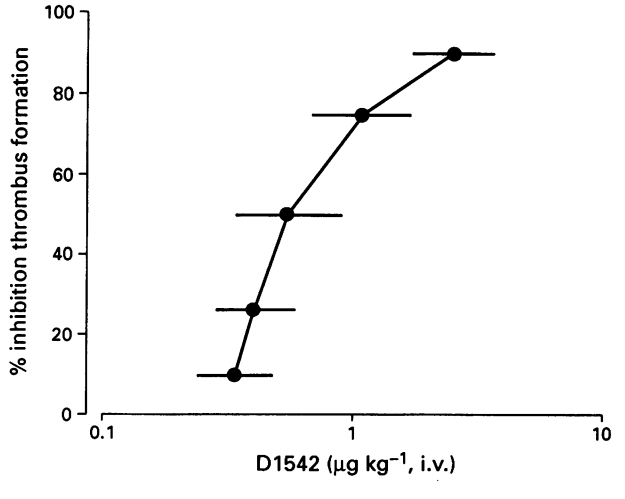


Figure 3 Mean dose (± s.e.mean)-response curve ($n = 8$) relating the % inhibition of the rate of thrombus formation to the dose of D1542 (μg kg^{-1}, i.v.). In each individual dog the dose required to give 20%, 30%, 50%, 70% and 90% was obtained by interpolation. The mean ED_{50} is 0.55 μg kg^{-1}, i.v. (95% CI 0.24–1.27). These initial effects of D1542 were obtained in the absence of an infusion of adrenaline.

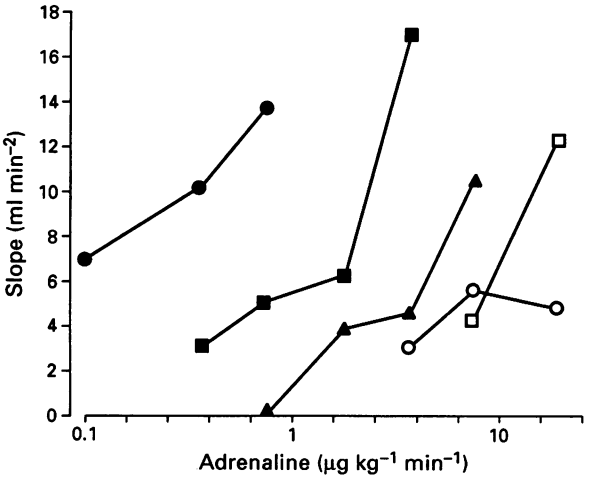


Figure 4 Dose-response curves obtained in one dog relating the dose of adrenaline (μg kg^{-1} min^{-1}, i.v.) to the rate of thrombus formation, as indicated by the rate of decline of coronary blood flow (slope, ml min^{-2}). Dose-response curves were obtained in the absence (●) and in the presence of increasing intravenous doses of D1542: (■) 5 μg kg^{-1}, (▲) 20 μg kg^{-1}, (○) 100 μg kg^{-1}, (□) 1000 μg kg^{-1}.

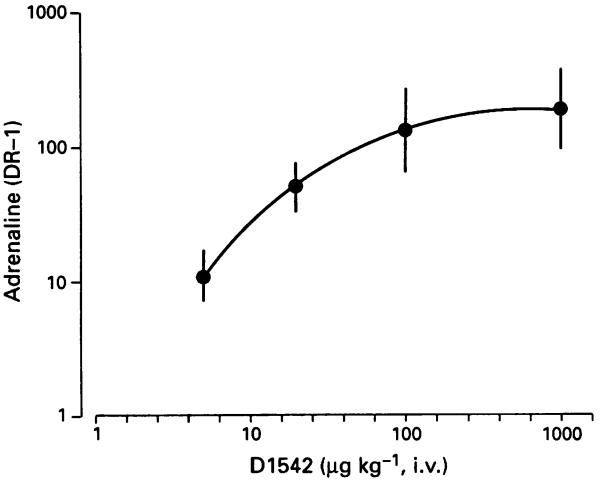


Figure 5 Mean inhibitory effect of D1542 ($n = 5$) on the ability of adrenaline to increase the rate of thrombus formation. The dose of D1542 is plotted against the dose-ratio (DR-1) increase in adrenaline required to restore thrombus formation to an equivalent rate. The maximum effective dose of D1542 required a 190 fold increase in the infusion rate of adrenaline to restore thrombus formation.

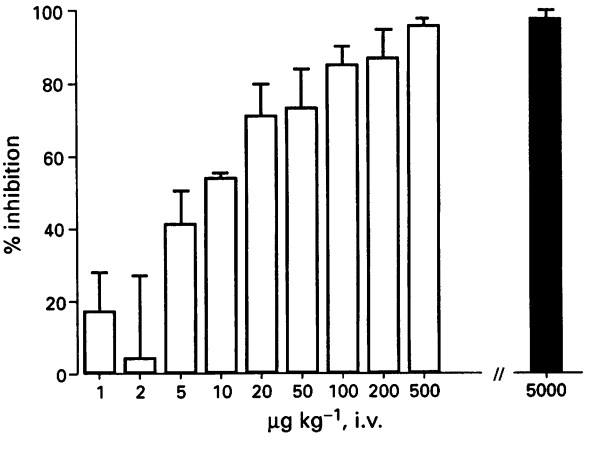


Figure 6 Mean effects (± s.e.mean, $n = 4$) of D1542 (open columns) on % inhibition of collagen-stimulated thromboxane B_2 (TXB_2) production, measured in whole blood *ex vivo*. TXB_2 production was 50% inhibited at a mean dose of 7.1 μg kg^{-1}, i.v. (95% CI 1.5–31). The inhibition of cyclo-oxygenase as aspirin (closed column) had no further effect on TXB_2 production.

Results

About 1 h after the completion of surgical procedures, reproducible records of CFRs were usually obtained, at which time heart rate, and mean systemic arterial blood pressure were respectively: 152 ± 25 beats min^{-1} (mean ± s.d.) and 121 ± 14 mmHg. The pH, P_{CO_2} and P_{O_2} of the arterial blood were respectively: 7.382 ± 0.03, 38.6 ± 4.0 mmHg and 229 ± 44 mmHg. The oesophageal temperature was 37.9 ± 0.3°C.

Records of coronary blood flow in one dog (Figure 2), show the effects of D1542 (20 μg kg^{-1}, i.v.), in the absence of an infusion of adrenaline. Thrombus formation was abolished. Dose-response curves were obtained in 8 dogs, and the mean dose-response curve is shown in Figure 3. In each dog complete elimination of CFRs was taken as 100% response. The mean ED_{50} for this initial effect of D1542, in the absence of an infusion of adrenaline, is 0.55 μg kg^{-1}, i.v. (95% confidence limits 0.24–1.27). The variation of the ED_{50} between individual dogs is large with an overall range from 0.1 to 5.5 μg kg^{-1}, i.v. The slope of the dose-response curve is unusually steep; from threshold to maximum effect takes place over a dose-range of less than one order of magnitude. D1542 had no effect upon heart rate or arterial blood pressure.

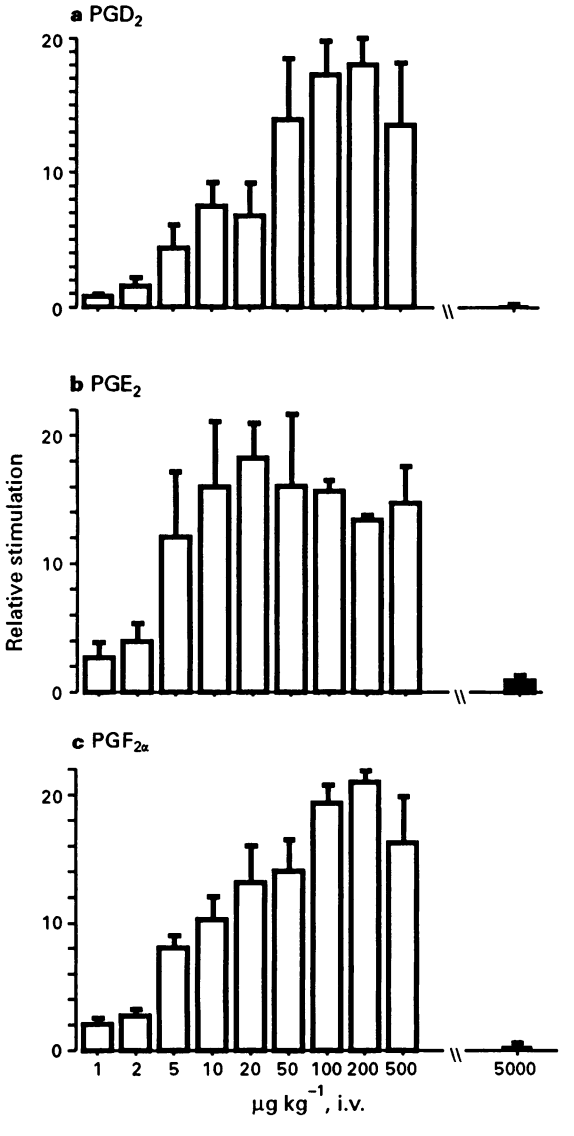


Figure 7 Mean effects (± s.e.mean, $n = 4$) of D1542 (open columns) on collagen-stimulated eicosanoid production, measured *ex vivo* in whole blood. (a) Prostaglandin D_2 (PGD_2), (b) PGE_2, (c) $PGF_{2\alpha}$. These increases in eicosanoids correspond to the inhibition of thromboxane B_2 (TXB_2) production, confirming redirection of arachidonic acid metabolism by inhibition of TXA_2 synthase, and are abolished by inhibition of cyclo-oxygenase by aspirin (closed columns).

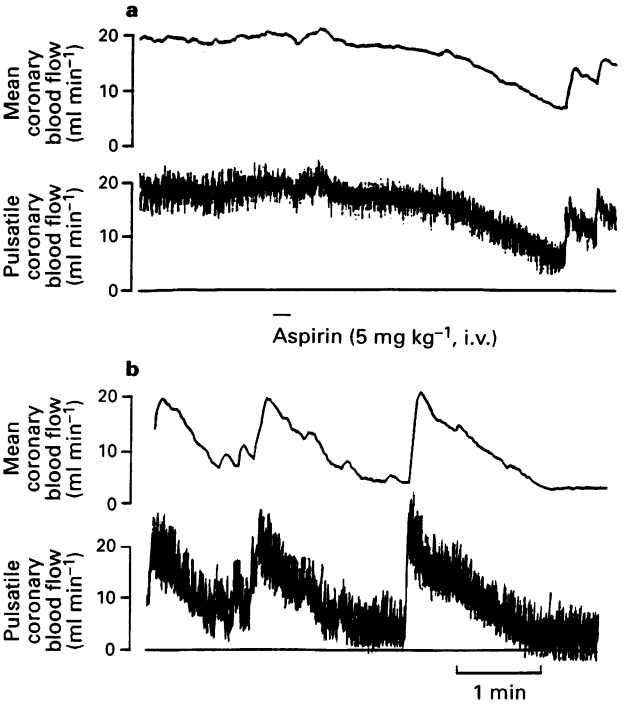


Figure 8 (a) Effects of aspirin (5 mg kg^{-1}, i.v.) on blood flow in the presence of D1542 (500 μg kg^{-1}, i.v.) and an infusion of adrenaline (1.9 μg kg^{-1} min^{-1}, i.v.). Within 2 min blood flow has begun to decline indicating thrombus formation. (b) Fifteen min after inhibition of cyclo-oxygenase by aspirin, a well defined pattern of cyclic flow reductions (CFRs) has been established.

The interaction between D1542 and adrenaline on thrombus formation was investigated by obtaining dose-response curves to adrenaline in the presence of increasing doses of D1542. Results obtained in one dog are shown in Figure 4. In the presence of D1542, increased amounts of adrenaline are required to restore equivalent rates of thrombus formation. In this experiment (Figure 4), at doses of D1542 above 100 μg kg^{-1}, i.v. the effects on the rightward shift of the adrenaline dose-response curve are reduced. The mean inhibitory effect of D1542 on the ability of adrenaline to increase the rate of thrombus formation in 5 dogs is shown in Figure 5. In each dog the dose-ratio (DR) of adrenaline at the same rate of thrombus formation was calculated and plotted as log(DR − 1) against the dose of D1542. All the slopes deviated from the theoretical slope of unity for competitive inhibition. There is a tendency for the inhibitory effects of D1542 to reach an upper limit at doses above 100 μg kg^{-1}, i.v., when the maximum dose-ratio shift observed was 190 (95%CI 380–96).

The effects of D1542 (dose range 1–1000 μg kg^{-1}, i.v.) on arachidonic acid metabolism in 4 dogs, measured *ex vivo* in collagen-activated platelets are shown in Figures 6 and 7. Thromboxane production measured as TXB_2, was 50% inhibited at a mean dose of 7.1 (95%CI 1.5–31) μg kg^{-1}, i.v. (Figure 6). There were corresponding increases in the production of PGE_2, $PGF_{2\alpha}$, and PGD_2 (Figure 7).

If the inhibitory effects of D1542 are in part because of the re-direction of arachidonic acid metabolism towards increased production of PGD_2 and PGI_2, then abolition of re-direction by inhibition of cyclo-oxygenase with aspirin (5 mg kg^{-1}, i.v.) should increase thrombus formation. Such an effect is seen in Figure 8, where, in the presence of an infusion of adrenaline (1.9 μg kg^{-1} min^{-1}, i.v.), D1542 has abolished thrombus formation. Within 1–2 min after the intravenous injection of aspirin (5 mg kg^{-1}, i.v.), thrombus

formation restarts and 15 min later a well defined pattern of CFRs is established. Similar results were obtained in 4 dogs and the corresponding effects on arachidonic acid metabolites are shown in Figures 6 and 7; inhibition of cyclo-oxygenase by aspirin maintains the abolition of TXB_2, but the increases in the metabolites of the endoperoxide, originally brought about by D1542, are now abolished.

Discussion

Activation of platelets by shear stress and exposure to a thrombogenic surface causes *de novo* synthesis and release from the platelet of the endoperoxide PGH_2 and TXA_2, metabolized from arachidonic acid via the enzymes cyclo-oxygenase and TXA_2 synthase. Both PGH_2 and TXA_2 act on the TP-receptor to cause further aggregation, and on smooth muscle to cause contraction. D1542 redirects arachidonic acid metabolism by inhibition of TXA_2 synthase resulting in an increase in PGH_2, which, in the platelet, degrades to PGD_2, and in the endothelium to PGI_2 (Gresele *et al.*, 1984). These latter two eicosanoids (PGD_2, PGI_2) inhibit platelet aggregation and relax smooth muscle. In addition D1542, by simultaneously blocking the TP-receptor, prevents the opposing aggregating and constrictor effects of PGH_2.

Watts *et al.* (1991) showed that inhibition of human platelet aggregation *ex vivo* by a combination of a TXA_2 synthase inhibitor (dazoxiben) and TP-receptor blocker (GR32191) was more effective than aspirin or either compound alone. Sheng-Kun *et al.* (1990) also showed that combined TXA_2 synthase inhibition and TP-receptor blockade was able to prevent thrombus formation in the dog coronary artery. We obtained similar results (McAuliffe *et al.*, 1992), in the dog, using dazoxiben and the TP-receptor antagonist ICI 192605 (Jessup *et al.*, 1988), in their presence PGD_2, a marker of re-direction was increased and its removal by the addition of aspirin increased the rate of thrombus formation.

Experiments carried out *in vitro* on human platelets showed that D1542 inhibited microsomal TXA_2 production (IC_{50} $0.018 \pm 0.003\ \mu M$, mean ± s.e.mean) and antagonized platelet aggregation by the TXA_2-mimetic U46619 (pA_2 8.43 ± 0.14, mean ± s.e.mean; Brownrigg *et al.*, 1992). Thus inhibition of TXA_2 synthase and antagonism of the TP-receptor occur within a fivefold concentration-range. Importantly there was no evidence of inhibition of cyclo-oxygenase by D1542 at a concentration of $10\ \mu M$. In the present series of experiments evidence of redirection was obtained by *ex vivo* measurement of arachidonic acid metabolites in collagen-activated platelets (Figure 7). The functional significance of this re-direction is illustrated by the ability of aspirin, through inhibition of cyclo-oxygenase, to restart thrombus formation. Thus removal of anti-aggregatory eicosanoids by aspirin results in an increase in the rate of thrombus formation.

During the initial period of these experiments, in anaesthetized dogs and before any infusion of adrenaline, it is apparent that prevention of thrombus formation by D1542 (ED_{50} $0.55\ \mu g\ kg^{-1}$, i.v.) occurs before complete inhibition of TXA_2 synthase (IC_{50} $7.1\ \mu g\ kg^{-1}$, i.v.), see Figures 2 and 7. This differential effect probably occurs because the initial stimulus for platelet aggregation in the dog is not maximal as opposed to stimulation *ex vivo* with collagen. Further the initial stimulus in the dog cannot be accurately quantitated. These two factors probably explain the steepness of the initial dose-response curve and the variability of the ED_{50} between dogs (CI $0.14-1.27\ \mu g\ kg^{-1}$).

Adrenaline is known to potentiate the ability of both TXA_2 and 5-hydroxytryptamine (5-HT) to aggregate platelets (Rao *et al.*, 1985; Cox *et al.*, 1991) and may also have a direct aggregating effect through an α_2-adrenoceptor (Grant & Scrutton, 1979). Therefore the relative effectiveness of inhibitors of platelet aggregation may be compared *in vivo* in terms of their ability to antagonize the aggregating effect of adrenaline (e.g. Figures 4 and 5). The relation between the dose-ratio of adrenaline (log DR − 1) and the dose of D1542 (log dose) is not linear and approaches a maximum value for the dose-ratio of adrenaline i.e. there is an upper limit to the effectiveness of D1542 which is indicated by the maximum dose-ratio of adrenaline (Figure 5). This result might be expected since adrenaline and D1542 are acting through different populations of receptors and even when the arachidonic acid pathway is completely inhibited, by aspirin or redirected by D1542, platelet aggregation is still able to take place via other agents (e.g. 5-HT), in response to a sufficiently strong stimulus. The maximum effect of D1542 occurred at a dose of $1\ mg\ kg^{-1}$, i.v., a dose that caused 95% inhibition of TXB_2 production and required a 190 fold increase in the rate of infusion of adrenaline to restore thrombus formation. In a similar series of experiments (Snow *et al.*, 1992), the $5\text{-}HT_2$ antagonist, ICI 170809, the TXA_2 receptor antagonist, ICI 192605, aspirin and dazoxiben all required significantly ($P<0.05$) smaller increases in adrenaline of 26, 40, 38 and 81 fold respectively to restore equivalent rates of thrombus formation.

In conclusion the above greater effectiveness of D1542 as an inhibitor of thrombus formation, coupled with the potential to redirect endoperoxide metabolism towards eicosanoids with the ability to cause vasodilatation and inhibition of smooth muscle cell proliferation, make D1542 a potentially useful drug in the treatment of both peripheral and coronary artery disease.

References

ANTI PLATELET TRIALLISTS' COLLABORATION (1988). Secondary prevention of vascular disease by prolonged antiplatelet treatment. *Br. Med. J.*, **296**, 320–331.

BROWNRIGG, N., GARCIA, R., JESSUP, R., LEE, V., TUNSTALL, S. & WAYNE, M. (1992). D1542; a potent thromboxane A_2 (TXA_2) synthase inhibitor and receptor antagonist. *Br. J. Pharmacol.*, (in press).

COX, B., MCAULIFFE, S.J.G., MOORS, J.A., NOBLE, M.I.M. & SNOW, H.M. (1991). The effects of adrenaline and serotonin on the rate of formation of platelet thrombi in a stenosed coronary artery of the anaesthetized dog. *J. Physiol.*, **438**, 169P.

FOLTS, J.D. (1982). Experimental arterial platelet thrombosis, platelet inhibitors, and their possible clinical relevance. *Cardiovasc. Rev. Rep.*, **3**, 370–382.

FOLTS, J.D., CROWELL, E.B. & ROWE, G.G. (1976). Platelet aggregation in partially obstructed vessels and its elimination with aspirin. *Circulation*, **54**, 365–370.

GRANT, J.A. & SCRUTTON, M.C. (1979). Novel alpha$_2$-adrenoceptors primarily responsible for inducing human platelet aggregation. *Nature*, **277**, 659–661.

GRESELE, P., VAN HOUTTE, P., ARNOUT, J., DECKMYN, H. & VERMYLEN, J. (1984). Thromboxane synthase inhibition combined with thromboxane receptor blockade: a step forward in antithrombotic strategy. *Thromb. Haemostas.*, **52**, 364.

JESSUP, C.L., JESSUP, R. & WAYNE, M. (1988). The effect of ICI 192605, a selective thromboxane A_2 receptor antagonist, on platelets. *Br. J. Pharmacol.*, **95**, 676P.

MCAULIFFE, S.J.G., SNOW, H.M., MOORS, J.A., WAYNE, M. & NOBLE, M.I.M. (1992). Inhibition of arachidonic acid metabolism by aspirin can promote thrombus formation in a stenosed coronary artery of the anaesthethized dog. *J. Physiol.*, **446**, 413P.

RAO, G.H.R., ESCOLAR, G. & WHITE, J.G. (1985). Epinephrine reverses the inhibitory influence of aspirin on platelet-vessel wall interactions. *Thrombosis Res.*, **44**, 65–74.

SHENG-KUN, YAO, ROSOLOWSY, M., ANDERSON, H.V., GOLINO, P., MCNATT, J.M., DE CLERCK, K., BUJA, L.M. & WILLERSON, J.T. (1990). Combined thromboxane A_2 synthase inhibition and receptor blockade are effective in preventing spontaneous and epinephrine induced canine coronary cyclic flow variations. *J. Am. Coll. Cardiol.*, **16**, 705–713.

SNOW, H.M., McAULIFFE, S.J.G., JESSUP, R., WAYNE, M. & NOBLE, M.I.M. (1992). Prevention of coronary thrombosis in the dog by inhibitors of platelet aggregation. *Br. J. Pharmacol.*, (in press).

THE RISC GROUP (1990). Risk of myocardial infarction and death during treatment with low dose aspirin and intravenous heparin in men with unstable coronary artery disease. *Lancet*, **336**, 827–830.

WATTS, I.S., WHARTON, K.A., WHITE, B.P. & LUMLEY, P. (1991). Thromboxane $(Tx)A_2$ receptor blockade and TxA_2 synthase inhibition alone and in combination: comparison of the anti-aggregatory efficacy in human platelets. *Br. J. Pharmacol.*, **102**, 497–505.

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The spinal antinociceptive activity of the α_2-adrenoceptor agonist, xylazine in sheep

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1 The intrathecal administration of xylazine (100 μg), via a chronic indwelling, cervical intrathecal catheter, produced a marked elevation of the mechanical nociceptive thresholds in the sheep. This antinociceptive effect was abolished by the prior intrathecal administration of the α_2-adrenoceptor antagonist, idazoxan.

2 The intrathecal administration of the selective α_2-antagonists, idazoxan (100 μg) and RX811059 (33 μg), significantly attenuated the antinociceptive activity of intravenous xylazine, with a 60–65% reduction in the area under the antinociceptive curve. The intrathecal administration of the antagonists alone had no significant effect on nociceptive thresholds.

3 Examination of the distribution of tritiated idazoxan (25 μCi in 100 μl) indicated that the site of action of the drug was limited to the cervical spinal cord after intrathecal administration.

4 These studies demonstrate that a significant proportion of the antinociceptive effect of systemically administered xylazine is mediated by spinal α_2-adrenoceptors.

Keywords: α_2-Adrenoceptors; antinociception; intrathecal; sheep; xylazine

Introduction

In 1904 Weber demonstrated that the application of adrenaline to the spinal cord attenuated the thermally-evoked withdrawal response in the cat. Some 70 years later, Paalzow (1974) showed that the systemic administration of the α_2-adrenoceptor agonist, clonidine, increased the vocalisation threshold produced by electrical stimulation of the tail in the rat, and Schmitt *et al.* (1974) reported that the α_2-agonists, xylazine and oxymetazoline, produced profound antinociception following intracerebroventricular administration.

A large number of reports have indicated that the spinal cord is an important site for the mediation of the antinociceptive effects of α_2-adrenoceptor agonists. Firstly, intrathecal studies have demonstrated that noradrenaline administration into the lumbar subarachnoid space of the rat produces dose-dependent antinociceptive activity in the tail flick, hot plate (Reddy & Yaksh, 1980) and tail pinch (Kuraishi *et al.*, 1979) tests. This antinociceptive effect was antagonized by the intrathecal administration of the α-adrenoceptor antagonists, phentolamine (Reddy & Yaksh, 1980) and phenoxybenzamine (Kuraishi *et al.*, 1979), but was unaffected by pre-treatment with the β-antagonist, propranolol. The intrathecal administration of the α_2-adrenoceptor agonists, clonidine, xylazine, guanfacine and dexmedetomidine, produced dose-dependent antinociception in the tail flick, hot plate and tail pinch tests in the rat (Post *et al.*, 1987; Ossipov *et al.*, 1988; Fisher *et al.*, 1991; Kalso *et al.*, 1991), and intrathecal clonidine and ST-91 elevated the shock titration threshold in the macaque monkey (Yaksh & Reddy, 1981).

Electrophysiological studies provide further evidence of the spinal antinociceptive effects of α_2-agonists. Exogenously applied noradrenaline and α_2-agonists produce selective suppression of spinal nociceptive neurones in the cat (Headley *et al.*, 1978; Murata *et al.*, 1989). In the rat, clonidine inhibited the neuronal responses in dorsal horn cells to both cutaneous (thermal) and visceral (colorectal distension) noxious stimuli (Ness & Gebhart, 1989) and reduced C fibre evoked activity (Sullivan *et al.*, 1987).

Finally, in the spinalized animal, the elimination of descending neuronal controls by spinal transection cranial to the region of the cord examined results in the removal of the supraspinal effects of a systemically administered analgesic drug and any remaining antinociceptive activity should represent a spinally or peripherally mediated effect. In spinalized rats, clonidine and xylazine produced a marked elevation of the tail flick latency (Zemlan *et al.*, 1979; 1980), and in mice, Spaulding *et al.* (1979) demonstrated that the antinociceptive activity of subcutaneously administered clonidine in the tail flick test was not significantly altered by spinal transection.

Although these studies demonstrate that α_2-adrenoceptor agonists can modify the perception of pain by an action at the spinal level, they do not indicate the relative importance of the spinal effects of the drug in the total antinociceptive actions of the systemically administered agent. After intrathecal administration, the local concentration of the drug is considerably higher than after systemic administration. In the spinalized animal, the antinociceptive activity could be mediated at either spinal or peripheral sites. In addition, spinal transection will remove tonic descending inhibition (Duggan & Morton, 1988) and could induce plastic changes. The observed effects may, therefore, not reflect the spinal actions of the drug in the intact animal. In order to assess the spinal contribution to the effects of a systemically administered α_2-agonist, we attempted to remove the spinal effect by the local, intrathecal administration of a specific antagonist. The difference between the antinociceptive activity of the systemically administered drug given alone, and following the intrathecal administration of the antagonist, should indicate the relative spinal contribution. In addition, the localization of the site of action of the antagonist was evaluated by assessing the distribution of radioactivity following the intrathecal administration of the radiolabelled antagonist.

These studies were undertaken in the sheep. In this species xylazine and clonidine have been shown to produce marked elevation of the nociceptive threshold responses after intravenous (Nolan *et al.*, 1987b), epidural (Eisenach *et al.*, 1987) and intrathecal (Waterman *et al.*, 1988) administration.

Methods

Ten adult, female Clun Forest, Swaledale and Swaledale cross sheep (weight range 45–72 kg) were used in this study.

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The sheep had chronic indwelling intrathecal catheters, terminating at the level of the fifth cervical vertebra, implanted under general anaesthesia (Kyles *et al.*, 1992). The sheep were housed in loose pens and fed hay and concentrates and were weighed prior to each experiment.

The sheep were placed individually in wooden crates (62 cm high × 120 cm long × 53 cm wide) and the skin on the cranial aspect of the antebrachium was clipped. The sheep were always kept in pairs since individual sheep became distressed when isolated from the flock, and they were allowed 20–30 min to become accustomed to the laboratory. In addition, the design of the nociceptive testing device (see below) allowed the operator to sit at least 2 metres from the sheep in order to minimize disturbance to the animals. Testing was carried out in a quiet environment at a constant ambient temperature of 64–68°F.

Drugs

Idazoxan and RX811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imadazoline) were kindly supplied by Reckitt and Colman Ltd and xylazine hydrochloride was supplied by Bayer UK Ltd. [^{3}H]-idazoxan (40 Ci mmol^{-1}) was supplied by Amersham International plc. All the drugs were prepared from the dry substance by dissolving them in either saline solution (0.9%) (for intravenous administration) or Ringer solution (for intrathecal administration). Drugs were administered intravenously to the sheep via a catheter inserted in the left jugular vein under local analgesia at least 30 minutes earlier. For intrathecal administration, the drugs were dissolved in a volume of 100 µl and flushed in with 350 µl of Ringer solution, the volume of the catheter.

Mechanical antinociceptive testing

Threshold responses to a mechanically applied stimulus were measured with a device based on that described by Nolan *et al.* (1987a) and modified to increase the accuracy of measurement of the force applied to the leg. The device was designed to push a blunt ended pin (2 mm diameter) against the cranial aspect of the antebrachium with increasing force and the threshold response was defined as the minimum force needed to elicit a clear raising of the forelimb. In order to prevent tissue damage, a maximum stimulus intensity of 18 Newtons was imposed. The pin was housed in a light-weight perspex box which was strapped to the sheep's forelimb and connected to a set of hand operated controls via a Bowden cable. The pin was attached to a spring which ensured its return to the housing when the hand controls were relaxed. The spring was also connected to a series of strain gauges which enabled the pressure exerted by the pin on the limb to be converted into an output in millivolts, displayed as a digital reading on the control box. The control box contained a 'sample and hold' circuit to record peak pressure readings and a discriminatory circuit which allowed the rapid change in pressure produced by the sheep raising its forelimb to be differentiated from the gradual increase in pressure produced prior to the response. The machine was calibrated using a beam type device which produced a read-out directly in Newtons; the leg unit was screwed firmly in position so that when the hand controls were squeezed, the pin pressed against a beam attached to a strain gauge bridge. The output of the strain gauge bridge was calibrated as a force in Newtons.

Protocol for antinociceptive testing

In all the experiments control readings were measured at 5 min intervals until at least four consistent threshold values were achieved before drug administration. An interval of at least seven days was allowed between successive experiments in each sheep to avoid the possibility of drug tolerance developing.

The operator was not 'blind' to the drug treatments which the animal was receiving.

Intrathecal xylazine studies (i) Intrathecal xylazine (total dose 100 µg) ($n = 6$): the mechanical thresholds were measured at 10 min intervals for 270 min after drug administration; (ii) intrathecal idazoxan (total dose 100 µg) administered 30 min before intrathecal xylazine (total dose 100 µg) ($n = 4$): the threshold responses were measured at 10 min intervals for 120 min after drug administration.

Intravenous xylazine studies The first requirement was to find a dose of intravenous xylazine which would elevate mechanical thresholds significantly, without exceeding the maximum cut-out level of 18 Newtons, for a reasonable time in each sheep. A dose of 25 µg kg^{-1} was arbitrarily chosen as the first dose to be tested in each sheep. Depending on the antinociceptive effect obtained at this dose, a lower or higher dose was tested subsequently. Thus, in each individual sheep, a number of different doses were evaluated until a suitable dose was found. The doses chosen for investigation were equally spaced on a logarithmic scale (12.5, 14.9, 17.7, 21.0, 25.0, 29.6, 35.0, 41.6 and 50.0 µg kg^{-1}).

The mechanical threshold responses were measured at 5 min intervals for 40 min after the administration of the drug or until they returned to pretest values, whichever was the longer. The area under the antinociceptive curve (AUC) was calculated by measuring the area under the time versus response curve above the pretest threshold baseline for the first 40 min after drug administration.

Intrathecal antagonism studies The dose of xylazine which was selected for the intrathecal antagonism experiments varied for each individual sheep (from 12.5 to 41.6 µg kg^{-1}), but the doses were equipotent, producing a marked, submaximal, antinociceptive effect and a similar AUC. The maximum threshold recorded in each individual sheep ranged from 13.9 to 17.9 Newtons. In each individual, the effects of the two treatments (intravenous xylazine alone and combined with an intrathecal antagonist) were always compared contemporaneously, usually within a 3–4 week period. If the interval was greater than 3 months, the initial treatment was repeated.

After control thresholds were measured, the antagonist was administered intrathecally and 30 min later, the intravenous dose of xylazine appropriate for that sheep was administered and the mechanical thresholds were measured at 5 min intervals for the next 40 min. The AUC was calculated as before. The following antagonists were used: (i) intrathecal idazoxan (total dose 100 µg) ($n = 5$); (ii) intrathecal RX811059 (total dose 33 µg) ($n = 5$).

Intrathecal antagonists The mechanical nociceptive thresholds were measured at 10 min intervals for 90 min after drug administration: (i) intrathecal idazoxan (total dose 100 µg) ($n = 5$); (ii) intrathecal RX811059 (total dose 33 µg) ($n = 5$).

Behavioural effects

The behavioural effects produced by the intravenous and intrathecal administration of xylazine, such as sedation, ataxia, stance and recumbency, were subjectively assessed during nociceptive testing. The effects of intrathecally administered antagonists alone, and on xylazine-induced behavioural changes were also examined.

Intrathecal [^{3}H]-idazoxan study

[^{3}H]-idazoxan (25 µCi) was diluted in Ringer solution to a total volume of 100 µl and administered intrathecally via an

indwelling catheter and flushed in with 350 μl of Ringer solution (the volume of the catheter). The animal was killed 30 min later by intravenous administration of pentobarbitone. The spinal cord was rapidly exposed and the position of the catheter tip was noted. The cord was divided into spinal segments (e.g. cervical (C1), cervical (C2), etc.), the nerve roots were cut and the dura mater carefully dissected off. The sections were washed three times in 50 mM Tris-HCl buffer (adjusted to pH 7.5), frozen on dry ice and stored at −70°C.

Tissue sections were cut from C1, C2, C3, C4, C5, C6, C7, C8, T1, T3, T5, T9, T13, L3 and S1 spinal segments. Three sections from each selected spinal segment were cut in the cryostat (2800 Frigocut, Reichert-Jung); each was thaw mounted onto a glass coverslip and placed in a separate scintillation vial. Each vial was filled with 20 ml of scintillation fluid (Emulsifier-safe, Packard), agitated with a vortex mixer and left for 24 h. Just before scintillation counting, the vials were vortexed again and the radioactivity present in the tissue sections was then counted by liquid scintillation spectrometry (Tri-carb Liquid Scintillation analyser, Packard). The scintillation counting was repeated after 24 h and the average radioactivity (d.p.m.) calculated.

Statistics

All the results presented in the text are expressed as the mean ± s.e.mean, unless one or more of the threshold responses of the sheep exceeded the maximal cut-out level of 18 Newtons. In the intrathecal antagonism studies, a three way analysis of variance was used to compared the effects of xylazine alone and in combination with each intrathecally administered antagonist separately. The data were first checked to ensure that they were normally distributed. The analysis of variance assessed the variation produced by each sheep, the drug treatment and time; by blocking the variation produced by each individual sheep, the effect of drug treatment and time course were evaluated. In addition, the paired Student's *t* test was used to compare the area under the antinociceptive curves for each treatment. Finally, the differences in the threshold responses at each time point were analysed by the paired Students *t* test.

Results

Intrathecal xylazine studies

The intrathecal administration of xylazine (100 μg) produced a marked elevation of the mechanical nociceptive thresholds from control readings of 1.5 ± 0.4 Newtons (Figure 1). The mechanical thresholds reached the maximum cut-out level in five of the six sheep 20 min after drug administration and remained at or near this level for 160 min after administration, whereupon they gradually declined towards baseline levels. The thresholds were still elevated at the end of the experiment, 270 min after drug administration, and one or more of the sheep failed to respond to the nociceptive stimulus below the cut-out level, at all the time points between 10 and 260 min.

The prior intrathecal administration of idazoxan (100 μg) completely abolished the antinociceptive activity of intrathecal xylazine, with no obvious change in the threshold responses after xylazine administration (Figure 1).

Intravenous xylazine studies

The dose-response relationship for the antinociceptive effects of intravenously administered xylazine differed markedly between individual sheep. At the initial dose of xylazine tested (25 μg kg^{-1}) there was marked variation in the elevation of the mechanical thresholds and in the area under the antinociceptive curve (AUC), which ranged from 60–18624 arbitrary units (Figure 2). At this dose, in three out of the eight sheep, thresholds exceeded the maximum cut-out level, while one individual demonstrated no change in the threshold responses. Hence, the dose of intravenous xylazine that produced a submaximal elevation of the threshold responses and roughly similar AUC varied between individual sheep and ranged from 12.5 to 41.6 μg kg^{-1} (Table 1). The doses used for the subsequent intrathecal antagonism studies were therefore evaluated separately for each individual sheep.

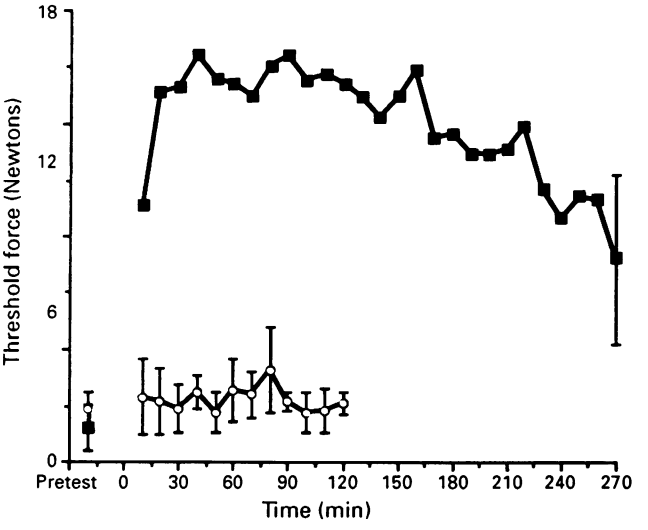


Figure 1 The effect of intrathecal administration of 100 μg xylazine (■) alone ($n = 6$) and 30 min after the intrathecal administration of 100 μg idazoxan (○) ($n = 4$) on the mechanical nociceptive thresholds. Intrathecal xylazine was administered at time = 0 min. The mechanical threshold responses (Newtons) are plotted against time (min) and each point represents the mean ± s.e.mean (vertical bars), unless the maximal threshold response was reached in one or more of the sheep at that time point, in which case only the mean is shown.

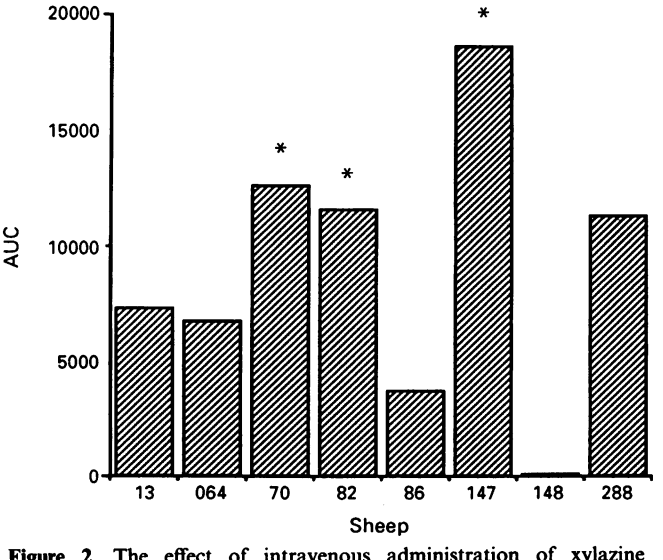


Figure 2 The effect of intravenous administration of xylazine (25 μg kg^{-1}) on the area under the antinociceptive curve. The area under the curve (arbitrary units) is plotted for each sheep tested. (* the maximal cut-out level of 18 Newtons was reached during nociceptive testing and, thus, the AUC will be underestimated).

Table 1 The doses of intravenous xylazine ($\mu g\ kg^{-1}$) selected for each individual sheep for the intrathecal antagonism experiments

Sheep number	*Breed*	*Weight* (kg)	*Dose* ($\mu g\ kg$)	*Area under the antinociceptive curve* (arbitrary units)
6	SwX	61	12.5	7861
13	SwX	61	25.0	7328
16	SwX	69	17.7	6313
064	CF	72	25.0	6734
70	Sw	45	17.7	9532
82	Sw	45	17.7	11010
86	Sw	47	25.0	3709
147	SwX	54	12.5	6278
148	SwX	66	41.6	7456
288	CF	64	21.8	6635
Mean ± s.e.mean		58.4 ± 3.2	21.6 ± 2.7	7286 ± 621

(Sw – Swaledale, SwX – Swaledale cross Leicester & CF – Clun Forest)

Intrathecal antagonism studies

(i) *Idazoxan* the antinociceptive effects of intravenous xylazine were significantly attenuated by the prior intrathecal administration of idazoxan (100 μg) ($P<0.01$) (Figure 3). The AUC for xylazine was significantly attenuated ($P<0.01$), with a reduction of 65.4 ± 5.6%. The intrathecal administration of idazoxan significantly reduced the mechanical thresholds for 20 min after intravenous xylazine.

(ii) *RX811059* The intrathecal administration of RX811059 produced a significant attenuation of the antinociceptive effects of intravenously administered xylazine ($P<0.01$) (Figure 4). The AUC for xylazine was significantly attenuated ($P<0.01$), with a reduction of 61.0 ± 6.0%. RX811059 significantly reduced the mechanical nociceptive thresholds for 10 min after xylazine administration.

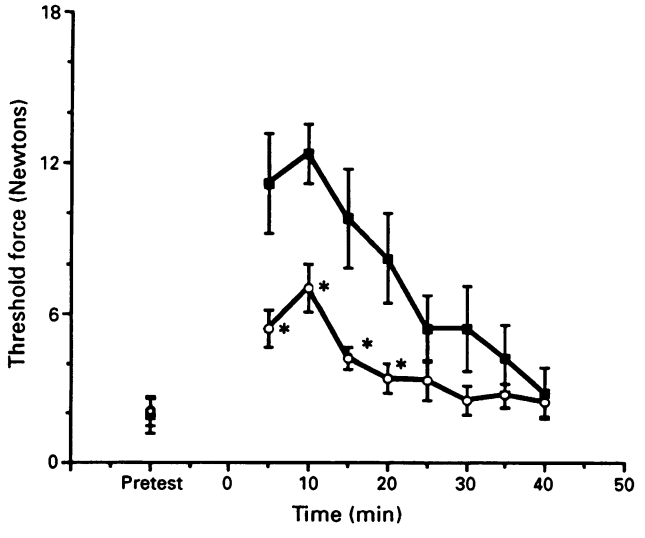


Figure 3 The effect of intravenous administration of xylazine (12.5–41.6 $\mu g\ kg^{-1}$) alone (■) and 30 min after the intrathecal administration of 100 μg idazoxan (○) on the mechanical threshold responses. Intravenous xylazine was administered at time = 0 min. The threshold response (Newtons) is plotted against time (min) and each time point represents the mean ± s.e.mean (vertical bars) for five sheep. Significant *($P<0.05$) reduction in the elevation of the mechanical threshold responses produced by intravenous xylazine after the intrathecal administration of idazoxan, compared to intravenous xylazine alone, determined by the paired Student's t test.

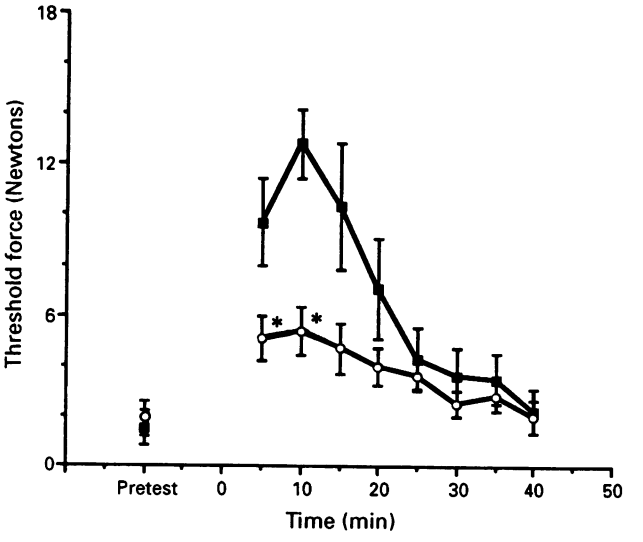


Figure 4 The effect of intravenous administration of xylazine (12.5–41.6 $\mu g\ kg^{-1}$) alone (■) and 30 min after the intrathecal administration of RX811059 (○) on the mechanical threshold responses. Intravenous xylazine was administered at time = 0 min. The threshold response (Newtons) is plotted against time (min) and each time point represents the mean ± s.e.mean (vertical bars) for five sheep. Significant *($P<0.05$) reduction in the elevation of the mechanical threshold responses produced by intravenous xylazine after the intrathecal administration of idazoxan, compared to intravenous xylazine alone, determined by the paired Student's t test.

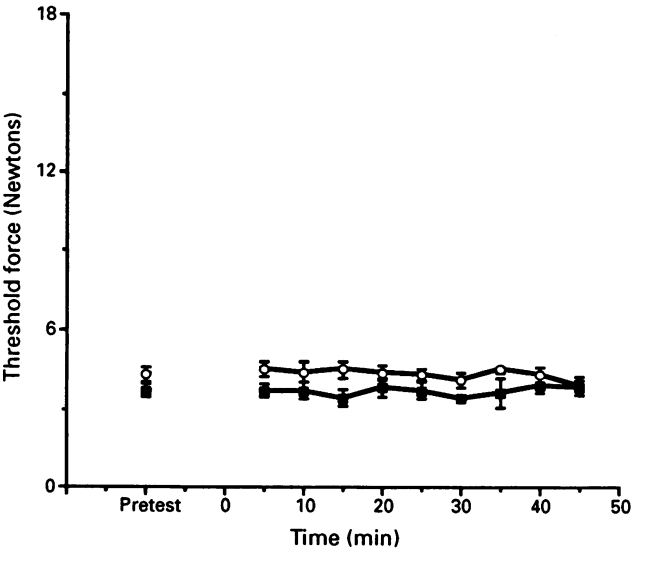


Figure 5 The effect of intrathecal administration of the antagonists idazoxan (○) and RX811059 (■) on mechanical threshold responses. The drug was administered at time = 0 min. The threshold response (Newtons) is plotted against time (min) and each time point represents the mean ± s.e.mean (vertical bars) for five sheep.

Intrathecal antagonists

The intrathecal administration of the antagonists idazoxan (100 μg) and RX811059 (33 μg) alone produced no significant alteration in the mechanical nociceptive thresholds of the sheep; the threshold responses remained close to control values (Figure 5). The control thresholds were higher in these studies (4.3 ± 0.3 Newtons for intrathecal idazoxan and 3.7 ± 0.2 Newtons for intrathecal RX811059), which were

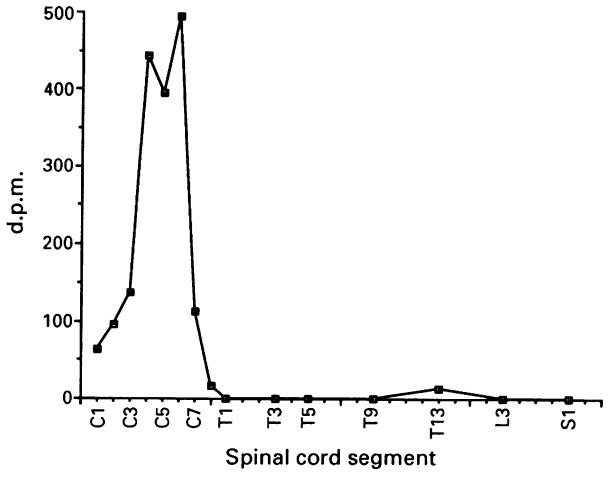


Figure 6 The distribution of radioactivity (d.p.m.) in individual spinal cord slices (25 μm thick) following the intrathecal administration of [^{3}H]-idazoxan (25 μCi) via an intrathecal catheter which terminates at the C5/C6 intervertebral disc space.

undertaken some months after the intrathecal antagonism experiments were completed. This difference in the control levels between the two studies probably reflects changes in the hair and skin thickness in the individual sheep used.

Behavioural effects

The intravenous administration of xylazine produced dose-dependent sedation in each individual sheep, with obvious drooping of the head and ears and reduced responsiveness to external stimuli. Subjective assessment suggested that the dose required to produce a similar degree of sedation varied markedly between sheep. A short period of sternal recumbency within a few minutes of drug administration, was often noted at high doses.

The intrathecal administration of xylazine produced no obvious behavioural changes. The intrathecal administration of idazoxan and RX811059 produced no behavioural effects when administered alone and had no moderating influence on the behavioural changes mediated by intravenous xylazine.

Intrathecal [^{3}H]-idazoxan study

The intrathecal administration of [^{3}H]-idazoxan (25 μCi) resulted in a peak radioactivity count of 495 d.p.m. at the level of the sixth cervical spinal segment (Figure 6), which corresponded to the position of the catheter tip at the level of the intervertebral disc space between C5 and C6. The levels of radioactivity dropped dramatically both cranially (forward from C4) and caudally (behind C6), so that the distribution of the radioactive drug was concentrated in the 5–6 vertebral segments around the catheter tip.

Discussion

Xylazine has been in veterinary use as a sedative for over twenty years, it is used in a wide variety of species, but is of particular value in the chemical restraint of ruminants (Clarke & Hall, 1969). Evaluation of its analgesic efficacy, therefore, has considerable clinical relevance.

The intrathecal administration of xylazine (100 μg) produced a marked, prolonged elevation of the mechanical nociceptive thresholds in the sheep, which was completely abolished by the prior intrathecal administration of idazoxan (100 μg). This indicates that the antinociceptive actions of intrathecal xylazine are probably mediated by α_2-adrenoceptors. Similarly, Waterman *et al.* (1988) reported a dose-dependent increase in the mechanical threshold responses with intrathecally administered xylazine (5–50 μg) which was completely antagonized by intravenously administered idazoxan (100 μg kg^{-1}).

The *in vivo* studies with tritiated idazoxan injected in a total volume of 100 μl, via chronic indwelling intrathecal catheters, showed that the spread of idazoxan after this route of administration was limited to 2–3 vertebral segments on either side of the catheter tip. This indicates that the effects of intrathecally administered idazoxan are mediated at the spinal level and, since intrathecal idazoxan completely abolished the antinociceptive activity of intrathecal xylazine, this suggests that the antinociceptive effects of intrathecal xylazine are mediated entirely at the spinal level (at least when the volume of injection is limited to 100 μl and the catheter tip is located at the level of the fifth cervical vertebra). The lack of behavioural changes following the intrathecal administration of xylazine would also tend to support the conclusion that there was no detectable redistribution of xylazine to supraspinal structures and that the antinociception is mediated spinally. It is interesting to note that intrathecally applied xylazine had a slower onset of action, but a much longer duration of effect than when it was given intravenously: a maximum degree of antinociception being achieved after 30 min (i.t.) versus 10 min (i.v.), and lasting for 270 min intrathecally, but only 40 min intravenously. These differences associated with the route of drug administration may well reflect the relative protection from hepatometabolism afforded by the intrathecal route. Livingston *et al.* (1992) noted a similar difference with clonidine, depending on its route of administration.

There was a consistent dose-response relationship for the antinociceptive actions of intravenous xylazine for each individual sheep, and when specific doses were repeated in an individual animal, the elevation of their mechanical thresholds and AUC were similar each time. However, the dose of xylazine needed to produce a marked, but submaximal elevation in the mechanical nociceptive thresholds (for the subsequent intrathecal antagonism studies) varied markedly between individual sheep. In addition, the sedative effects of a given dose of xylazine varied between sheep. Raptopoulos and Weaver (1984) noted that the sedative effects of intravenous xylazine in steers varied in different breeds of cattle, with Herefords being more sensitive than Friesians to the effects of the drug. Similarly, Ley *et al.* (1990) demonstrated that the antinociceptive effects of intravenous xylazine (50 μg kg^{-1}) varied with the breed of the sheep tested, with a greater analgesic response being observed in Clun Forest ewes compared to either Swaledale or Welsh Mountain sheep. In this study, Clun Forest, Swaledale and Swaledale cross Leicester ewes were represented. Although the numbers involved are too few for meaningful statistical analysis to be possible, there was no obvious correlation between the breed of sheep and the dose selected. The individual variation probably reflects intrinsic factors which alter each animal's inherent sensitivity to α_2-adrenoceptor agonists, such as differences in drug metabolism and excretion and the subtypes, number and distribution of α_2-adrenoceptors. In contrast, the intrathecal administration of xylazine produced a relatively consistent antinociceptive effect in all the sheep tested.

Despite the variation in the intravenous dose of xylazine used in each sheep, the analgesic activity of xylazine given alone, and in combination with an intrathecally administered antagonist, was directly compared in each individual animal. Hence, each sheep acted as its own control and thus, by using paired data, the inherent difficulties in analysing the effects of the intrathecal antagonists on the individually variable analgesic activity of intravenous xylazine were overcome.

The intrathecal administration of idazoxan significantly attenuated, but did not abolish, the antinociceptive effect of

intravenous xylazine. Intrathecally injected idazoxan completely abolished the analgesic effects of intrathecally administered xylazine and the distribution of idazoxan after this route of administration was limited to the spinal cord. Hence, it is reasonable to assume that this dose of idazoxan should also antagonize the spinal component of the antinociceptive effects of intravenous xylazine in the forelimbs, without producing significant attenuation of supraspinally mediated drug effects. Intrathecally administered idazoxan reduced the AUC for intravenous xylazine by 65%, indicating that a substantial proportion of the antinociceptive activity of intravenous xylazine is mediated at the spinal level.

Idazoxan was originally considered to be a selective α_2-adrenoceptor antagonist, with low affinity for α_1-adrenoceptors (Doxey *et al.*, 1985). However, recent evidence has shown that a number of α_2-adrenoceptor ligands which contain an imidazoline structure, including idazoxan, also bind to sites which are not competed for by catecholamines, indicating that they are not adrenoceptors (Michel & Insel, 1989). At least three non-adrenoceptor imidazoline binding sites have been described (Michel & Insel, 1989), including the non-adrenoceptor idazoxan binding site (NAIBS), which has been demonstrated in the brain (Mallard *et al.*, 1991) and spinal cord (Tracey & Livingston, 1991) of the rat and the *para*-aminoclonidine (PAC) binding site which has been shown to mediate the anti-hypertensive actions of clonidine in the ventro-lateral medulla (Ernsberger *et al.*, 1988; Tibirica *et al.*, 1991) which were previously assumed to be produced by a α_2-adrenoceptor mediated effect. Hence, the actions of imidazoline α_2-adrenoceptor ligands could be associated with either an action at α_2-adrenoceptors or at imidazoline preferring sites. In order to investigate the relative importance of these two sites in mediating the attenuation of the spinal activity of xylazine, the effects of intrathecal administration of the imidazoline α_2-adrenoceptor antagonist, RX811059, which has low affinity at NAIB sites (Mallard *et al.*, 1991) were also evaluated.

The intrathecal administration of RX811059 produced a marked attenuation of the antinociceptive activity of intravenous xylazine and reduced the AUC by 61%, similar to the reduction observed with intrathecal idazoxan (65%). This suggests that the spinal antinociceptive actions of xylazine and the modifying effects of intrathecal idazoxan on xylazine mediated analgesia were mediated at α_2-adrenoceptors.

The intrathecal administration of idazoxan or RX811059 did not abolish the antinociceptive actions of intravenous xylazine completely, although intrathecal idazoxan did antagonize the spinal analgesia mediated by intrathecal xylazine totally. Hence, part of the antinociceptive effect of intravenous xylazine is not mediated at the spinal level. Although a minor peripheral antinociceptive effect against prostaglandin-induced pain in acute inflammation has been suggested (Ferreria, 1980), most of the residual, non-spinal analgesic effect is probably mediated at the supraspinal level. The intracerebroventricular administration of α_2-agonists has been shown to produce antinociception in the mouse and rat (Schmitt *et al.*, 1974; Lipman & Spencer, 1979) indicating that supraspinal structures may well be involved in mediating the antinociceptive effects of systemically administered α_2-adrenoceptor agonists. Fujimoto & Arts (1990) have suggested that the supraspinal injection of clonidine in mice activates two descending spinal systems, an antanalgesic system involving the release of dynorphin and a second analgesic pathway. However, the existence or importance of a descending supraspinally mediated antanalgesic effect of α_2-agonists has not been demonstrated in other species.

The intrathecal administration of the α_2-adrenoceptor antagonists, idazoxan and RX811059, produced no significant alteration in mechanical nociceptive thresholds in the sheep. In contrast, the intrathecal administration of the α_2-antagonist yohimbine in the rat has been reported to produce a dose-dependent decrease in nociceptive thresholds (Sagen & Proudfit, 1984), and the depletion of spinal noradrenaline by the intrathecal administration of 6-hydroxydopamine produced a decrease in thermal nociceptive thresholds (Howe & Yaksh, 1982). Both of these reports are consistent with the proposal that there are tonically active, bulbospinal, noradrenergic pathways which inhibit spinal nociceptive processing. However, in the cat there is little evidence that tonic descending inhibition involves the release of noradrenaline; noradrenaline depleted cats showed no reduction in tonic descending inhibition and intravenous idazoxan had no effect on tonic inhibition of spinal nociceptive transmission in the anaesthetized cat (Duggan & Morton, 1988). The failure of intrathecal α_2-antagonists to produce hyperalgesia in the sheep may indicate either that spinal α_2-adrenoceptors are not involved in descending inhibition in this species or may reflect the insensitivity of the mechanical nociceptive testing system used to detect decreases in the threshold responses below normal control values. The pretest mechanical thresholds were reasonably high during these experiments, so that a decrease in threshold responses could potentially have occurred, but the mechanical thresholds remained close to control levels in all the sheep after intrathecal idazoxan or RX811059. In addition, significant hyperalgesia was demonstrated with the mechanical leg device following the intrathecal administration of a high dose of naloxone (5 mg) in the sheep (Kyles *et al.*, 1990). Thus, the lack of hyperalgesia with intrathecally administered α_2-antagonists is probably a genuine indication that spinal α_2-adrenoceptors are not involved in descending inhibition in the sheep.

These results indicate that a substantial proportion of the antinociceptive activity of intravenously administered xylazine in the conscious, unrestrained sheep are mediated by spinal α_2-adrenoceptors.

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References

CLARKE, K.W. & HALE, L.W. (1969). Xylazine – a new sedative for horses and cattle. *Vet. Rec.*, **85**, 512–517.

DOXEY, J.C., LANE, A.C., ROACH, A.G., SMITH, C.F.C. & WALTERS, D.S. (1985). Selective α_2 adrenoceptor agonists and antagonists. In *Pharmacology of Adrenoceptors*. ed. Szabadi, E., Bradshaw, C.M. & Nahoishi, S.R. pp. 13–22. New York: MacMillan Press.

DUGGAN, A.W. & MORTON, C.R. (1988). Tonic descending inhibition and spinal nociceptive transmission. *Progr. Brain Res.*, **77**, 193–207.

EISENACH, J.C., DEWAN, D.M., ROSE, J.C. & ANGELO, J.M. (1987). Epidural clonidine produces antinociception but not hypotension in sheep. *Anesthesiology*, **66**, 496–501.

ERNSBERGER, P., GINLIANO, R., WILLETTE, R.N., GRANATA, A.R. & REISS, R.J. (1988). Hypotensive action of clonidine analogues correlates with binding affinity of imidazole and not α_2 adrenergic receptors. *J. Hypertens.*, **6**, S554–S557.

FERREIRA, S.H. (1980). Peripheral analgesia: mechanism of analgesic action of aspirin-like drugs and opiate-antagonists. *Br. J. Clin. Pharmacol.*, **10**, 237s.

FISHER, B., ZORNOW, M.H., YAKSH, T.L. & PETERSON, B.M. (1991). Antinociceptive properties of dexmedetomidine in rats. *Eur. J. Pharmacol.*, **192**, 211–225.

FUJIMOTO, J.M. & ARTS, K.M. (1990). Clonidine, administered intracerebroventricularly in mice produces an anti-analgesic effect which may be mediated spinally by dynorphin A (1-17). *Neuropharmacol.*, **29**, 351–358.

HEADLEY, P.M., DUGGAN, A.W. & GRIERSMITH, B.T. (1987). Selective reduction by noradrenaline and 5-HT of nociceptive responses of cat dorsal horn neurones. *Brain Res.*, **145**, 185–189.

HOWE, J.R. & YAKSH, T.L. (1982). Changes in the sensitivity to intrathecal norepinephrine and serotonin after 6-hydroxydopamine (60HDA), 5,6-dihydroxytryptamine (5,6DHT) or repeated monoamine administration. *J. Pharmacol. Exp. Ther.*, **220,** 311–321.

KALSO, E.A., POYLIA, R. & ROSENBERG, P.H. (1991). Spinal antinociception by dexmedetomidine, a highly selective α_2 adrenergic agonist. *Pharmacol. Toxicol.*, **68,** 140–143.

KURAISHI, Y., HARADA, Y. & TAKAGI, H. (1979). Noradrenaline regulation of pain transmission in the spinal cord mediated by alpha adrenoceptors. *Brain Res.*, **174,** 333–336.

KYLES, A.E., WATERMAN, A.E. & LIVINGSTON, A. (1990). Analgesic effects of low-dose intrathecal naloxone in the sheep. In *New Leads in Opioid Research*, ed. Van Ree, J., Mulder, A.H., Wiegant, V.N. & Van Wimersma Greidanus, T.B. pp. 52–54. Amsterdam: Excerpta Medica.

KYLES, A.E., WATERMAN, A.E. & LIVINGSTON, A. (1992). Chronic intrathecal catheterization of sheep. *J. Pharmacol. Methods*, **27,** 177–183.

LEY, S., WATERMAN, A. & LIVINGSTON, A. (1990). Variation in the analgesic effects of xylazine in different breeds of sheep. *Vet. Rec.*, **126,** 508.

LIPMAN, J.J. & SPENCER, P.S.J. (1979). Further evidence for a central site of action for the antinociceptive effect of clonidine-like drugs. *Neuropharmacol.*, **18,** 731–733.

LIVINGSTON, A., WATERMAN, A.E., BOUCHENFA, O. & KYLES, A.E. (1992). Antinociceptive effects of intrathecal opioids and alpha $_2$ agonists in the sheep. In *Animal Pain*. ed. Short, C.E. & Van Poznak, A. pp. 281–290. New York: Churchill Livingstone.

MALLARD, N.J., TGACKE, R., HUDSON, A.L. & NUTT, D.J. (1991). Comparative binding studies of [^{3}H] idazoxan and [^{3}H] RX821001 in the rat brain. *Br. J. Pharmacol.*, **101,** 221P.

MICHEL, M.C. & INSEL, P.A. (1989). Are there multiple imidazoline binding sites? *Trends Pharmacol. Sci.*, **10,** 342–344.

MURATA, K., NAKAGAWA, I., KUMETA, Y., KITAHATA, L.M. & COLLINS, J.G. (1989). Intrathecal clonidine suppresses noxiously evoked activity of spinal wide dynamic range neurons in cats. *Anesth. Analg.*, **69,** 185–191.

NESS, T.J. & GEBHART, G.F. (1989). Differential effects of morphine and clonidine on the visceral and cutaneous spinal nociceptive transmission in the rat. *J. Neurophysiol.*, **62,** 220–230.

NOLAN, A., LIVINGSTON, A., MORRIS, R. & WATERMAN, A. (1987a). Techniques for comparison of thermal and mechanical nociceptive stimuli in sheep. *J. Pharmacol. Methods*, **17,** 39–49.

NOLAN, A., LIVINGSTON, A. & WATERMAN, A.E. (1987b). Antinociceptive actions of intravenous alpha$_2$ adrenoceptor agonists in sheep. *J. Vet. Pharmacol. Ther.*, **10,** 202–209.

OSSIPOV, M.H., SUAREZ, L.H. & SPAULDING, T.C. (1988). A comparison of the antinociceptive and behavioural effects of intrathecally administered opiates, α-2-adrenergic agonists and local anaesthetics in mice and rats. *Anesth. Analg.*, **67,** 616–624.

PAALZOW, L. (1974). Analgesia produced by clonidine in mice and rats. *J. Pharm. Pharmacol.*, **26,** 361–363.

POST, C., GORDH, T. Jr., MINOR, B.G., ARCHER, T. & FREEMAN, J. (1987). Antinociceptive effects and spinal cord tissue concentrations after intrathecal injections of guanfacine or clonidine into rats. *Anesth. Analg.*, **66,** 317–324.

RAPTOPOULOS, D. & WEAVER, B.M.Q. (1984). Observations following intravenous xylazine administration to steers. *Vet. Rec.*, **114,** 567–569.

REDDY, S.V.R. & YAKSH, T.L. (1980). Spinal noradrenergic terminal system mediates antinociception. *Brain Res.*, **189,** 391–401.

SAGEN, J. & PROUDFIT, J.K. (1984). Effect of intrathecally administered noradrenergic antagonists on nociception in the rat. *Brain Res.*, **310,** 295–301.

SCHMITT, H., LEDOUAREC, J.C. & PETILLOT, N. (1974). Antinociceptive effect of some α-sympathomimetic agents. *Neuropharmacol.*, **13,** 289–294.

SPAULDING, T.C., VENAFRO, J.J., MA, M.G. & FIELDING, S. (1979). The dissociation of the antinociceptive effect of clonidine from supraspinal structures. *Neuropharmacol.*, **18,** 103–105.

SULLIVAN, A.F., DASHWOOD, M.R. & DICKENSON, A.H. (1987). α_2 adrenoceptor modulation of nociception in the rat spinal cord: location, effects and interactions with morphine. *Eur. J. Pharmacol.*, **128,** 169–177.

TIBIRICA, E., FELDMAN, J., MERMET, C., GONON, F. & BOUSQUET, P. (1991). An imidazoline-specific mechanism for the hypotensive effect of clonidine: a study with yohimbine and idazoxan. *J. Pharmacol. Exp. Ther.*, **256,** 606–613.

TRACEY, H. & LIVINGSTON, A. (1991). [^{3}H]-idazoxan binding to α_2 adrenoceptors and 'non-adrenergic idazoxan binding sites' (NAIBS) in the rat spinal cord. *Br. J. Pharmacol.*, **104,** 352P.

WATERMAN, A., LIVINGSTON, A. & BOUCHENFA, O. (1988). Analgesic effects of intrathecally applied α_2 adrenoceptor agonists in conscious, unrestrained sheep. *Neuropharmacol.*, **27,** 213–216.

WEBER, H. (1904). Uber Anasthesie durch Adrenalin. *Verh. Dtsch. Ges. Med.*, **21,** 616–619.

YAKSH, T.L. & REDDY, S.V.R. (1981). Studies in the primate on the analgesic effects associated with intrathecal actions of opiates, adrenergic agonists and baclofen. *Anesthesiology*, **54,** 451–467.

ZEMLAN, F.R., CORRIGEN, S.A. & PFAFF, D.W. (1980). Noradrenergic and serotonergic mediation of spinal analgesic mechanisms. *Eur. J. Pharmacol.*, **61,** 111–124.

ZEMLAN, F.R., KOW, L.M. & PFAFF, D.W. (1979). Effect of noradrenergic and serotonergic agonists and antagonists on nociceptive and non-nociceptive reflexes in the rad. *Fed. Proc.*, **37,** 323.

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Degradation of acetylcholine in human airways: role of butyrylcholinesterase

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1 Neostigmine and BW284C51 induced concentration-dependent contractions in human isolated bronchial preparations whereas tetraisopropylpyrophosphoramide (iso-OMPA) was inactive on airway resting tone.

2 Neostigmine (0.1 μM) or iso-OMPA (100 μM) increased acetylcholine sensitivity in human isolated bronchial preparations but did not alter methacholine or carbachol concentration-effect curves.

3 In the presence of iso-OMPA (10 μM) the bronchial rings were more sensitive to neostigmine. The pD_2 values were, control: 6.05 ± 0.15 and treated: 6.91 ± 0.14.

4 Neostigmine or iso-OMPA retarded the degradation of acetylcholine when this substrate was exogenously added to human isolated airways. A marked reduction of acetylcholine degradation was observed in the presence of both inhibitors. Exogenous butyrylcholine degradation was prevented by iso-OMPA (10 μM) but not by neostigmine (0.1 μM).

5 These results suggest the presence of butyrylcholinesterase activity in human bronchial muscle and this enzyme may co-regulate the degradation of acetylcholine in this tissue.

Keywords: Human bronchus; butyrylcholinesterase; acetylcholinesterase; acetylcholine; butyrylcholine; tetraisopropylpyrophosphoramide; neostigmine

Introduction

The inherent tone observed in human airways is maintained by the local actions of neuronal inputs derived from the parasympathetic nervous system. This system regulates airway calibre by activation of the post-synaptic muscarinic sites on airway smooth muscle via the release of the neurotransmitter, acetylcholine (ACh). Enzymatic degradation of ACh occurs through the action of cholinesterases. Two different cholinesterases have been described in many tissues (Mann, 1971; Aas *et al.*, 1986; Chatonnet & Lockridge, 1989; Small *et al.*, 1990) namely, acetylcholinesterase (AChE: EC 3.1.1.7) and butyrylcholinesterase (BChE: EC 3.1.1.8). Previous investigators (Mittag *et al.*, 1971; Small *et al.*, 1990; Adler *et al.*, 1991) have shown that BChE represents a considerable fraction of the cholinesterase pool in smooth muscle. While the function of AChE at the cholinergic neuronal synapse in airway muscles is to regulate the duration of action of ACh (Adler & Filbert, 1990), the physiological role of BChE has not been systematically examined in human airways. *In vivo*, treatment with cholinesterase inhibitors leads to an increase in resistance to airflow in animals (Pauluhn *et al.*, 1987) and in man (Haber *et al.*, 1987). Recently, Adler and co-workers (1991) proposed that BChE may participate in the co-regulation of the duration of action of ACh in dog airways *in vitro*. These investigators demonstrated that the contractions induced by electrical field stimulation were enhanced and prolonged in tissues treated with the specific BChE inhibitor, tetraisopropylpyrophosphoramide (iso-OMPA; Atack *et al.*, 1989). While Ito and co-workers (1989) have demonstrated that physostigmine, a non-specific cholinesterase inhibitor, enhanced the response to electrical field stimulation in human isolated bronchial muscles, few studies have been performed to examine the relative contribution of these two enzymes on the functional response of human airways.

The aim of this study was to determine the sensitivity of human bronchial preparations to exogenous and endogenous ACh in the presence of specific cholinesterase inhibitors. A measurement of the degradation of exogenous ACh or butyrylcholine (BuCh: specific substrate for BChE) were also performed in order to compare the activity of AChE and BChE in human isolated bronchial preparations. These studies were undertaken to test the hypothesis that BChE may be a co-regulator of ACh activity in human airways.

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Methods

Isolated preparations

Human lung tissue was obtained from 34 male and 6 female patients who had undergone surgery for lung carcinoma. The mean age was 56.20 ± 1.15 years. After the resection of a lung or a lobe, parts of the bronchus were dissected free from parenchymal lung tissue and placed in Tyrode solution at 4°C for 12 h. All experiments were performed on 161 subsegmental bronchial preparations derived from 40 lung samples (n); these bronchial ring preparations were of 2–4 mm internal diameter and weighed 63.09 ± 2.84 mg. Values are means ± s.e.mean.

Physiological studies (contractions)

Bronchial ring preparations were set up in the 10 ml organ baths containing Tyrode solution (concentration mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4, 0.4 and glucose 5.5; pH 7.4; gassed with 95% O_2/5% CO_2 and maintained at 37°C. The preparations were placed under initial loads (2–3 g) depending on their size.

These loads ensured that responses to contractile agonists were maximal. Isometric force displacement transducers (Narco F-60) and physiographs (Linseis) were used to record the changes in force. The tissues were allowed to equilibrate for 90 min and the bath fluid was exchanged every 15 min with fresh Tyrode solution.

After this period, all bronchial preparations were contracted with ACh (100 μM). The tissues were washed with

fresh Tyrode solution and allowed to return passively to their resting tone. When resting tone was established, two different protocols were followed. In the first protocol, each cholinesterase inhibitor (neostigmine, BW284C51 or iso-OMPA) was added to the bath fluid at 10 min intervals in a cumulative fashion, beginning with the lowest concentration. In the second protocol, the preparations were incubated for 30 min in Tyrode solution containing either neostigmine or iso-OMPA, and subsequently a cholinoceptor agonist-response relationship was determined. Cumulative concentration-effect curves to neostigmine were also produced in the tissues after a 30 min incubation with iso-OMPA. Atropine 1 μM was added 30 min before or at the end of the cholinesterase inhibitors concentration-effect curves.

Biochemical studies (enzyme activities)

Bronchial rings were placed in 10 ml tubes containing 5 ml Tyrode solution gassed with 95% O_2/5% CO_2 and maintained at 37°C. After a 60 min equilibration period, the preparations were pre-incubated for 30 min in Tyrode solution or Tyrode solution containing cholinesterase inhibitors (neostigmine or iso-OMPA) at different concentrations. ACh or BuCh (0.5 μmol) was then added to each tube: 1 h and 3 h after this substrate challenge, a 30 μl sample of supernatant was collected and stored at −20°C. The cholinesterase inhibitors were always present in appropriate tubes during the 3 h following the pre-incubation period. Measurements of choline, ACh and BuCh in the supernatants were performed according to the technique of Israël & Lesbats (1985). This assay involved hydrolysis of ACh by AChE to give choline, which was oxidized to betaine by choline oxidase with H_2O_2 production. Using this technique for measurement of BuCh, the AChE was markedly less efficient in degrading this substrate. In the presence of luminol, H_2O_2 and horseradish peroxidase (HRP), light emission was generated and recorded by a photomultiplier (1250 LKB-Wallac) connected to a potentiometric recorder (2210-032 LKB-Wallac). The assay was carried out in a microcuvette containing 250 μl of phosphate buffer (Na_2HPO_4/NaH_2PO_4 0.2 M; pH 8.6) and 10 μl of a mixture containing choline oxidase (62.5 u ml^{-1}), horseradish peroxidase (0.5 g l^{-1}) and luminol (0.5 mM). The baseline level of luminescence was obtained using these enzymes and buffer. When the samples or choline standards (10 μl) were added to this mixture a peak of luminescence was induced. After the baseline had been re-established, 10 μl of purified AChE (less than 250 u ml^{-1}) was added to determine the ACh or BuCh present in samples or standards. Standards were made up daily, and were tested for each lung sample; the relationship between peak luminometer deflection and amount of added standard in the assay system was linear over the range (4 pmol–2 nmol). Sample values were derived from standard curves. In each lung sample experiment, stock solutions of ACh or BuCh were prepared twice: initially for the tissue incubations in the tubes and again for standards in the chemiluminescence technique.

Data analysis

Physiological studies (contractions) The changes in force were measured from isometric recordings and expressed in grams (g) or as a percentage of the initial contraction induced by ACh (100 μM). The maximal response (E_{max}) was induced with a cholinoceptor agonist, neostigmine or BW284 C51 and the EC_{50} values were interpolated from the individual concentration-effect curves. The EC_{50} values were transformed into pD_2 values, that is, negative logarithms of EC_{50} values.

Biochemical studies (enzyme activities) The amounts of ACh or BuCh remaining in the supernatants after 1 or 3 h as well as the quantities obtained after treatment of tissues with enzyme inhibitors were expressed in μmol. The protection of ACh or BuCh degradation by the different inhibitors was calculated from the following formula: $100 \times (S_c - S_b)/(S_a - S_b)$ where S_a = 0.5 μmol of ACh or BuCh, S_b = the quantity (μmol) of ACh or BuCh remaining after 3 h of incubation with Tyrode solution, S_c = μmol of ACh or BuCh remaining after 3 h of incubation with a cholinesterase inhibitor.

All results are expressed as means ± s.e.mean. Statistical analysis was performed using Multirange ANOVA followed by a post-hoc test (Least Significant Difference) with a confidence level of 95% and taking into account the preparations derived from the same or different lung samples.

Drugs

The drugs and their sources were: acetylcholine chloride, butyrylcholine chloride, choline chloride (three times crystallized), methacholine chloride (acetyl-β-methylcholine chloride), carbachol (carbamylcholine chloride), atropine sulphate, iso-OMPA (tetraisopropylpyrophosphoramide), BW284C51 (1, 5-*bis* (4-allyldimethylammoniumphenyl) pentane-3-one dibromide), Trizma buffer solution [Tris(hydroxymethyl) aminomethane], choline oxidase from *Alcaligenes* species, peroxidase HRP II (Horseradish peroxidase type II) (Sigma Chemical Co., St. Louis, MO, U.S.A.). Neostigmine methylsulphate (Prostigmine; Roche, 92521 Neuilly sur Seine, France). Luminol (5-amino-1,2,3,4 tetrahydrophtalazindion-1,4) (Merck, Schuchardt 8011 Hohenbrunn bei München, Germany). Acetylcholinesterase from *Electrophorus electricus* was obtained from Boehringer Mannheim GmbH (Germany) and purified by passing through a Sephadex G50 coarse column (Pharmacia, Uppsala, Sweden).

All cholinoceptor agonists and BW284C51 were dissolved in Tyrode solution and enzymes were dissolved in distilled water. Iso-OMPA (0.1 M) was dissolved in 100% ethanol. Subsequent dilutions of each drug and neostigmine were made in Tyrode solution. A stock solution of luminol (1 mM) was prepared by dissolving 18 mg in a few drops of 1 M NaOH, and the volume was adjusted to 100 ml with 0.2 M Tris buffer (final pH of 8.6).

Results

Physiological studies (contractions)

The data presented in Figure 1 demonstrate that neostigmine produced concentration-dependent contractions in human isolated bronchial preparations whereas iso-OMPA did not provoke contractions under similar experimental conditions. The pD_2 values for neostigmine and BW284C51 on human bronchial preparations were: 6.46 ± 0.23 and 6.73 ± 0.22, respectively, while the maximal effect of neostigmine was two fold greater than that of BW284C51. These data were obtained in paired preparations derived from 4 lung samples. The neostigmine and BW284C51 responses were completely reversed or inhibited by atropine (1 μM). Pretreatment of human bronchial muscles with neostigmine (0.1 μM; 30 min; Figure 2a and Table 1) or iso-OMPA (100 μM; 30 min; Figure 2b and Table 1) significantly shifted to the left the ACh curves in these preparations without modifying the reactivity (E_{max}). In contrast, these inhibitors at the same concentrations failed to alter the methacholine or carbachol concentration-effect curves (Table 1). Iso-OMPA (10 μM) did not shift ACh curves (Table 1).

The concentration-effect curves obtained with neostigmine were shifted to the left when the bronchial preparations were pretreated with iso-OMPA (30 min; 10 μM; Figure 3 and Table 1).

Biochemical studies (enzyme activities)

Three hours after the addition of exogenous ACh (0.5 μmol) or BuCh (0.5 μmol) to human isolated bronchial ring preparations, a marked reduction in the quantities of these substrates

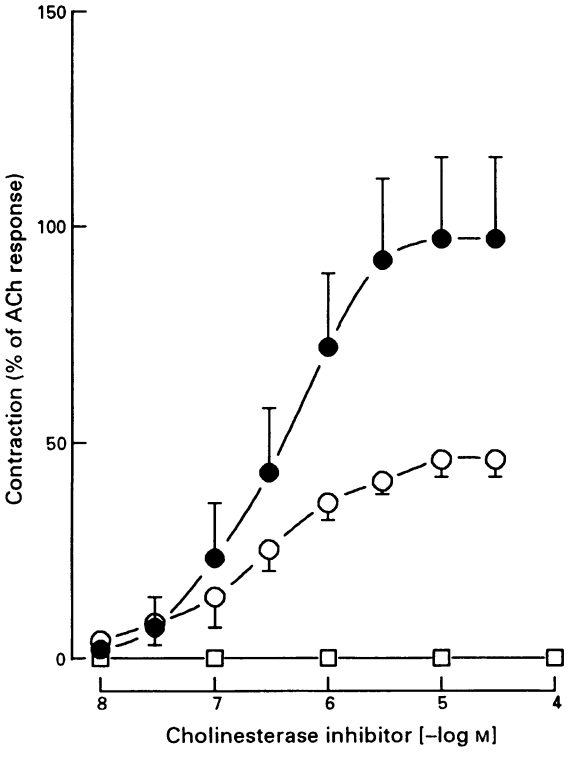


Figure 1 Effect of cholinesterase inhibitors on basal tone in human isolated bronchial preparations. Cumulative concentration-effect curves were constructed with neostigmine (●; $n = 4$), BW284C51 (○, $n = 4$) or tetraisopropylpyrophosphoramide (iso-OMPA, □; $n = 5$). Each response was expressed as a percentage of the acetylcholine (ACh, 100 μM) contraction induced prior to the cumulative-curve. Values are means with s.e.mean shown by vertical bars.

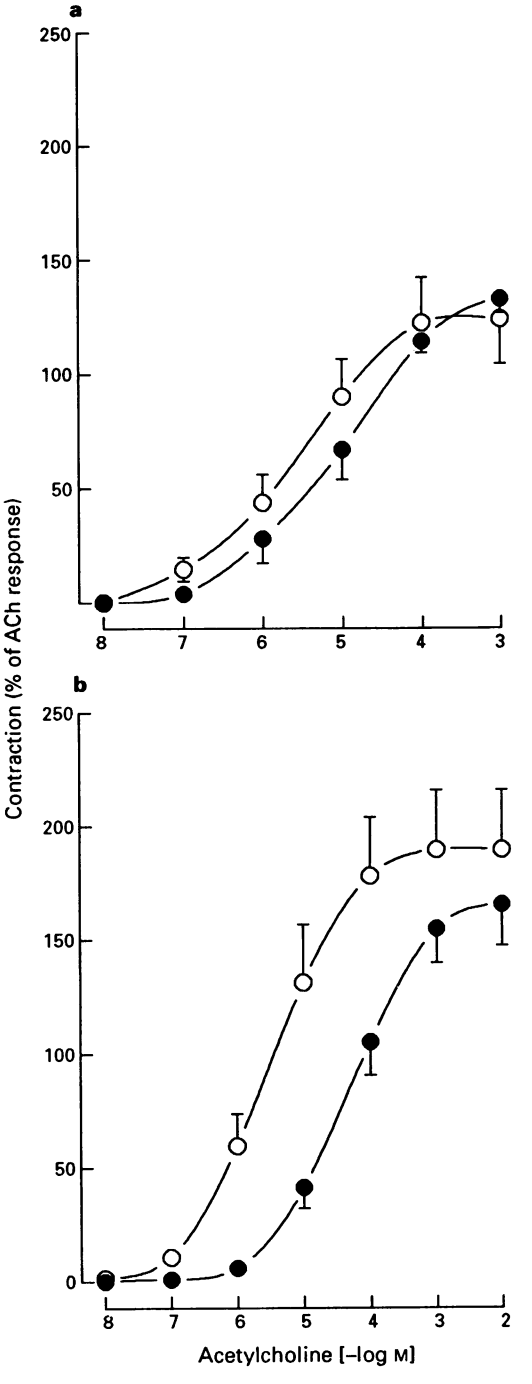


Figure 2 Acetylcholine (ACh) concentration-effect curves produced in human isolated bronchial preparations after 30 min incubation with Tyrode solution (●) or cholinesterase inhibitors (○). (a) Neostigmine (0.1 μM; $n = 6$) (b) tetraisopropylpyrophosphoramide (iso-OMPA, 100 μM; $n = 4$). Each response was expressed as a percentage of the ACh (100 μM) contraction induced prior to the incubation. Values are means ± s.e.mean from paired lung samples.

was observed: ACh (0.10 ± 0.04 μmol; $n = 7$) and BuCh (0.05 ± 0.03 μmol; $n = 4$). This reduction was also detectable at 1 h: ACh (0.25 ± 0.07 μmol; $n = 7$) and BuCh (0.17 ± 0.06 μmol; $n = 4$).

In paired preparations which had been pretreated for 30 min with neostigmine (0.1 μM), prior to the addition of ACh, or in tissues treated with iso-OMPA (10 μM), before the BuCh challenge, a small or no reduction in the quantities of these substrates was detected. Thus at these concentrations, the enzyme inhibitors prevented the hydrolysis of the appropriate substrate (Table 2). In experiments with BuCh, this substrate was degraded even if tissues were treated with neostigmine (Figure 4 and Table 2). The protection of ACh or BuCh by each inhibitor at the concentrations used and neostigmine 10 μM are shown in Table 2. The ACh remaining at 1 h after treatment with neostigmine (0.1 μM) or iso-OMPA (100 μM) were: 0.49 ± 0.05 μmol and 0.50 ± 0.06 μmol, respectively. However, in tissues treated with both neostigmine (0.1 μM) and iso-OMPA (100 μM), the quantity of ACh detected was 0.59 ± 0.05 μmol ($n = 7$ paired lung samples). This quantity was significantly greater than those detected when tissues were treated with the individual inhibitors. The ACh results obtained after 3 h with the inhibitors were: 0.32 ± 0.06 μmol and 0.43 ± 0.06 μmol for neostigmine (0.1 μM) and iso-OMPA (100 μM), respectively. The ACh remaining after 3 h in tissues treated with a combination of both inhibitors at these same concentrations was: 0.51 ± 0.05 μmol. These latter data were greater than results obtained in supernatants in the presence of one inhibitor. When lower concentrations of these enzyme inhibitors were used under the same experimental conditions, a more pronounced alteration was observed, that is, both inhibitors in combination significantly protected ACh against degradation at 1 h (Figure 5a) and at 3 h (Figure 5b).

Choline measurements for each of the preceding experiments were exactly the reverse of all measurements of ACh or BuCh. The choline content increased in supernatants when degradation of ACh or BuCh occurred specifically when tissues were not exposed to cholinesterase inhibitors (data not shown).

Table 1 Effects of cholinesterase inhibitors on acetylcholine, carbachol, methacholine and neostigmine concentration-effect curves in human isolated bronchial preparations

Treatment	n	*ACh* (100 μM) *response* (g)	*Agonist* E_{max} (g)	pD_2 *value*
			Acetylcholine	
Tyrode	17	1.74 ± 0.29	2.42 ± 0.36	4.70 ± 0.36
Iso-OMPA (10 μM)	5	1.42 ± 0.28	2.30 ± 0.55	4.91 ± 0.12
Iso-OMPA (100 μM)	6	1.78 ± 0.48	3.14 ± 0.69	5.57 ± 0.26*
Neostigmine (0.1 μM)	6	2.30 ± 0.56	2.90 ± 0.84	5.68 ± 0.20*
			Carbachol	
Tyrode	4	1.86 ± 0.50	2.88 ± 0.74	6.34 ± 0.03
Iso-OMPA (100 μM)	4	1.90 ± 0.38	2.65 ± 0.55	6.50 ± 0.12
			Methacholine	
Tyrode	3	0.95 ± 0.40	1.22 ± 0.43	6.76 ± 0.03
Neostigmine (0.1 μM)	3	1.47 ± 0.53	1.63 ± 0.49	6.60 ± 0.26
			Neostigmine	
Tyrode	5	1.46 ± 0.38	1.42 ± 0.27	6.05 ± 0.15
Iso-OMPA (10 μM)	5	1.77 ± 0.27	2.41 ± 0.18	6.91 ± 0.14*

Acetylcholine (ACh, 100 μM) response was obtained prior to treatment. Tissues were exposed to Tyrode solution or to Tyrode solution containing cholinesterase inhibitors (30 min). E_{max} indicates the maximal contraction produced by the agonist expressed in grams (g). Values are means ± s.e.mean, *n* indicates the number of lung samples.
*Values significantly different when results from control and treated tissues were compared ($P<0.05$, ANOVA).

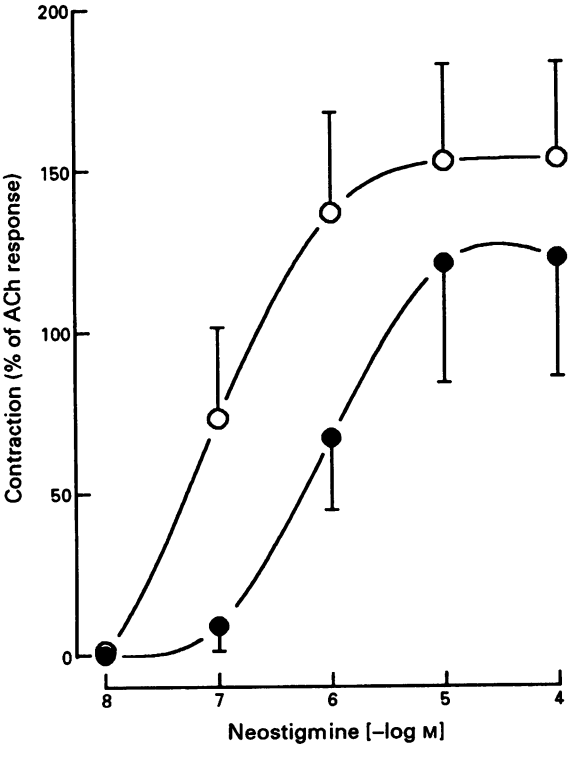


Figure 3 Effect of tetraisopropylpyrophosphoramide (iso-OMPA) on neostigmine concentration-effect curves in human isolated bronchial preparations. Data are derived from curves produced after a 30 min incubation with Tyrode solution (●) or Tyrode solution containing iso-OMPA (10 μM; ○). Each response was expressed as a percentage of the acetylcholine (ACh, 100 μM) contraction induced prior to the incubation. Values are means ± s.e.mean from paired lung samples. Preparations were derived from 5 lungs.

Table 2 Protection against the degradation of acetylcholine (ACh) and butyrylcholine (BuCh) in human isolated bronchial preparations

	Neostigmine (μM) 0.01	0.1	10	*Iso-OMPA* (μM) 10	100
Substrate					
ACh	21 ± 16 (4)	66 ± 17 (7)	102 ± 13 (4)	19 ± 05 (4)	96 ± 22 (7)
BuCh	3 ± 10 (3)	25 ± 04 (4)	52 ± 06 (3)	94 ± 09 (4)	106 ± 10 (7)

Results are expressed as percent protection against the degradation of ACh or BuCh. Protection was evaluated 3 h after the addition of 0.5 μmol of substrate. Values are means with s.e.mean. Number of lung samples used is indicated in parentheses.

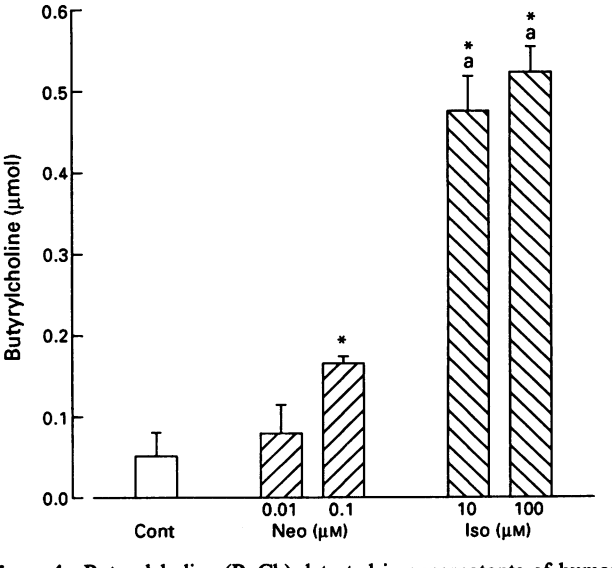


Figure 4 Butyrylcholine (BuCh) detected in supernatants of human isolated bronchial preparations after 3 h incubation with Tyrode solution (Cont) or Tyrode solution containing neostigmine (Neo) or tetraisopropylpyrophosphoramide (Iso). The quantity of BuCh initially added (time zero) was 0.5 μmol. Values are means ± s.e.mean of tissues derived from 3–4 lung samples. Values significantly different from (*) control data or (a) neostigmine (0.1 μM) results ($P<0.05$, ANOVA).

Discussion

The results presented in this paper demonstrate that two functional cholinesterase enzyme pools are present in human bronchial muscle preparations. Hydrolysis of ACh is achieved by the action of endogenous AChE and is inhibited by neostigmine or BW284C51 (Atack *et al.*, 1989; this paper). In addition, BuCh degradation occurs via BChE (Mittag *et al.*, 1971), an activity which is prevented by pretreatment of the tissues

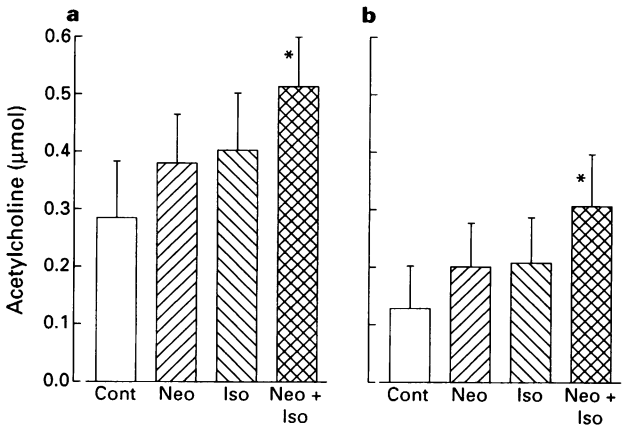


Figure 5 Acetylcholine (ACh) detected in supernatants of human isolated bronchial preparations after 1 h (a) or 3 h incubation (b) in Tyrode solution (Cont) or Tyrode solution containing: neostigmine (Neo; 0.01 μM); tetraisopropylpyrophosphoramide (Iso; 10 μM) or a combination of both inhibitors at these concentrations (Neo + Iso). The quantity of ACh initially added (time zero) was 0.5 μmol. Values shown are means ± s.e.mean from paired lung samples. Preparations were derived from 4 lungs. (*) Values significantly different when compared with the three other values ($P<0.05$, ANOVA).

with iso-OMPA (Austin & Berry, 1953; Thomsen *et al.*, 1991; this paper). In bronchial preparations, the responses induced with BW284C51 were approximately one half of those responses observed with neostigmine (Figure 1). These results support the anti-muscarinic effect of BW284C51 as described by Ambache & Lessin (1955) and Adler *et al.* (1991). Therefore, we used neostigmine as the AChE inhibitor in all other protocols. The data obtained in human airways (this paper) are similar to those results derived from canine tracheal smooth muscle preparations (Adler & Filbert, 1990; Adler *et al.*, 1991). These functional studies support the histochemical findings in which both cholinesterases have been detected in respiratory smooth muscles from a variety of animal species (Mann, 1971; Small *et al.*, 1990) including man (Partanen *et al.*, 1982).

Initial studies dealing with the effects of AChE activity in airway preparations have shown that inhibition of this enzyme by agents such as physostigmine and neostigmine increases the contractile response to exogenous ACh in airway preparations (Douglas, 1951; De Candole *et al.*, 1953; Daly, 1957; Carlyle, 1963) or to electrical field stimulation (Kirkpatrick & Rooney, 1982; Ito *et al.*, 1989). However, none of these reports distinguished between the relative contributions of the different cholinesterases involved in the contractions to cholinoceptor agonists in airway muscles. Recently, Adler and co-workers (1991) demonstrated that both AChE and BChE play a role in the co-regulation of canine airways response to electrical field stimulation. These observations are supported by the results obtained in human airways (this paper), where the role of BChE in the ACh contraction can be observed in tissues which were challenged with increasing concentrations of neostigmine in the presence of iso-OMPA (see Figure 3). These functional data (contractions) are supported by the biochemical measurements in bronchial tissues treated with exogenous ACh in the presence of iso-OMPA (see Figure 5).

While the activities of both enzymes were detected in canine and human isolated airways, few reports have described the distribution of these enzymes in respiratory tissues. The suggestion that these enzymes were distributed differently in different tissues, was based on an early report (Koelle & Koelle, 1959) which showed that AChE in autonomic ganglia of the cat was confined to the neural elements, whereas BChE was restricted to the glial cells. Appleyard & Smith (1989) showed that BChE was present in ileal smooth muscle, whereas AChE was detected in nerves of Auerbach's plexus. Recent evidence derived from the guinea-pig trachea (Small *et al.*, 1990) indicated that BChE was mainly detected in smooth muscle cells while AChE was found in nerve fibres running within the smooth muscle. These data suggest a tissue distribution for the cholinesterases with AChE exhibiting a close association with neuronal elements, while BChE has an extra-neuronal location. Our physiological results (Figure 1) are in agreement with these reports. Muscarinic M_3-receptors are uniformly distributed on smooth muscle cells of human distal bronchi (Mak & Barnes, 1990). A dense nerve supply has been reported throughout the smooth muscle in human airways (Partanen *et al.*, 1982; Daniel *et al.*, 1986). These reports and our physiological results suggest that cholinergic nerve endings are co-localized with AChE, but not with BChE. Excess ACh (present in tissues treated with neostigmine) may lead to a diffusion of this neurotransmitter from the parasympathetic varicosities to the smooth muscle regions where hydrolysis may occur via BChE. This may explain the increased sensitivity to neostigmine which was observed in human airways in the presence of iso-OMPA. These data suggest that neurotransmitter hydrolysis may be controlled by a dual enzymatic process: the primary regulatory, namely AChE, responsible for the local neuronal synaptic regulation of ACh and BChE a secondary system, to protect against an increased release of ACh or against extra-neuronal appearance of ACh.

The results derived from human bronchial preparations, confirm previously published data in animal tissues concerning the different substrates for cholinesterases (Mittag *et al.*, 1971; Adler & Filbert, 1990). ACh is degraded by both AChE and BChE while BuCh is principally hydrolysed by BChE (Table 2). In some protocols the supernatant samples obtained following cholinesterase inhibitor treatments contained slightly higher quantities of the exogenously added substrates (0.5 μmol ACh or BuCh). This may have been due to protection of the endogenous production of ACh in presence of cholinesterases inhibitors.

Kirkpatrick & Rooney (1982) have suggested that in bovine tracheal muscle, neostigmine may act not only by preventing endogenous ACh degradation but also by stimulating release of endogenous ACh from nerve terminals. This notion was supported by the potentiation of maximal contractions to histamine, carbachol and acetylcholine in the bovine tracheal muscle. However, the sensitivity to these agents were unaffected. In contrast, the results presented here demonstrate that a higher concentration of neostigmine shifted the ACh concentration-effect curves to the left, while the methacholine curves were unaltered.

Iso-OMPA (10 μM) was a selective inhibitor of BChE. However, a higher concentration of iso-OMPA (100 μM) appeared not to be selective, since subsequent to 3 h of incubation, the protection against ACh enzymatic degradation was maximal (see Table 2). However, treatment of tissues for a shorter period (30 min or 1 h) with iso-OMPA (100 μM) selectively inhibited BChE, and these results from human airways were similar to those previously described (Traina & Serpietri, 1984; Thomsen *et al.*, 1991).

The data derived from the physiological studies (contractions) as well as results obtained in the biochemical protocols suggest that the BChE activity detected in human bronchi is associated with the enzymatic degradation of acetylcholine in these tissues.

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References

AAS, P., VEITEBERG, T. & FONNUM, F. (1986). In vitro effects of soman on bronchial smooth muscle. *Biochem. Pharmacol.*, **35**, 1793–1799.

ADLER, M. & FILBERT, M.G. (1990). Role of butyrylcholinesterase in canine tracheal smooth muscle function. *FEBS Lett.*, **267**, 107–110.

ADLER, M., REUTTER, S.A., MOORE, D.H. & FILBERT, M.G. (1991). Regulation of acetylcholine hydrolysis in canine tracheal smooth muscle. *Eur. J. Pharmacol.*, **205**, 73–79.

AMBACHE, N. & LESSIN, A.W. (1955). Classification of intestinomotor drugs by means of type D botulinum toxin. *J. Physiol.*, **127**, 449–478.

APPLEYARD, M.E. & SMITH, A.D. (1989). Secretion of acetylcholinesterase and butyrylcholinesterase from the guinea-pig isolated ileum. *Br. J. Pharmacol.*, **97**, 490–498.

ATACK, J.R., YU, Q., SONCRANT, T.T., BROSSI, A. & RAPOPORT, S.I. (1989). Comparative inhibitory effects of various physostigmine analogs against acetyl- and butyrylcholinesterases. *J. Pharmacol. Exp. Ther.*, **249**, 194–202.

AUSTIN, L. & BERRY, W.K. (1953). Two selective inhibitors of cholinesterase. *Biochem. J.*, **54**, 695–700.

CARLYLE, R.F. (1963). The mode of action of neostigmine and physostigmine on the guinea-pig trachealis muscle. *Br. J. Pharmacol.*, **21**, 137–149.

CHATONNET, A. & LOCKRIDGE, O. (1989). Comparison of butyrylcholinesterase and acetylcholinesterase. *Biochem. J.*, **260**, 625–634.

DALY, M. DE BURGH (1957). The effects of anticholinesterases on the bronchioles and pulmonary blood vessels in isolated perfused lungs of the dog. *Br. J. Pharmacol.*, **12**, 504–512.

DANIEL, E.E., KANNAN, M., DAVIS, C. & POSEY-DANIEL, V. (1986). Ultrastructural studies on the neuromuscular control of human tracheal and bronchial muscle. *Respir. Physiol.*, **63**, 109–128.

DE CANDOLE, C.A., DOUGLAS, W.W., EVANS, C.L., HOLMES, R., SPENCER, K.E.V., TORRANCE, R.W. & WILSON, K.M. (1953). The failure of respiration in death by anticholinesterase poisoning. *Br. J. Pharmacol.*, **8**, 466–475.

DOUGLAS, W.W. (1951). The effect of some anticholinesterase drugs on the isolated tracheal muscle of the guinea-pig. *J. Physiol.*, **112**, 20P.

HABER, P., HARMUTH, P., WOLF, C., MAYR, N. & ZEITLHOFER, J. (1987). Acute effects of prostigmin on lung function and gas exchange in patients with myasthenia gravis during rest and ergometric exercise. *Respiration*, **52**, 59–68.

ISRAEL, M. & LESBATS, B. (1985). *Bioluminescence and Chemiluminescence: Instruments and Applications*. ed. Van Dyke, K. Volume 2, pp. 1–11. Boca Raton: CRC Press, Inc.

ITO, Y., SUZUKI, H., AIZAWA, H., HAKODA, H. & HIROSE, T. (1989). The spontaneous electrical and mechanical activity of human bronchial smooth muscle: its modulation by drugs. *Br. J. Pharmacol.*, **98**, 1249–1260.

KIRKPATRICK, C.T. & ROONEY, P.J. (1982). Contractures produced by carbamate anticholinesterases in bovine tracheal smooth muscle. *Clin. Exp. Pharmacol. Physiol.*, **9**, 603–611.

KOELLE, W.A. & KOELLE, G.B. (1959). The localization of external or functional acetylcholinesterase at the synapses of autonomic ganglia. *J. Pharmacol. Exp. Ther.*, **126**, 1–8.

MAK, J.C.W. & BARNES, P.J. (1990). Autoradiographic visualization of muscarinic receptor subtypes in human and guinea-pig lung. *Am. Rev. Respir. Dis.*, **141**, 1559–1568.

MANN, S. (1971). The innervation of mammalian bronchial smooth muscle: the localization of catecholamines and cholinesterases. *Histochem. J.*, **3**, 319–331.

MITTAG, T.W., EHRENPREIS, S. & PATRICK, P. (1971). Some properties of cholinesterases in intact guinea-pig ileum in vitro. *Arch. Int. Pharmacodyn.*, **191**, 270–278.

PARTANEN, M., LAITINEN, A., HERVONEN, A., TOIVANEN, M. & LAITINEN, L.A. (1982). Catecholamine- and acetylcholinesterase-containing nerves in human lower respiratory tract. *Histochemistry*, **76**, 175–188.

PAULUHN, J., MACHEMER, L. & KIMMERLE, G. (1987). Effects of inhaled cholinesterase inhibitors on bronchial tonus and on plasma and erythrocyte acetylcholine esterase activity in rats. *Toxicology*, **46**, 177–190.

SMALL, R.C., GOOD, D.M., DIXON, J.S. & KENNEDY, I. (1990). The effects of epithelium removal on the actions of cholinomimetic drugs in opened segments and perfused tubular preparations of guinea-pig trachea. *Br. J. Pharmacol.*, **100**, 516–522.

THOMSEN, T., ZENDEH, B., FISCHER, J.P. & KEWITZ, H. (1991). In vitro effects of various cholinesterase inhibitors on acetyl- and butyrylcholinesterase of healthy volunteers. *Biochem. Pharmacol.*, **41**, 139–141.

TRAINA, M.E. & SERPIETRI, L.A. (1984). Changes in the levels and forms of rat plasma cholinesterases during chronic diisopropylphosphorofluoridate intoxication. *Biochem. Pharmacol.*, **33**, 645–653.

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Arterial endothelial barrier dysfunction: actions of homocysteine and the hypoxanthine-xanthine oxidase free radical generating system

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1 Endothelial barrier function was assessed by use of an *in vitro* model in which transfer of trypan blue-labelled albumin was measured across monolayers of bovine aortic endothelial cells grown on polycarbonate membranes.

2 Addition of either hypoxanthine (0.2 mM) or xanthine oxidase (20 mu ml^{-1}) alone during a 90 min incubation did not affect albumin transfer across endothelial cell monolayers, but a combination of both increased transfer.

3 The increase in albumin transfer induced by hypoxanthine and xanthine oxidase was abolished by catalase (3 u ml^{-1}), reduced by allopurinol (4 mM), but unaffected by superoxide dismutase (6000 u ml^{-1}), the hydroxyl radical scavengers, mannitol (15 mM), dimethylthiourea (10 mM) and N-(2-mercaptopropionyl)-glycine (1 mM), the iron chelator, deferoxamine (0.5 mM), ferric chloride (50 μM), an inhibitor of nitric oxide synthase, N^G-nitro-L-arginine (30 μM), or the antioxidant, dithiothreitol (3 mM).

4 Hydrogen peroxide (0.1–30 mM) itself increased albumin transfer across endothelial cell monolayers, exhibiting a biphasic concentration-response curve. The increase induced by 0.1 mM hydrogen peroxide was abolished in the presence of 0.3 u ml^{-1} catalase whilst that induced by 10 mM hydrogen peroxide was abolished by 3000 u ml^{-1} catalase.

5 Homocysteine (0.5–1.5 mM) did not affect albumin transfer across endothelial monolayers when added alone, but when added in combination with copper sulphate (50 μM), which catalyses its oxidation, a significant increase in albumin transfer was observed.

6 The increase in albumin transfer induced by the combination of homocysteine (1.5 mM) and copper sulphate was abolished by catalase (1 u ml^{-1}), but was unaffected by superoxide dismutase (6000 u ml^{-1}), mannitol (15 mM), dimethylthiourea (1 mM) or deferoxamine (0.5 mM).

7 The data suggest that the endothelial barrier dysfunction induced by the combination of hypoxanthine and xanthine oxidase is mediated solely by the action of hydrogen peroxide and not by superoxide anion, hydroxyl radical, peroxynitrite anion or hypochlorous acid. The copper-catalysed oxidation of homocysteine also induces endothelial barrier dysfunction through the generation of hydrogen peroxide. These findings may have relevance to the endothelial barrier dysfunction associated with ischaemia-reperfusion injury and the atherogenic actions of homocysteine.

Keywords: Endothelial barrier function; reactive oxygen species; oxygen-derived free radicals; hydrogen peroxide; xanthine oxidase; hypoxanthine; homocysteine; catalase

Introduction

The vascular endothelium plays a key role in the maintenance of homeostasis within the body by controlling the transport of fluid, solutes and cells between vascular and extravascular sites. Inflammatory mediators, such as histamine and 5-hydroxytryptamine, have been shown to increase the permeability of the endothelial barrier at the post-capillary venule through contraction of individual endothelial cells. This leads to the formation of intercellular gaps, so causing vascular leakage and the formation of oedema (Majino & Palade, 1961; Shasby *et al.*, 1982). In the arterial circulation, impairment of endothelial barrier function leads to increased entry into the blood vessel wall of high molecular weight solutes, including cholesterol-rich low-density lipoproteins (Ross & Harker, 1976), and contributes to the subsequent development of atherosclerosis (Munro & Cotran, 1988).

Reactive oxygen species, including hydrogen peroxide, superoxide anion and hydroxyl radical, may cause endothelial barrier dysfunction through cytotoxic actions leading to the formation of intercellular gaps (Shasby *et al.*, 1985). This may occur, for example, following oxidation of low-density lipoproteins or free fatty acids (Zilversmit, 1973; Morel *et al.*, 1983). It is also believed to occur on reperfusion following a period of ischaemia during which anaerobic catabolism of adenosine triphosphate leads to an accumulation of hypoxanthine (Werns & Lucchesi, 1990). During subsequent reperfusion, xanthine oxidase is formed by conversion from its native state of xanthine dehydrogenase, either through limited proteolysis or oxidation of essential thiol groups (Phan *et al.*, 1989) and catalyses the breakdown of hypoxanthine to xanthine and xanthine to uric acid. In both of these reactions, superoxide radicals are produced which undergo dismutation to hydrogen peroxide (Fridovich, 1970) and possible further conversion, catalysed by ferrous iron, to hydroxyl radical (Beauchamp & Fridovich, 1970; Kvietys *et al.*, 1989). Hypochlorous acid, a product of the polymorphonuclear leukocyte, is another factor implicated in the injury caused by ischaemia-reperfusion and is primarily believed to cause damage through interaction with sulphydryl-containing structures (Eley *et al.*, 1989).

Another condition in which reactive oxygen species have been implicated in causing endothelial barrier dysfunction is that of homocystinuria (Starkebaum & Harlan, 1986). This condition is characterized by elevated circulating levels of the

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amino acid, homocysteine, an intermediate product formed during the metabolism of both methionine and cysteine. Defects in the metabolism of homocysteine, lead to its accumlation, accompanied by an increased incidence of atherosclerosis (Boers *et al.*, 1985; Refsum & Ueland, 1990).

Using an *in vitro* model of the arterial endothelial barrier (Gudgeon & Martin, 1989), we wished to identify the damaging species produced by the well characterized hypoxanthine-xanthine oxidase free radical generating system. Furthermore, we wished to determine if the atherogenic action of homocysteine could be explained by induction of endothelial barrier dysfunction and if so investigate the involvement of reactive oxygen species.

Methods

Endothelial cell culture

Bovine aortic endothelial cells were isolated and grown in 80 cm^2 tissue culture flasks as previously described (Buchan & Martin, 1991). When these primary cultures achieved confluency (3–7 days), the cells were removed by treatment with 0.05% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA) and seeded onto 48 Transwell (Costar) polycarbonate membranes (6.5 mm diameter, 3 μm pore size). These membrane assemblies were, in turn, placed in wells of 24-well cluster plates (Costar), each of which contained 1 ml of Dulbecco's modified Eagle's medium containing foetal calf serum (10%), newborn calf serum (10%), glutamine (4 mM), benzyl penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}), and allowed to grow for a further 3 days to reach confluency again. All tissue culture reagents were obtained from Gibco (Paisley, UK) except trypsin-EDTA which was obtained from Flow Laboratories (Irvine, UK).

Albumin transfer experiments

Albumin transfer experiments were conducted as previously described (Gudgeon & Martin, 1989). Briefly, membrane assemblies with endothelial monolayers attached were initially washed twice by immersion in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 2.4, glucose 11 and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 5 at 37°C and pH 7.4, before subsequent transfer to 24-well cluster plates. Krebs solution (600 μl) was placed in each well, these forming the lower chamber of the two-chamber system and 100 μl of Krebs containing 4% trypan blue-labelled albumin was placed in the upper chamber of each well, above the cell monolayer, these volumes having been chosen so as to avoid the generation of a hydrostatic gradient. Drugs were then added in volumes of 7 μl to both upper and lower chambers, and the plates were transferred to an orbital shaker, which ensured gentle, continuous agitation, and incubated for 90 min at 37°C. At the end of this period, samples were removed from the lower chamber of each preparation, and the absorbance at 590 nm measured to establish the content of trypan blue-labelled albumin. The extent of equilibration of albumin across the monolayers into the lower chamber in each case was then determined by expressing these results as a percentage where 100% equilibration was the maximum which would be achieved at equilibrium.

For the experiments involving overnight pretreatment with the iron chelator, deferoxamine (0.5 mM), the drug was added to the endothelial cell monolayers 18 h before the start of the experiment. On transfer of these monolayers to wells containing Krebs solution and following addition of trypan blue-labelled albumin, deferoxamine was added again to both upper and lower chambers.

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

Drugs

Allopurinol, catalase (bovine liver), deferoxamine mesylate, DL-dithiothreitol, DL-homocysteine, hydrogen peroxide (30%), hypoxanthine, D-mannitol, N-(2-mercaptopropionyl)-glycine, N^G-nitro L-arginine, superoxide dismutase (bovine erythrocyte), xanthine and xanthine oxidase (buttermilk) were obtained from Sigma (Poole, Dorset) whilst 1,3-dimethyl-2-thiourea was obtained from Aldrich (Gillingham, Dorset). All drugs were dissolved in distilled water except for hypoxanthine and xanthine which were dissolved in sodium hydroxide. The final concentration of sodium hydroxide in experiments did not exceed 0.2 mM which, in itself, did not affect albumin transfer across monolayers.

Statistical analysis

Results are expressed as means ± s.e.mean and were compared by one-way analysis of variance to determine if any significant differences existed between treatment groups. This was followed by Fisher's test to determine where differences lay. Due to the variability encountered in resting transfer of albumin amongst different batches of cells, each experiment was performed with its own internal controls. A probability of 0.05 or less was taken to be significant.

Results

Effects of hypoxanthine and xanthine oxidase on albumin transfer

Neither hypoxanthine (0.2 mM) nor xanthine oxidase (20 mu ml^{-1}) when added alone had any effect on the equilibration of albumin across monolayers of bovine aortic endothelial cells after a 90 min exposure, but when both were added in combination, a significant increase in albumin transfer was observed (Figure 1). Moreover, in the presence of a fixed concentration of hypoxanthine (0.2 mM), xanthine oxidase (3–300 u ml^{-1}) was found to exhibit a concentration-dependent increase in albumin transfer (data not shown). Sub-

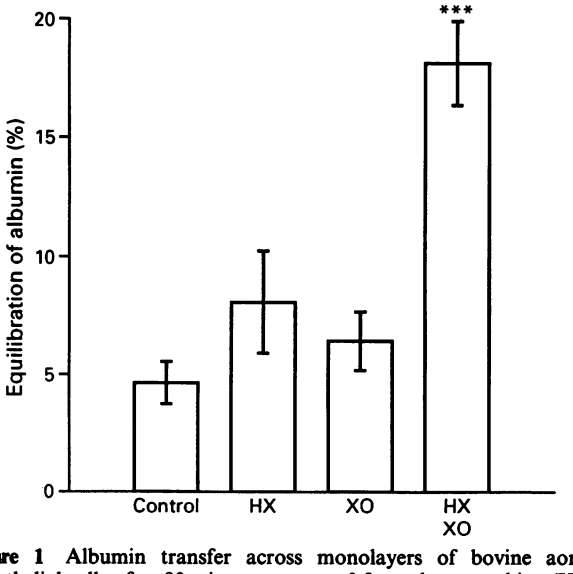


Figure 1 Albumin transfer across monolayers of bovine aortic endothelial cells after 90 min exposure to 0.2 mM hypoxanthine (HX) and 20 mu ml^{-1} xanthine oxidase (XO) added alone and in combination. Each column is the mean of 6 observations with vertical bars representing s.e.mean. ***$P<0.001$, indicates a significant difference from control.

stituting xanthine (0.2 mM) for hypoxanthine similarly gave an increase in albumin transfer from 6.5 ± 0.5% to 29.2 ± 1.2% ($n = 6$) when added in combination with xanthine oxidase.

The increase in albumin transfer seen on addition of 0.2 mM hypoxanthine and 20 mu ml^{-1} xanthine oxidase was abolished in the presence of catalase at concentrations of 3 u ml^{-1} and above (Figure 2) and was significantly reduced in the presence of the xanthine oxidase inhibitor, allopurinol (4 mM) (Table 1). This increase was, however, unaffected by superoxide dismutase (6000 u ml^{-1}), mannitol (15 mM), dimethylthiourea (10 mM), N-(2-mercaptopropionyl)-glycine (3 mM), N^G-nitro-L-arginine (30 μM) or dithiothreitol (3 mM) (Table 1). Overnight pretreatment of monolayers with the iron chelator, deferoxamine (0.5 mM), similarly failed to affect this increase, whilst addition of ferric chloride (50 μM) during the 90 min incubation, failed to enhance it (Table 1).

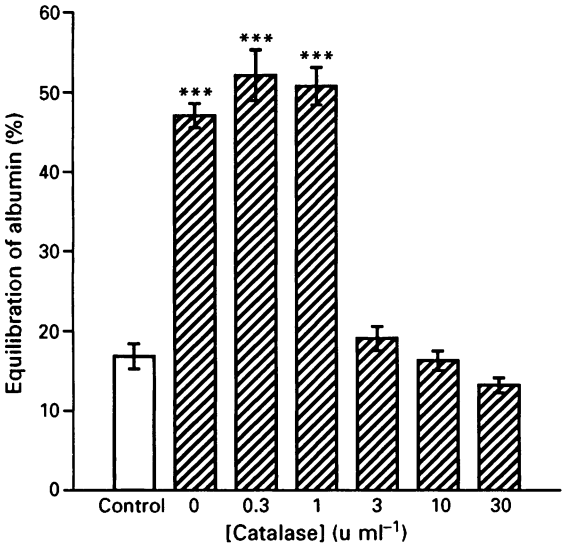


Figure 2 Albumin transfer across monolayers of bovine aortic endothelial cells after 90 min incubation both in the presence (hatched columns) and absence (open columns) of 0.2 mM hypoxanthine and 20 u ml^{-1} xanthine oxidase. The effects of concomitant addition of 0.3–30 u ml^{-1} catalase are also shown. Each column is the mean of 6–9 observations with vertical bars representing s.e.mean. ***$P <$ 0.001, indicates a significant difference from control.

Effects of hydrogen peroxide on albumin transfer

Hydrogen peroxide itself (0.1–30 mM) induced a biphasic increase in albumin transfer across monolayers, exhibiting peaks at around 0.1–0.3 mM and again at 10–30 mM (Figure 3). The increase in albumin transfer induced by 0.1 mM hydrogen peroxide was abolished in the presence of 0.3 u ml^{-1} catalase whilst the increase induced by 10 mM hydrogen peroxide was abolished in the presence of 3000 u ml^{-1} catalase (Table 2).

Effects of homocysteine and copper sulphate on albumin transfer

Homocysteine when added alone at either 0.5 mM or 1.5 mM had no effect on albumin transfer. However, when added in the presence of copper sulphate (5 or 50 μM) which itself had no effect on albumin transfer, homocysteine significantly increased equilibration of albumin at both 0.5 and 1.5 mM (Figure 4).

The increase in albumin transfer seen on addition of homocysteine (1.5 mM) and copper sulphate (50 μM) was

Table 2 Effect of catalase on the increase in albumin transfer across monolayers of bovine aortic endothelial cells stimulated by hydrogen peroxide (H_2O_2)

[H_2O_2] (mM)	[Catalase] (u ml^{-1})	*Equilibration of albumin* (%)
0 (control)	0	10.8 ± 1.5
0.1	0	21.9 ± 2.8***
0.1	0.3	12.29 ± 1.8
0.1	1	10.5 ± 1.4
0 (control)	0	6.1 ± 1.2
10	0	25.1 ± 2.2***
10	30	30.9 ± 1.6***
10	100	26.4 ± 0.9***
10	300	22.5 ± 4.0***
10	1000	11.3 ± 4.1
10	3000	7.9 ± 1.6

Albumin transfer across monolayers of bovine aortic endothelial cells after 90 min incubation in the presence and absence of hydrogen peroxide. The effect of concomitant addition of catalase is also shown. Results are expressed as mean ± s.e.mean of 6–9 observations.
***$P <$ 0.001, indicates a significant difference from control.

Table 1 Effects of superoxide dismutase (SOD), mannitol (Man), dimethylthiourea (DMTU), N-(2-mercaptopropionyl)-glycine (MPG), deferoxamine (Def), ferric chloride ($FeCl_3$), N^G-nitro-L-arginine (L-NOARG), dithiothreitol (DTT) and allopurinol (Allo) on the increase in albumin transfer across monolayers of bovine aortic endothelial cells stimulated by 0.2 mM hypoxanthine (HX) and 20 mu ml^{-1} xanthine oxidase (XO)

		Equilibration of albumin (%)		
Treatment	*Control*	*HX/XO*	*Treatment alone*	*HX/XO + treatment*
SOD, 6000 u ml^{-1}	22.3 ± 4.3	41.5 ± 4.1**	19.9 ± 3.1	39.8 ± 3.0**
Man, 15 mM	11.5 ± 1.4	26.7 ± 1.9***	8.8 ± 1.0	27.3 ± 1.5***
DMTU, 10 mM	27.7 ± 3.5	45.0 ± 4.0**	31.1 ± 2.5	48.4 ± 2.9***
MPG, 3 mM	13.1 ± 1.1	37.4 ± 3.4***	18.6 ± 1.7	33.1 ± 2.3***
Def, 0.5 mM	9.2 ± 1.2	37.7 ± 2.0***	9.4 ± 1.8	36.3 ± 1.6***
$FeCl_3$, 50 μM	13.9 ± 0.9	23.3 ± 2.7***	14.5 ± 1.0	22.3 ± 1.1**
DTT, 3 mM	13.2 ± 1.4	42.4 ± 5.2***	10.8 ± 0.8	31.4 ± 1.6***
L-NOARG, 30 μM	9.9 ± 1.7	24.3 ± 3.8***	15.7 ± 1.5	27.5 ± 1.4***
Allo, 4 mM	7.0 ± 1.8	24.6 ± 2.7***	6.63 ± 1.1	16.0 ± 3.5*‡

Albumin transfer across monolayers of bovine aortic endothelial cells after 90 min incubation in the absence of drugs (control) and in the presence of hypoxanthine and xanthine oxidase. Effects of concomitant addition of superoxide dismutase, mannitol, dimethylthiourea, N-(2-mercaptopropionyl)-glycine, ferric chloride, N^G-nitro-L-arginine, dithiothreitol and allopurinol, and of overnight pretreatment with deferoxamine, are also shown. Results are expressed as the mean ± s.e.mean of 6 observations.
*$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.001, indicates a significant difference from control. ‡$P <$ 0.05, indicates a significant difference from HX/XO.

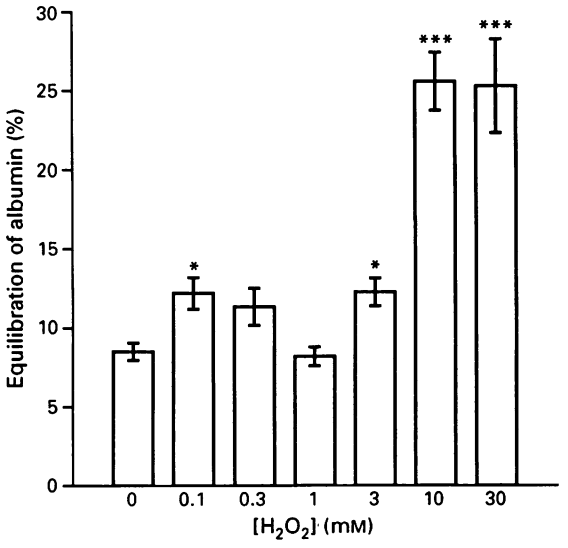


Figure 3 Albumin transfer across monolayers of bovine aortic endothelial cells after 90 min exposure to 0–30 mM hydrogen peroxide (H_2O_2). Each column is the mean of 6 observations with vertical bars representing s.e.mean. $*P<0.05$; $***P<0.001$, indicates a significant difference from control.

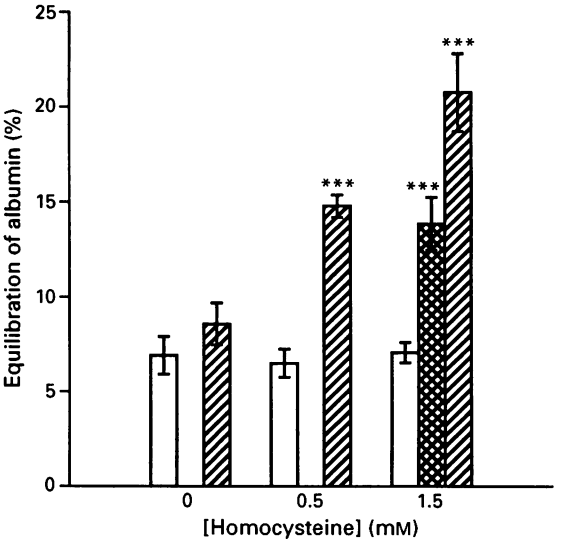


Figure 4 Albumin transfer across monolayers of bovine aortic endothelial cells following 90 min incubation in the presence (open columns) of 0, 0.5 and 1.5 mM homocysteine. The effects of concomitant addition of copper sulphate at 5 μM (cross-hatched column) and 50 μM (hatched columns) are also shown. Each column is the mean of 6 observations with vertical bars representing s.e.mean. $***P<0.001$, indicates a significant difference from control.

abolished in the presence of catalase (1 u ml^{-1}) but was unaffected by superoxide dismutase (600 u ml^{-1}), mannitol (15 mM), dimethylthiourea (1 mM) or deferoxamine (0.5 mM) (Table 3).

Discussion

The use of an *in vitro* model of the endothelial barrier employing endothelial cell monolayers grown on polycarbonate membranes is well established (Shasby *et al.*, 1985; Rotrosen & Gallin, 1986; Wilson *et al.*, 1990). Hypoxanthine and xanthine oxidase have been implicated in the endothelial cell injury induced by ischaemia-reperfusion (Granger *et al.*, 1981). In our study, no increase in albumin transfer across monolayers of bovine aortic endothelial cells was seen when either hypoxanthine or xanthine oxidase was added alone. Only when hypoxanthine and xanthine oxidase were added in combination, was a significant increase in albumin transfer observed, indicating that the full free radical generating system is required to induce endothelial barrier dysfunction. Moreover, experiments with allopurinol, an inhibitor of xanthine oxidase, confirmed the need for the complete enzyme-substrate system for the induction of barrier dysfunction.

When xanthine was substituted for hypoxanthine and added in combination with xanthine oxidase, no difference was seen in the increase in albumin transfer stimulated across endothelial monolayers. Thus, both hypoxanthine and xanthine can equally well be used as a substrate for xanthine oxidase in mediating endothelial barrier dysfunction in this system.

Superoxide dismutase, which rapidly converts superoxide anion to hydrogen peroxide, had no effect on the increase in albumin transfer seen on addition of hypoxanthine and xanthine oxidase to endothelial monolayers. In contrast, this increase was abolished in the presence of catalase which breaks down hydrogen peroxide to water and oxygen. Thus, in accord with the findings of Kvietys *et al.* (1989), these results indicate that hydrogen peroxide, and not superoxide anion plays a key role in mediating the endothelial barrier dysfunction induced by the hypoxanthine-xanthine oxidase free radical generating system. Indeed, when we examined the effects of hydrogen peroxide itself, we found that it also induced an increase in albumin transfer across endothelial monolayers, exhibiting a biphasic concentration-response curve. The lower peak of this curve (0.1–0.3 mM) closely

Table 3 Effects of catalase (Cat), superoxide dismutase (SOD), mannitol (Man), dimethylthiourea (DMTU) and deferoxamine (Def) on the increase in albumin transfer across monolayers of bovine aortic endothelial cells stimulated by 1.5 mM homocysteine (HC) and 50 μM copper sulphate ($CuSO_4$)

	Equilibration of albumin (%)			
Treatment	*Control*	*HC/$CuSO_4$*	*Treatment alone*	*HC/$CuSO_4$ + treatment*
Cat, 1 u ml^{-1}	7.2 ± 0.7	19.7 ± 2.6***	–	6.1 ± 0.8
SOD, 6000 u ml^{-1}	9.2 ± 1.1	27.1 ± 4.4***	8.7 ± 1.1	23.4 ± 2.0***
Man, 15 mM	9.8 ± 1.1	18.1 ± 1.1***	9.8 ± 1.1	18.4 ± 1.0***
DMTU, 1 mM	8.0 ± 0.9	24.2 ± 3.2***	9.9 ± 1.1	24.2 ± 3.7***
Def, 0.5 mM	17.7 ± 3.4	35.3 ± 5.3**	18.0 ± 3.0	31.4 ± 3.4*

Albumin transfer across monolayers of bovine aortic endothelial cells after 90 min incubation in the absence of drugs (control) and in the presence of homocysteine and copper sulphate. Effects of concomitant addition of catalase, superoxide dismutase, mannitol, dimethylthiourea, and of overnight pretreatment with deferoxamine, are also shown. Results are expressed as mean ± s.e.mean of 6 observations.

$*P<0.05$; $**P<0.01$; $***P<0.001$, indicates a significant difference from control.

correlates with the maximum amount of hydrogen peroxide (0.2 mM) which could theoretically be generated by hypoxanthine and xanthine oxidase at the concentrations used.

Since xanthine oxidase was added extracellularly and would be unable to cross the cell membrane, it can be assumed that the superoxide anion is generated extracellularly. It is not clear, though, if its subsequent conversion to hydrogen peroxide is mediated by endogenous superoxide dismutase or occurs spontaneously. The fact that exogenously added superoxide dismutase does not potentiate the barrier dysfunction induced by hypoxanthine and xanthine oxidase suggests that conversion of superoxide anion to hydrogen peroxide is already sufficiently rapid to produce a maximal effect for a given concentration of hypoxanthine and xanthine oxidase.

The precise reason for the biphasic-nature of the hydrogen peroxide concentration-response curve is unclear. One possible explanation is that at low concentrations (around 0.1–0.3 mM), enough hydrogen peroxide is available to cause damage to the endothelial cell monolayer, but when this concentration is increased, some form of protective mechanism (e.g. release of intracellular catalase) is triggered within the endothelial cells and this counters any damaging effect. As the concentration is raised still further, enough hydrogen peroxide then becomes available to overcome this protective effect and so a second wave of endothelial cell damage is observed. Alternatively, it is possible that hydrogen peroxide causes cell injury at two separate target sites in the endothelial cell, one with low sensitivity and one with higher sensitivity, with each corresponding to a peak in the concentration-response curve.

Hydrogen peroxide, however, may not be the sole damaging species produced by the hypoxanthine-xanthine oxidase free radical generating system. It may, for example, act as a precursor for some other mediator of endothelial cell damage. Several studies indicate involvement of hydroxyl radical in the endothelial damage induced by activated neutrophils (Ward *et al.*, 1983; Varani *et al.*, 1985; Gannon *et al.*, 1987), ischaemia-reperfusion (Dauber *et al.*, 1991; Karwatowska-Prokopczuk *et al.*, 1992) or the hypoxanthine-xanthine oxidase free radical generating system (Kvietys *et al.*, 1989). In order to investigate this possibility, we looked at the effects of a number of hydroxyl radical scavengers on the increased albumin transfer induced by hypoxanthine and xanthine oxidase. These included mannitol (Halliwell & Gutteridge, 1989), a membrane-impermeant scavenger, as well as dimethylthiourea (Fox, 1984) and N-(2-mercaptopropionyl)-glycine (Bolli *et al.*, 1989), two membrane-permeant scavengers. None of these agents showed any inhibitory effect on the increase in albumin transfer induced by the hypoxanthine-xanthine oxidase free radical generating system in this study, indicating against hydroxyl radical involvement.

Hydroxyl radical production is believed to rely on the availability of ferrous iron which catalyses its formation from hydrogen peroxide. Intracellular iron is found predominantly in the ferric form (Halliwell & Gutteridge, 1984). It has been proposed (Starke & Farber, 1985), that superoxide anion, produced by the hypoxanthine-xanthine oxidase system, acts as a reductant to promote the formation of ferrous iron from its stored ferric form and that once produced, ferrous iron is then able to promote hydroxyl radical formation by the Fenton reaction. It was found, however, that deferoxamine, an iron chelator, had no effect on the increase in albumin transfer stimulated by hypoxanthine and xanthine oxidase either when present only during the 90 min incubation, or when it had been added overnight to allow extensive accumulation into cells (Starke & Farber, 1985). Also consistent with the apparent lack of involvement of hydroxyl radical is the finding that addition of ferric chloride, as an exogenous source of ferric iron to drive the Fenton reaction (Thomas *et al.*, 1984), failed to enhance the increase in albumin transfer seen to the hypoxanthine-xanthine oxidase free radical generating system.

Our findings therefore differ from those of other groups who have studied oxidant- (Kvietys *et al.*, 1989) and neutrophil- (Martin, 1984; Varani *et al.*, 1985; Gannon *et al.*, 1987) induced injury to endothelial cell monolayers where both hydroxyl radical scavengers and iron chelators were reported to be effective in inhibiting cell damage. However, in these four studies, endothelial cell damage was assessed by a ^{51}Cr release assay, a method which only detects cell damage over a considerably longer time-course (4–8 h) than that seen when measuring albumin transfer across cell monolayers (90 min). Thus it is possible that whilst hydroxyl radical production may be necessary for the extensive damage required for ^{51}Cr release, it is not required for the more rapidly developing increase in permeability of endothelial cell monolayers.

As an alternative to the Fenton reaction theory of hydroxyl radical production, Beckman *et al.* (1990) proposed that an interaction takes place between superoxide anion and nitric oxide, released from endothelial cells, leading to the formation of the peroxynitrite anion. This anion is itself a powerful oxidant which undergoes decomposition to hydroxyl radical and nitrogen dioxide radical by a mechanism not involving iron catalysation. In our study, however, N^G-nitro-L-arginine, an inhibitor of nitric oxide synthase (Moore *et al.*, 1990), had no effect on the increase in albumin transfer induced by the hypoxanthine-xanthine oxidase free radical generating system. Although we did not measure production of peroxynitrite, this finding suggests it plays no role in the induction of barrier dysfunction.

The antioxidant, dithiothreitol, has been demonstrated to reverse the damaging effects of hypochlorous acid on Ca^{2+} homeostasis in rabbit isolated ventricular myocytes (Eley *et al.*, 1991) and on cardiac muscle contractile function during ischaemia-reperfusion damage (Eley *et al.*, 1989). We found, however, that dithiothreitol had no effect on the increase in albumin transfer induced by the hypoxanthine-xanthine oxidase free radical generating system suggesting that involvement of hypochlorous acid is unlikely.

Taking all of these observations into account, it would seen likely that hydrogen peroxide and not superoxide anion, hydroxyl radical, hypochlorous acid or peroxynitrite is the major species which mediates the hypoxanthine-xanthine oxidase-induced increase in albumin transfer across endothelial monolayers.

Homocystinuria is a known risk factor for the development of atherosclerosis (Boers *et al.*, 1985), but the effects of homocysteine on endothelial barrier function have not been established. In this study, we found that homocysteine was unable to increase albumin transfer when added alone to endothelial cell monolayers. Starkebaum & Harlan (1986) demonstrated, however, that in the presence of copper, either free in solution or bound to caeruloplasmin, homocysteine will undergo oxidation, with concomitant production of hydrogen peroxide, resulting in injury to endothelial cells. Thus, we examined the effects of homocysteine in combination with copper sulphate and found that an increase in albumin transfer was stimulated. This is consistent with the view that it is the copper-induced oxidation of homocysteine, rather than homocysteine itself, which causes endothelial cell damage.

Copper is present in the circulation in the form of caeruloplasmin and in homozygotic homocystinuria sufferers, the circulating level of homocysteine may be as high as 100–200 μM (Boers *et al.*, 1985). Whilst this level of homocysteine is around 2.5 to 5 fold less than that found to induce an increase in albumin transfer across cell monolayers in this study during a 90 min incubation, it is conceivable that over a longer time-course in the circulation, these levels of homocysteine could be sufficient to induce endothelial barrier dysfunction.

As with the hypoxanthine-xanthine oxidase free radical generating system, the increase in albumin transfer seen on addition of homocysteine and copper sulphate was unaffected by superoxide dismutase but was abolished by catalase. Thus,

as was found by Starkebaum & Harlan (1986), hydrogen peroxide, and not superoxide anion, is implicated in the injury caused by copper-catalysed oxidation of homocysteine. Co-incubation with mannitol or dimethylthiourea, and also overnight pretreatment with deferoxamine, failed to prevent the increase in albumin transfer stimulated by homocysteine and copper sulphate, suggesting that hydroxyl radical is not involved in mediating this endothelial barrier dysfunction.

In conclusion, we have shown using an established *in vitro* model of the arterial endothelial barrier, that hypoxanthine and xanthine oxidase can induce endothelial barrier dysfunction as measured by an increase in trypan blue-labelled albumin across monolayers of bovine aortic endothelial cells. We have demonstrated that the mediator of this dysfunction is probably hydrogen peroxide and not superoxide anion, hydroxyl radical, peroxynitrite or hypochlorous acid. We have further demonstrated that homocysteine, following copper-catalysed oxidation, can cause dysfunction of the endothelial barrier and that this is mediated by hydrogen peroxide. This finding may explain the higher incidence of atherosclerosis observed in homocystinuria.

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References

BEAUCHAMP, C. & FRIDOVICH, I. (1970). A mechanism for the production of ethylene from methional. The generation of the hydroxyl radical by xanthine oxidase. *J. Biol. Chem.*, **245**, 4461–4646.

BECKMAN, J.S., BECKMAN, T.W., CHEN, J., MARSHALL, P.A. & FREEMAN, B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1620–1624.

BOERS, G.H.J., SMALS, A.G.H., TRIJBELS, F.J.M., FOWLER, B., BAKKEREN, J.A.J.M., SCHOONDERWALDT, H.C., KLEIJER, W.J. & KLOPPENBORG, P.W.C. (1985). Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. *N. Engl. J. Med.*, **313**, 709–715.

BOLLI, R., JEROUDI, M.O., PATEL, B.S., ARUOMA, O.I., HALLIWELL, B., LAI, E.K. & MCCAY, P.B. (1989). Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial 'stunning' is a manifestation of reperfusion injury. *Circ. Res.*, **65**, 607–622.

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

DAUBER, I.M., LESNEFSKY, E.J., VAN BENTHUYSEN, K.M., WEIL, J.V. & HORWITZ, L.D. (1991). Reactive oxygen metabolite scavengers decrease functional coronary microvascular injury due to ischaemia-reperfusion. *Am. J. Physiol.*, **260**, H42–H49.

ELEY, D.W., KORECKY, B. & FLISS, H. (1989). Dithiothreitol restores contractile function to oxidant-injured cardiac muscle. *Am. J. Physiol.*, **257**, H1321–H1325.

ELEY, D.W., KORECKY, B., FLISS, H. & DÉSILETS, M. (1991). Calcium homeostasis in rabbit ventricular myocytes. Disruption by hypochlorous acid and restoration by dithiothreitol. *Circ. Res.*, **69**, 1132–1138.

FOX, R.B. (1984). Prevention of granulocyte-mediated oxidant lung injury in rats by an hydroxyl radical scavenger, dimethylthiourea. *J. Clin. Invest.*, **74**, 1456–1464.

FRIDOVICH, I. (1970). Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J. Biol. Chem.*, **245**, 4053–4057.

GANNON, D.E., VARANI, J., PHAN, S.H., WARD, J.H., KAPLAN, J., TILL, G.O., SIMON, R.H., RYAN, U.S. & WARD, P.A. (1987). Source of iron in neutrophil-mediated killing of endothelial cells. *Lab. Invest.*., **57**, 37–44.

GRANGER, D.N., RUTILI, G., MCCORD, J.M. (1981). Superoxide radicals and feline intestinal ischaemia. *Gastroenterology*, **81**, 22–29.

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

HALLIWELL, B. & GUTTERIDGE, J.M.C. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.*, **219**, 1–14.

HALLIWELL, B. & GUTTERIDGE, J.M.C. (1989). The chemistry of oxygen radicals and other oxygen derived species. In *Free Radicals in Biology and Medicine*. pp. 22–85. Oxford: Clarendon Press.

KARWATOWSKA-PROKOPCZUK, E., CZARNOWSKA, E. & BERESWICZ, A. (1992). Iron availability and free radical induced injury in the isolated ischaemic/reperfused rat heart. *Cardiovasc. Res.*, **26**, 58–66.

KVIETYS, P.R., INAUEN, W., BACON, B.R. & GRISHAM, M.B. (1989). Xanthine oxidase-induced injury to endothelium: role of intracellular iron and hydroxyl radical. *Am. J. Physiol.*, **257**, H1640–H1646.

MAJNO, G. & PALADE, G.E. (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.

MARTIN, W.J. (1984). Neutrophils kill pulmonary endothelial cells by a hydrogen-peroxide-dependent pathway. *Am. Rev. Respir. Dis.*, **130**, 209–213.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro arginine, a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

MOREL, D.W., HESSLER, J.R. & CHISOLM, G.M. (1983). Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J. Lipid Res.*, **24**, 1076–1979.

MUNRO, J.M. & COTRAN, R.S. (1988). The pathogenesis of atherosclerosis: atherogenesis and inflammation. *Lab. Invest.*, **58**, 249–261.

PHAN, S.H., GANNON, D.E., VARANI, J., RYAN, U.S. & WARD, P.A. (1989). Xanthine oxidase activity in rat pulmonary artery endothelial cells and its alteration by activated neutrophils. *Am. J. Pathol.*, **134**, 1201–1211.

REFSUM, H. & UELAND, P.M. (1990). Clinical significance of pharmacological modulation of homocysteine metabolism. *Trends Pharmacol. Sci.*, **11**, 411–416.

ROSS, R. & HARKER, L. (1976). Hyperlipidemia and atherosclerosis. Chronic hyperlipidemia initiates and maintains lesion by endothelial cell desquamation and lipid accumulation. *Science*, **193**, 1094–1100.

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

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

SHASBY, D.M., SHASBY, S.S., SULLIVAN, J.M. & PEACH, M.J. (1982). Role of endothelial cell cytoskeleton in control of endothelial permeability. *Circ. Res.*, **51**, 657–661.

STARKE, P.E. & FARBER, J.L. (1985). Ferric iron and superoxide ions are required for the killing of cultured hepatocytes by hydrogen peroxide. *J. Biol. Chem.*, **260**, 10099–10104.

STARKEBAUM, G. & HARLAN, J.M. (1986). Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. *J. Clin. Invest.*, **77**, 1370–1376.

THOMAS, C.E., MOREHOUSE, L.A. & AUST, S.D. (1984). Ferritin and superoxide-dependent lipid peroxidation. *J. Biol. Chem.*, **260**, 3275–3280.

VARANI, J., FLIGIEL, S.E.G., TILL, G.O., KUNKEL, R.G., RYAN, U.S. & WARD, P.A. (1985). Pulmonary endothelial cell killing by human neutrophils; possible involvement of hydroxyl radical. *Lab. Invest.*, **53**, 656–663.

WARD, P.A., TILL, G.O., KUNKEL, R. & BEACHAMP, C. (1983). Evidence for role of hyroxyl radical in complement and neutrophil-dependent tissue injury. *J. Clin. Invest.*, **72**, 789–801.

WERNS, S.W. & LUCCHESI, B.R. (1990). Free radicals and ischaemic tissue injury. *Trends Pharmacol. Sci.*, **11**, 161–166.

WILSON, J., WINTER, M. & SHASBY, D.M. (1990). Oxidants, ATP, and endothelial permeability to macromolecules. *Blood*, **76**, 2578–2582.

ZILVERSMIT, D.B. (1973). A proposal linking atherogenesis to the interaction of endothelial lipoprotein lipase with triglyceride-rich lipoproteins. *Circ. Res.*, **33**, 633–638.

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α_2-Adrenoceptor antagonist potencies of two hydroxylated metabolites of yohimbine

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1 The α_2-adrenoceptor antagonist capacities of two hydroxylated metabolites of yohimbine in man (10-OH-yohimbine and 11-OH-yohimbine) were investigated on the α_2-adrenoceptors of human platelets and adipocytes and compared to those of yohimbine.

2 Yohimbine and 11-OH-yohimbine exhibited similar α_2-adrenoceptor affinity in biological studies i.e. inhibition of adrenaline-induced platelet aggregation and inhibition of UK14304-induced antilipolysis in adipocytes.

3 Yohimbine and the two metabolites displaced [^{3}H]-RX 821002 binding with equivalent affinities in platelet and adipocyte membranes with the following order of potency: yohimbine $>$ 11-OH-yohimbine $>$ 10-OH-yohimbine. However, when binding studies were carried out in binding buffer supplemented with 5% albumin, the apparent affinity of yohimbine was reduced about 10 fold and was similar to that of 11-OH-yohimbine.

4 Yohimbine and its metabolites were bound to different extents to plasma proteins, the bound fraction being 82%, 43% and 32% respectively for yohimbine, 11-OH-yohimbine and 10-OH-yohimbine.

5 These results show that the main hydroxylated metabolite of yohimbine in man (11-OH-yohimbine) possesses α_2-adrenoceptor antagonist properties. The discrepancies found in binding studies (i.e. 10 fold lower affinity of 11-OH-yohimbine than yohimbine for α_2-adrenoceptors but similar capacities in blocking biological α_2-adrenoceptor effects in cells) are attributable to the higher degree of binding of yohimbine to plasma protein.

Keywords: Yohimbine: human hydroxylated yohimbine metabolites; α_2-adrenoceptors; adipocyte; platelet

Introduction

Yohimbine is an indole alkaloid obtained from a variety of botanical sources. It is a selective α_2-adrenoceptor antagonist and has been a reference for differentiating α-adrenoceptor subtypes (Goldberg & Robertson, 1983). Due to the widespread locations of α-adrenoceptors in the central and peripheral nervous system (at the pre- or post-synaptic level) yohimbine has a variety of autonomic and psychic effects related to an increase in noradrenaline release. These are presumed to result from a blockade of the presynaptic α_2-adrenoceptors (Peskind *et al.*, 1989; Grunhaus *et al.*, 1989). Various endocrine and metabolic effects observed after yohimbine administration can be due to a direct inhibition of α_2-adrenoceptors or to an effect of the released noradrenaline on other adrenoceptors located on target tissues i.e. β- or α_1-adrenoceptors (Goldberg *et al.*, 1983; Goldberg & Robertson, 1983; Galitzky *et al.*, 1988; Nichols & Ruffolo, 1991).

Yohimbine could be a useful agent in the management of autonomic failure (Onrot *et al.*, 1987) and orthostatic hypotension due to tricyclic antidepressant administration. In combination with desipramine, yohimbine has been claimed to be of value in the treatment of refractory depression (Charney *et al.*, 1986). Recently, yohimbine was shown to induce lipid mobilization in obese subjects, related to activation of the adrenergic nervous system (Berlan *et al.*, 1991) and to enhance saliva secretion in patients with dry mouth after antidepressant therapy (Rispail *et al.*, 1990).

Pharmacokinetic studies carried out in man indicate that yohimbine is rapidly eliminated from the plasma ($t_{\frac{1}{2}}$ of 0.6 h), has a low renal excretion (about 1%), a high plasma clearance, and that its bioavailability after oral administration is low (about 20–30%) and variable (Owen *et al.*, 1987; Guthrie *et al.*, 1990). These data suggested that the low bioavailability of yohimbine may result from a variable first-pass effect. Moreover, in human use, the relatively long-lasting pharmacodynamic effects of yohimbine are inconsistent with its short apparent plasma half-life (Charney *et al.*, 1986; Berlin *et al.*, 1989; Berlan *et al.*, 1991).

Recently, the existence of two hydroxylated metabolites of yohimbine has been demonstrated in human subjects. They were identified as being hydroxylated in the C-10 and the C-11 positions (Le Verge *et al.*, 1992). They were extracted from plasma, urine and detected by a fluorimetric method and then identified by mass spectral and n.m.r. spectra. This new bioavailability study demonstrated that the 11-OH-yohimbine metabolite was largely present in the plasma and exhibited a longer elimination half-life than the parent drug (6 h compared with 1 h).

The present study was conducted in order to evaluate the properties of the recently synthesized hydroxylated metabolites of yohimbine (Levy *et al.*, personal communication) on human α_2-adrenoceptors from platelets and subcutaneous adipose tissue. The binding capacities of the two hydroxylated metabolites to human platelet and adipocyte membrane α_2-adrenoceptors were investigated. The efficacy of 11-OH-yohimbine in inhibiting adrenaline-induced platelet aggregation and the antilipolytic effect of the α_2-adrenoceptor agonist, UK 14304, on isolated adipocytes is also described.

Methods

Adipocyte preparation and lipolysis measurement

Human adipose tissue was obtained from 4 patients, submitted to dermolipectomia, who had been fasted overnight

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before tissue sampling. Fat biopsies (20–30 g) were taken immediately after the induction of general anaesthesia by pentobarbitone.

Isolated adipocytes were obtained as previously described (Mauriege *et al.*, 1987) by collagenase digestion of adipose fragments in Krebs Ringer bicarbonate buffer containing albumin (3.5 g 100 ml^{-1}) (KRB) and glucose (6 mM) at pH 7.4 and 37°C with gentle shaking at around 60 cycles min^{-1}. At the end of the incubation, the adipocytes were filtered through a silk screen and washed three times with KRB buffer to eliminate collagenase. Isolated adipocytes were incubated in 1 ml KRB (pH 7.4) containing glucose (6 mM) at 37°C in polyethylene tubes under a 95% O_2/5% CO_2 gas phase with gentle shaking (60 cycles min^{-1}) in a water bath. Pharmacological agents at suitable dilutions were added to the cell suspension just before the beginning of the assay. After 90 min of incubation, the tubes were placed in an ice bath and 200 µl aliquots of the infranatant were taken for enzymatic determination of glycerol (Wieland, 1957) released in the incubation medium which was used as the index of fat-cell lipolysis.

Platelet preparation and aggregation studies

Blood was taken by antecubital venopuncture from 4 healthy male volunteers. The platelet membranes were prepared as follows: 20–30 ml of blood was collected over 2 ml of 0.16 mM sodium citrate solution and immediately centrifuged at 160 *g* for 10 min at room temperature. The platelet rich plasma (PRP) was collected and immediately used for aggregation tests.

The platelet aggregation technique was described previously (Villeneuve *et al.*, 1985). Briefly, 10 ml of venous blood from 3 healthy male subjects were collected over 2 ml of 0.16 mM sodium citrate solution and immediately centrifuged at 160 *g* for 10 min at room temperature to obtain platelet rich plasma (PRP). The platelet number was adjusted to 350,000–400,000 mm^{-3} with autologous platelet-poor plasma obtained after centrifugation (10,000 *g* for 5 min of the residual blood collected after the first centrifugation) and platelet aggregatory responses to adrenaline (6 µM) were examined by a turbidimetric method using a Coultronic apparatus. The effect of increasing concentrations of yohimbine or 11-OH-yohimbine (0.1, 1 and 10 µM) was studied on the same fresh platelet preparation. Drugs were added to the PRP 5 min before the addition of adrenaline. The aggregatory response was followed until the complete aggregation of the platelets. The velocity of platelet aggregation was measured as the variation of the optical transmission recorded on the chart of the aggregometer (mm per unit time).

Cell membrane preparation and binding studies

Isolated adipocytes, obtained after collagenase digestion, or platelets were washed twice in a hypotonic lysing medium composed of 2.5 mM $MgCl_2$, 1 mM $KHCO_3$, 2 mM Tris-HCl (pH 7.5) and containing ATP (0.2 mM) and several protease inhibitors: benzamidine (100 µM), phenylmethylsulphonyl fluoride (100 µM), leupeptine (1 µg ml^{-1}) and EGTA (3 mM). Crude adipocyte or platelet ghosts were pelleted by centrifugation (45,000 *g*, 15 min) at 4°C and washed twice in the lysing buffer. At the end of the washing procedure, they were resuspended in the same buffer and immediately frozen. The membrane preparation was stored at − 80°C and generally used within 1–2 days.

Binding experiments on membranes from adipocytes or platelets were carried out as previously described (Galitzky *et al.*, 1990a,b) using [^{3}H]-RX 821002 as a ligand. Briefly, thawed frozen membranes were rehomogenized and washed in 50 mM Tris-HCl, 5 mM EDTA buffer before centrifugation (40,000 *g*, 15 min at 4°C). The pellet was washed once in Tris-Mg^{2+} buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) followed by a second centrifugation. The resulting pellet was finally resuspended in buffer and immediately used. Binding studies were carried out in two different buffers; one containing 50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5 and the second was supplemented with 5% bovine albumin. Incubations were conducted in a final volume of 400 µl and carried out at 25°C in a water bath for 40 min under constant shaking at 140 cycles min^{-1}. Non-specific binding was evaluated in the presence of 200 µM adrenaline. At the end ot the incubation, the reaction was stopped by the addition of 4 ml ice-cold incubation buffer followed by rapid filtration under reduced pressure through Whatmann GF/C glass fibre filters placed on a Millipore manifold. The filters were then washed twice with 10 ml of ice-cold incubation buffer, dried and placed in minivials containing 4 ml of liquid scintillation medium (Emulsifier safe, Packard INC, U.S.A.). The radioactivity retained on the filters was measured in a Packard beta counter at an efficiency of 50%. Specific binding was defined as the total binding minus the non-specific binding. In competition experiments, yohimbine, 10-OH-yohimbine and 11-OH-yohimbine were dissolved in the binding buffer, diluted and added to the assay (100 µl) just before the experiments.

Binding to plasma proteins

The binding of yohimbine, 11-OH-yohimbine and 10-OH-yohimbine to plasma proteins was evaluated by the determination of the unbound fraction obtained after ultrafiltration. The unbound fractions were obtained from pooled human plasma aliquots spiked individually with yohimbine, 11-OH-yohimbine and 10-OH-yohimbine at concentrations of 50, 100 and 200 ng ml^{-1}. Ultrafiltration was carried out in a Ultrafree-CL system, model UFC4 LTK 25, with a nominal molecular weigh cut-off of 30,000 g mol^{-1} (Millipore, Paris, France). The lack of non specific drug binding to the ultrafiltration system was checked by ultrafiltration of standard solution of yohimbine, 11-OH-yohimbine and 10-OH-yohimbine at concentrations of 50, 100 and 200 ng ml^{-1} in 0.1 M phosphate buffer saline, pH 7.4. A 2 ml aliquot of plasma or buffer was poured into the upper reservoir cup and the device was centrifuged at 25°C for 45 min at 4,000 *g* (plasma) or 5 min at 1,000 *g* (buffer). Using these conditions, 0.6 ml filtrates were obtained for both plasma and buffer.

Ultrafiltrate concentrations analysis was performed at 30°C by a high performance liquid chromatography (h.p.l.c.) method (Le Verge *et al.*, 1992), with physostigmine as internal standard. The analytical column was a Merck (Darmstadt, Germany) Lichrocart cartridge (250 mm × 4 mm) packed with Lichrosorb Si 60 (7 µm particle size) and the mobile phase was methanol, 0.02 M sodium acetate aqueous solution (95:5; v/v) at a flow rate of 1 ml min^{-1}. Fluorimetric detection was effected by a Schoeffel (Waters, Paris, France) GM 970 spectrophotofluorimeter (excitation at 280 nm, emission above 320 nm at a cell temperature of 15°C). Sample preparation: to 0.5 ml ultrafiltrate were added 5 µg ml^{-1} physostigmine chlorhydrate solution (0.1 ml), 0.5 M disodium phosphate solution pH 11 (0.25 ml) and chloroform (2 ml). The tube was shaken for 5 min and then centrifuged at 5,000 *g* for 5 min. A 1.5 ml aliquot of organic phase was evaporated under nitrogen and the residue was dissolved in 100 µl of a methanol-ethanol (85:15, v/v) mixture; 80 µl samples were injected into the chromatographic system.

Drugs and chemicals

[^{3}H]-RX 821002 (2-(2-methoxy-1,4 benzodioxan-2yl)-2-imidazoline, 43–57 Ci $mmol^{-1}$) was obtained from Amersham (England), (−)-adrenaline bitartrate and bovine albumin (fraction V) from Sigma Chemical CO. (U.S.A.), yohimbine hydrochloride from Houdé (France) and UK 14304 (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline) from Pfizer (U.K.). 10-OH-yohimbine and 11-OH-yohimbine (Figure 1) were

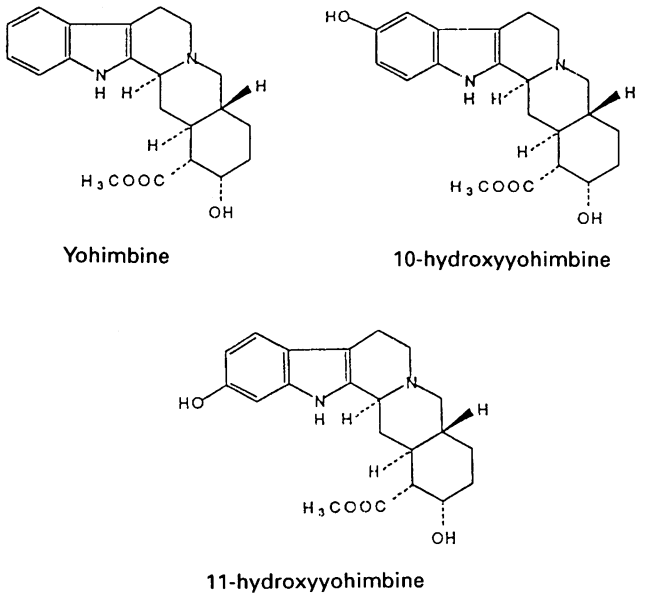


Figure 1 Structural formulae of yohimbine, 10-hydroxyyohimbine and 11-hydroxyyohimbine.

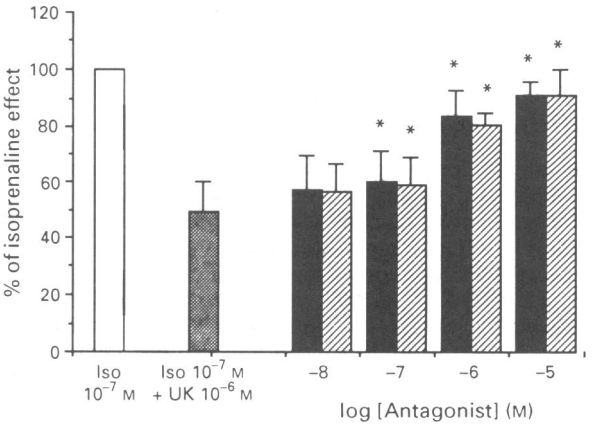


Figure 2 Effect of 11-OH-yohimbine or yohimbine on UK 14304-induced antilipolytic effects in isolated human adipocytes. Lipolysis was stimulated with 10^{-7} M isoprenaline (Iso) and in the presence of 10^{-6} M UK14304 (UK), a selective α_2-adrenoceptor agonist. The antilipolytic effect was then reversed by yohimbine (solid columns) or 11-OH-yohimbine (hatched columns). Values are expressed as percentage of the effect of spontaneous release of glycerol (i.e. basal lipolysis). Results are mean ± s.e.mean (vertical bars) of 4 separate experiments. *Significantly different when compared to isoprenaline plus UK 14304 values.

prepared by chemical synthesis from natural alkaloids and their purity was assessed by u.v. spectrometry, mass spectrometry, ^{13}C n.m.r. and ^{1}H n.m.r. (Le Verge *et al.*, 1992).

Data analysis

Values are means ± s.e.mean. Statistical analysis was made by a paired *t* test and differences were considered significant when *P* was less than 0.05.

Results

Effects of yohimbine and 11-OH-yohimbine on UK 14304-induced inhibition of lipolysis in human adipocytes

The potency of yohimbine and 11-OH-yohimbine was tested on the basis of their ability to suppress *in vitro* the inhibiting effect of 1 μM UK 14304, a selective α_2-adrenoceptor agonist inducing antilipolytic effects, on isoprenaline-induced lipolysis in human isolated adipocytes. This protocol has been described previously and offers a suitable evaluation of the antagonistic capacities of α_2-adrenoceptor antagonists (Galitzky *et al.*, 1988). Isoprenaline stimulated spontaneous lipolysis by about 254% and 1 μM UK 14304 inhibited 51% of the isoprenaline effect. The inhibitory effect of UK 14304 was significantly reduced to 40%, 14.6% and 9% in the presence of 10^{-7} M, 10^{-6} M and 10^{-5} M yohimbine or 11-OH-yohimbine respectively (Figure 2).

Effect of yohimbine and 11-OH-yohimbine on adrenaline-induced aggregation of human platelets

The aggregatory velocity of adrenaline was concentration-dependently reduced by increasing concentrations of yohimbine and 11-OH-yohimbine indicating a progressive occupation of platelet α_2-adrenoceptors (Figure 3). For 0.1 and 1 μM of each compound, the velocity of the aggregatory response to adrenaline was reduced in a concentration-dependent manner. The velocity of the adrenaline-induced aggregation was similarly reduced in the presence of yohimbine and 11-OH-yohimbine (2.1 and 2.3 fold with 0.1 μM and 3.1 and 3.4 fold with 1 μM of yohimbine or 11-OH-yohimbine respectively). For the higher concentration

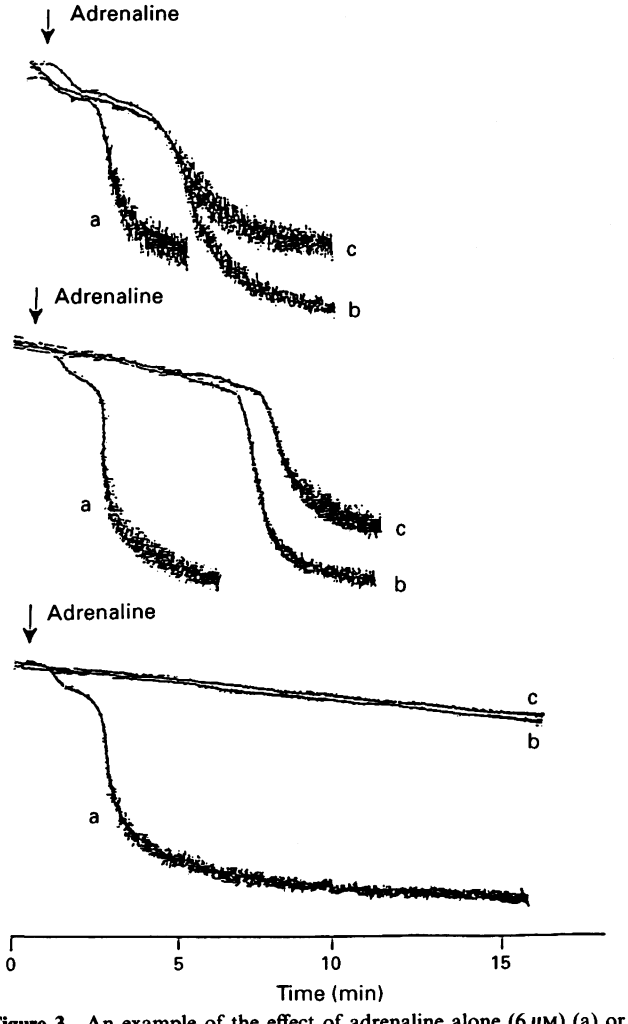


Figure 3 An example of the effect of adrenaline alone (6 μM) (a) or in the presence of various concentrations (0.1, 1 and 10 μM in the upper, central and lower panels respectively) of 11-OH-yohimbine (b) or yohimbine (c) on human platelet aggregation. The antagonists were added 5 min before the addition of adrenaline (indicated by the arrows).

(10 μM) of yohimbine or 11-OH-yohimbine, the aggregatory response to adrenaline was completely blocked.

Binding of yohimbine, 10-OH-yohimbine and 11-OH-yohimbine to adipocyte and platelet membranes

The affinity for α_2-adrenoceptors of the two hydroxylated metabolites of yohimbine was tested in binding studies on membranes from human adipocytes or platelets (two cells bearing α_2-adrenoceptors accepted as prototypes of the α_2-adrenoceptor). The competition studies were performed with 2–3 nM [^{3}H]-RX 821002, a selective radiolabelled α_2-adrenoceptor antagonist suitable for the identification of human platelet of adipocyte α_2-adrenoceptors (Galitzky *et al.*, 1990a,b). The competition curves obtained with increasing concentrations (10^{-11} M to 10^{-5} M) of yohimbine or the two hydroxylated metabolites of yohimbine on adipocyte membranes are shown in Figure 4 and the K_i values (obtained on adipocyte and platelet membranes) after computerized analysis of the curves are shown in Table 1. Yohimbine and its two hydroxylated metabolites impaired [^{3}H]-RX 821002 binding. The K_i values were found to be not significantly different in platelet or adipocyte membranes and the following order of potency was found when binding was realised in a buffer deprived of albumin: yohimbine > 11-OH-yohimbine > 10-OH-yohimbine. In buffer containing 5% albumin, the competition curve of yohimbine was shifted in a log order to the right whereas that of 11-OH-yohimbine and 10-OH-yohimbine were not significantly modified. As shown in Table 1, in the presence of albumin, K_i values for yohimbine were significantly different from those obtained in buffer supplemented with albumin.

Binding of yohimbine, 10-OH-yohimbine and 11-OH-yohimbine to plasma proteins

The concentrations of yohimbine and its two metabolites found in ultrafiltrate from plasma or buffer are given in Table 2. In the range of concentration of yohimbine used (50, 100 and 200 ng ml^{-1}, similar to that usually found in human plasma after oral yohimbine administration), the proportions of each drug bound on plasma proteins were 80.8 to 84%, 22.4 to 39.2% and 39.6 to 46.4% for yohimbine, 10-OH-yohimbine and 11-OH-yohimbine respectively. When buffer was used, the ultrafiltrate concentrations were similar for each drug to the initial added concentrations.

Discussion

The aim of the study was to define the α_2-adrenoceptor blocking potency of the two metabolites of yohimbine in man. Previous results showed that yohimbine has at least two metabolites (Figure 1) that have been identified as hydroxylated metabolites at the C-10 and C-11 positions (Le Verge *et al.*, 1992). One of these metabolites (11-OH-yohimbine) is largely present in the plasma after oral administration of

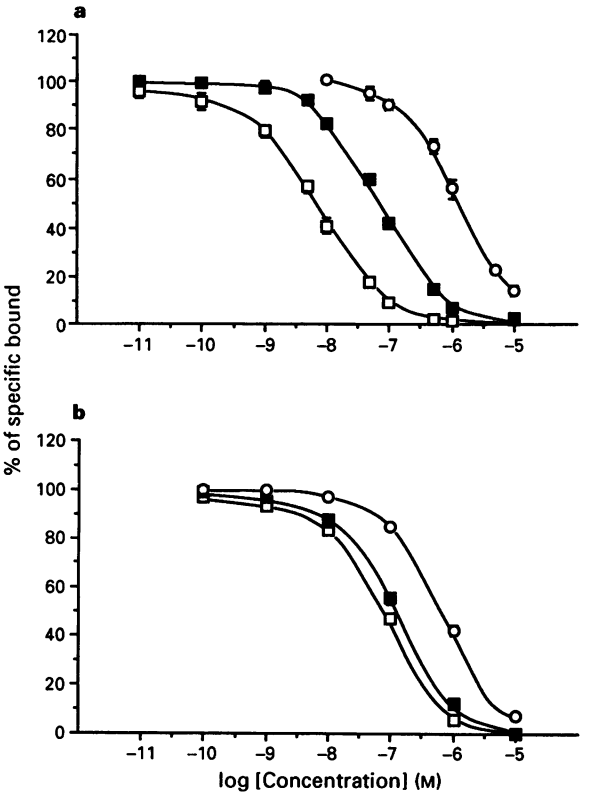


Figure 4 Inhibition of [^{3}H]-RX821002 binding on adipocyte membranes by yohimbine (□), 11-OH-yohimbine (■) and 10-OH-yohimbine (○). Competitions were performed in buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$) without (a) or containing 5% albumin buffer (b) in presence of 2–3 nM [^{3}H]-RX821002. Concentration curves reported are means of 5 experiments.

Table 2 Determination of yohimbine, 10-OH-yohimbine and 11-OH-yohimbine binding to plasma proteins

Plasma concentrations (ng ml^{-1})	50	100	200
	Filtrate concentrations (ng ml^{-1})		
Yohimbine	9.6	17.5	32.1
10-OH-yohimbine	38.8	66.4	121.7
11-OH-yohimbine	30.2	55.9	107.0
Buffer concentrations (ng ml^{-1})	50	100	200
	Filtrate concentrations (ng ml^{-1})		
Yohimbine	50.1	104.7	194.4
11-OH-yohimbine	45.5	94.7	193.9
10-OH-yohimbine	46.4	103.5	192.5

Values are the concentrations in the ultrafiltrate. The unbound fraction was evaluated by the difference between plasma (or buffer) and ultrafiltrate concentration.

Table 1 Inhibition constants of yohimbine, 11-OH-yohimbine and 10-OH-yohimbine on [^{3}H]-RX 821002 binding to human platelet or adipocyte membranes in the absence or presence of albumin

	Platelets; K_i (nM)		*Adipocytes; K_i* (nM)	
	(−)	+ *Albumin*	(−)	+ *Albumin*
Yohimbine	1.65 ± 0.11	15.6 ± 1.82*	2.74 ± 0.67	25.3 ± 2.1*
11-OH-yohimbine	25.1 ± 2.3	28.3 ± 4.7	26.6 ± 3.1	32.6 ± 4.3
10-OH-yohimbine	267 ± 62	241 ± 47	313 ± 19	278 ± 17

Competition experiments were performed in buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$) deprived (−) or containing 5% albumin in the presence of 2–3 nM [^{3}H]-RX821002. The antagonist inhibition data were analysed using INHIBITION, a computerized programme for curve fitting to a one-site inhibition model (Barlow, 1983). Inhibition constants (K_i) were calculated from the equation: $K_i = EC_{50}/(1 + [[^3H]\text{-RX821002}]/K_D)$, where EC_{50} is the concentration of drug displacing 50% of the bound radioligand and K_D the affinity of [^{3}H]-RX821002 determined in saturation experiments. The values reported are means ± s.e.mean of 4–5 determinations. *Significantly different when compared to values obtained in albumin-free medium.

yohimbine and exhibits a longer elimination half-life than the parent drug (6 h versus 1 h, respectively). This supports the hypothesis of a first pass effect and consequently low oral bioavailability of yohimbine in man.

The present study demonstrates that 10-OH-yohimbine or 11-OH-yohimbine have affinity for α_2-adrenoceptors since they displaced [^{3}H]-RX 821002 binding on human adipocyte and platelet membranes. However, the order of potency found (yohimbine > 11-OH-yohimbine > 10-OH-yohimbine) and the calculated K_i (Table 1) require some comment. The affinity of the 11-OH-yohimbine derivative for adipocyte or platelet α_2-adrenoceptors is within the range currently found for various α_2-adrenoceptor antagonists (Lafontan *et al.*, 1992) which induce various pharmacodynamic effects (increase in plasma noradrenaline and non-esterified fatty acid levels). However, 10-OH-yohimbine does not exhibit an affinity compatible with putative pharmacodynamic effects. This point is supported by the lack of pharmacodynamic effects of nicergoline or SKF 104078, two α_2-adrenoceptor antagonists exhibiting a low affinity (K_i > 90 nM) for α_2-adrenoceptors (Lafontan *et al.*, 1992). The interest of 11-OH-yohimbine is that after oral administration of yohimbine in man its concentration is 10 fold higher than that of the parent drug (Le Verge *et al.*, 1992).

Biological measurements of the α_2-adrenoceptor antagonist properties of 11-OH-yohimbine revealed that, whatever the cell model used (human platelets or adipocytes), this compound suppressed, in a concentration-dependent manner, the effect of the stimulation of the α_2-adrenoceptors (i.e. antagonism of the UK 14304-induced antilipolysis in adipocytes or the adrenaline-induced aggregation in platelets). Surprisingly, its potency in suppressing the agonist-induced effect is quite similar to that of yohimbine. This discrepancy between affinities for α_2-adrenoceptors (evaluated in binding studies carried out in medium deprived of proteins) and the α_2-adrenoceptor blocking capacities of yohimbine and 11-OH-yohimbine (evaluated in biological studies carried out in plasma or in medium supplemented with albumin) may be explained by a difference in the protein binding of the substances. The plasma protein binding study of yohimbine, 10-OH-yohimbine and 11-OH-yohimbine showed that yohimbine exhibited a higher affinity for proteins than its two metabolites (in the range of 50 to 200 ng per ml plasma which corresponds to usual concentrations found after oral yohimbine administrations; Le Verge *et al.*, 1992; Berlan *et al.*, 1991). So, in biological studies, the weaker affinity of 11-OH-yohimbine for α_2-adrenoceptors could be counterbalanced by the 3–4 fold higher concentration of its free form. This is supported by binding studies carried out in medium supplemented with protein (albumin) where the apparent affinities (K_i, Table 1) of yohimbine and 11-OH-yohimbine for α_2-adrenoceptors became approximately equivalent.

In addition, yohimbine is a highly lipid-soluble compound which enters the brain (Lambert *et al.*, 1978; Brannan *et al.*, 1991). The 11-hydroxylated metabolite is a less lipid-soluble compound eliminated through renal excretion (Le Verge *et al.*, 1992) and it is likely that this product may have a lower capacity to enter the brain. This compound may be useful (in comparison with yohimbine) in evaluating the relative part of the peripheral and central α_2-adrenoceptors involved in the pharmacodynamic effects of α_2-adrenoceptor antagonists.

The results presented here show that the two hydroxylated metabolites of yohimbine exhibit α_2-adrenoceptor antagonist properties. The main metabolite found in plasma after oral administration in man, 11-OH-yohimbine, has higher affinity for α_2-adrenoceptors in binding studies than the 10-OH-metabolite and is able to suppress the functional effects induced by α_2-adrenoceptor stimulation with the same efficacy as yohimbine in plasma or in medium containing protein. In conclusion, these results give a better understanding of the pharmacodynamic effects of yohimbine in man because the effects of yohimbine are frequently found to be longer lasting than those predicted by pharmacokinetic data and may indicate a measure of peripheral selectivity at later time intervals. This could be also a relevant explanation for its lack of efficacy in some cases correlated with its variable oxidative metabolism. For this purpose a complete pharmacokinetic study of yohimbine and its metabolites is to be undertaken.

References

BARLOW, R.B. (1983). *Biodata Handling with Microcomputers.* Amsterdam: Elsevier Science publishers.

BERLAN, M., GALITZKY, J., RIVIERE, D., FOUREAU, M., TRAN, M.A., FLORES, R., LOUVET, J.P., HOUIN, G. & LAFONTAN, M. (1991). Plasma catecholamine levels and lipid mobilization induced by yohimbine in obese and non-obese women. *Int. J. Obesity*, **15**, 305–315.

BERLIN, I., CRESPO-LAUMONNIER, B., TURPIN, G. & PUECH, A.J. (1989). The alpha-2 adrenoceptor antagonist yohimbine does not facilitate weight loss but blocks adrenaline-induced platelet aggregation in obese subjects. *Therapie*, **44**, 301.

BRANNAN, T., MARTINEZ-TICA, J. & YAHR, M.D. (1991). Effect of yohimbine on brain monoamines: an in vivo study. *J. Neural. Transm.*, **3**, 81–87.

CHARNEY, D.S., PRICE, L.H. & HENINGER, G.R. (1986). Desipramine-yohimbine combination treatment of refractory depression. *Arch. Gen. Psychiatry.*, **43**, 1155–1161.

GALITZKY, J., LARROUY, D., BERLAN, M. & LAFONTAN, M. (1990a). New tools for human fat cell alpha-2 A-adrenoceptor characterization. Identification on membranes and on intact cells using the new antagonist [^{3}H]-RX 821002. *J. Pharmacol. Exp. Ther.*, **252**, 312–319.

GALITZKY, J., SENARD, J.M., LAFONTAN, M., STILLINGS, M., MONTASTRUC, J.L. & BERLAN, M. (1990b). Identification of human platelet α_2-adrenoceptors with a new antagonist [^{3}H]-RX 821002, a 2-methoxy derivative of idazoxan. *Br. J. Pharmacol.*, **100**, 862–866.

GALITZKY, J., TAOUIS, M., BERLAN, M., RIVIERE, D., GARRIGUES, M. & LAFONTAN, M. (1988). Alpha2-antagonist compounds and lipid mobilization: evidence for a lipid mobilizing effect of oral yohimbine in healthy male volunteers. *Eur. J. Clin. Invest.*, **18**, 587–594.

GOLDBERG, M.R., HOLLISTER, A.S. & ROBERTSON, D. (1983). Influence of yohimbine on blood pressure, autonomic reflexes and plasma catecholamines in humans. *Hypertension*, **5**, 772–778.

GOLDBERG, M.R. & ROBERTSON, D. (1983). Yohimbine: a pharmacological probe for study of the alpha$_2$-adrenoceptors. *Pharmacol. Rev.*, **35**, 143–180.

GRUNHAUS, L., TIONGCO, D., ZELNIK, T., FLEGEL, P., HOLLINGSWORTH, P.M. & SMITH, C.B. (1989). Intravenous yohimbine: selective enhancer of norepinephrine and cortisol secretion and systolic blood pressure in humans. *Clin. Neuropharmacol.*, **12**, 106–114.

GUTHRIE, S.K., HARIHARAN, M. & GRUNHAUS, L. (1990). Yohimbine bioavailability in humans. *Eur. J. Clin. Pharmacol.*, **39**, 409–411.

LAFONTAN, M., BERLAN, M., GALITZKY, J. & MONTRASTRUC, J.L. (1992). Alpha2 adrenoceptors in lipolysis: alpha$_2$ antagonists and lipid mobilizing strategies. *Am. J Clin. Nutr.*, **55**, S219–S227.

LAMBERT, G.A., LANG, W.J., FRIEDMAN, E., MELLER, E. & GERSHON, S. (1978). Pharmacological and biochemical properties of isometric yohimbine alkaloids. *Eur. J. Pharmacol.*, **49**, 39–48.

LE VERGE, R., LE CORRE, P., CHEVANNE, F., DÖE DE MAINDREVILLE, M., ROYER, D. & LEVY, J. (1992). Determination of yohimbine and its two hydroxylated metabolites in humans by high-performance liquid chromatography and mass spectral analysis. *J. Chromatogr.*, **574**, 283–292.

MAURIEGE, P., GALITZKY, J., BERLAN, M. & LAFONTAN, M. (1987). Heterogenous distribution of beta- and alpha$_2$-adrenoceptor binding sites in human fat cells from various deposits: functional consequences. *Eur. J. Clin. Invest.*, **17**, 156–165.

NICHOLS, A.J. & RUFFOLO, R.R. (1991). Functions mediated by α-adrenoceptors. In *α-Adrenoceptors: Molecular Biology, Biochemistry and Pharmacology*. ed. Ruffolo, R.R. *Prog. Basic. Clin. Pharmacol.*. Vol. 8. pp. 115–179: Basel: Kager.

ONROT, J., GOLDBERG, M.R., BIAGGIONI, I., WILEY, R.G., HOLLISTER, A.S. & ROBERTSON, D. (1987). Oral yohimbine in autonomic failure. *Neurology*, **37**, 215–220.

OWEN, J.A., NAKATSU, S.L., FENEMORE, J., CONDRA, M., SURRIDGE, D.H.C. & MORALES, A. (1987). The pharmacokinetics of yohimbine in man. *Eur. J. Pharmacol.*, **32**, 577–582.

PESKIND, E.R., VEITH, R.C., DORSA, D.M., GRUMBRECHT, G. & RASKING, M.A. (1989). Yohimbine increases cerebrospinal fluid and plasma norepinephrine but not arginine vasopressine in humans. *Neuroendocrinol.*, **50**, 286–291.

RISPAIL, Y., SCHMITT, L., BERLAN, M., MONTASTRUC, J.L. & MONTASTRUC, P. (1990). Yohimbine increases salivary secretion in depressed patients treated by tricyclic antidepressants. *Eur. J. Clin. Pharmacol.*, **39**, 425–426.

VILLENEUVE, A., BERLAN, M., LAFONTAN, M., CARANOBE, C., BONEU, B., RASCOL, A. & MONTASTRUC, J.L. (1985). Platelet alpha$_2$-adrenoceptors in Parkinson's disease: decrease number in untreated patients and recovery after treatment. *Eur. J. Clin. Invest.*, **15**, 403–407.

WIELAND, O. (1957). Eine enzymatische Methode zur Bestimmung von Glycerin. *Biochem. Z.*, **239**, 313–319.

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The effects of N$^{\omega}$-nitro-L-arginine methyl ester, sodium nitroprusside and noradrenaline on venous return in the anaesthetized cat

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1 The vascular actions of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside and noradrenaline were investigated in cats under chloralose anaesthesia with controlled vascular tone and ventilation. Cardiac output, heart rate, vascular pressures and mean circulatory filling pressure (MCFP) were measured. Total peripheral resistance (TPR) and resistance to venous return (R_{vr}) were calculated from steady-state readings.

2 L-NAME (37 μmol kg^{-1}, i.v.) administered to ten cats receiving noradrenaline (6 nmol kg^{-1} min^{-1}, i.v.) increased aortic pressure by 47.5 ± 7.1 mmHg from 106 mmHg, and MCFP by 1.56 ± 0.36 mmHg from 10.0 mmHg (means ± s.e.means). Mean changes in portal venous pressure, RAP and heart rate were not significant. Cardiac output fell by 29.7 ± 3.3% from 130 ml min^{-1} kg^{-1}. TPR rose by 108 ± 7.2% from 796 mmHg l^{-1} min kg and R_{vr} by 58.4 ± 4.5% from 64 mmHg l^{-1} min kg.

3 Infusion of sodium nitroprusside into cats receiving noradrenaline evoked dose-related falls in aortic pressure, MCFP, TPR and R_{vr}. Changes in portal venous pressure, RAP and heart rate were not significant, and cardiac output fell slightly. After L-NAME, sensitivity to nitroprusside was increased by 139 ± 34% for MCFP, 176 ± 19% for TPR and 351 ± 39% for R_{vr}, and cardiac output rose slightly. The nitroprusside infusion required to restore TPR after L-NAME was estimated to be $5.8 \times 10^{\pm 0.41}$ nmol kg^{-1} min^{-1}, which was approximately three times more than that required to restore MCFP.

4 Infusion of noradrenaline evoked dose-related increases in aortic and portal venous pressures, heart rate, cardiac output, MCFP, TPR and R_{vr}. After L-NAME and nitroprusside (4.4 nmol kg^{-1} min^{-1}, i.v.), TPR and R_{vr} were not significantly different, but MCFP was reduced by 1.76 ± 0.24 mmHg, and cardiac output by 22 ± 1.9%. After subsequent expansion of the circulating blood volume (5–7.5 ml kg^{-1} dextran-saline), mean values for all parameters were restored to their previous levels. Sensitivity to noradrenaline was not significantly altered for heart rate, TPR and R_{vr}, but was reduced by 31.8 ± 12% for MCFP and by 66.5 ± 18% for cardiac output.

5 The depression of cardiac output by L-NAME is attributed to the increase in R_{vr}, partly compensated by the rise in MCFP. For a given rise in MCFP, the increase in R_{vr} was seven times greater after L-NAME than after noradrenaline, and the difference in the relative actions of the two drugs on resistance and capacitance vessels largely accounts for their contrasting effects on venous return. A procedure is suggested for replacement of vascular nitric oxide by nitroprusside infusion and blood volume expansion.

Keywords: Cardiac output; venous return; haemodynamics; vascular capacitance; vascular resistance; nitric oxide; N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME); nitroprusside; noradrenaline

Introduction

Vascular endothelial cells have been shown to release several vasoactive agents either spontaneously or in response to chemical or mechanical stimuli, and an important endothelium-derived relaxing factor is nitric oxide (NO) derived from L-arginine (Ignarro, 1989; Moncada *et al.*, 1991a). Synthesis of NO can be blocked by analogues of L-arginine, such as N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME; Moore *et al.*, 1990; Rees *et al.*, 1990). L-Arginine analogues administered *in vivo* increase peripheral resistance and aortic pressure and reduce cardiac output, evidently by preventing a normal, continuous release of NO from endothelial cells (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Gardiner *et al.*, 1990a,b; Vargas *et al.*, 1991; Klabunde *et al.*, 1991; van Gelderen *et al.*, 1991). Subsequent administration of nitrodilators reduces resistance and aortic pressure but does not restore cardiac output, and the reduction in cardiac output has not yet been explained (Gardiner *et al.*, 1990a; Klabunde *et al.*, 1991; van Gelderen *et al.*, 1991; Widdop *et al.*, 1992).

Cardiac output depends in part on regulation of venous return by the balance between capacitance and resistance in the peripheral circulation (Guyton *et al.*, 1973). Changes in total vascular capacitance can be observed by measurement of mean circulatory filling pressure (MCFP; Bower & O'Donnell, 1991). Resistance to venous return (R_{vr}) is the combined resistance of all vessels up to the right atrium from the venous locations where the intravascular pressure is equal to MCFP, and it accounts for about 5% of the total peripheral resistance (TPR). It depends not only on venous calibre, but also on the positions of the MCFP values, which will migrate if blood volume is redistributed by changes in arterial resistance (Guyton *et al.*, 1959). Both MCFP and R_{vr} depend on the veins, and available evidence indicates that venous endothelium is less efficient than arterial endothelium as a source of relaxing factor. Isolated veins showed only weak responses to endothelium-dependent relaxing agents which were effective in arteries, although both types of vessel were relaxed by sodium nitroprusside and by relaxing factor released from arterial endothelium (De Mey & Vanhoutte,

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1982; Gruetter & Lemke, 1986; Seidel & La Rochelle, 1987; Ignarro *et al.*, 1988). Abolition of NO synthesis in a perfused cat hindlimb increased resistance mainly in large-bore resistance arteries, with much less effect on venous resistance (Ekelund & Mellander, 1990). However, changes in MCFP and R_{vr}, and hence in cardiac output, are responses of the whole body which cannot be extrapolated from behaviour of isolated vessels or perfused organs, and there are no reports on how they are affected by endogenous NO.

Investigation of the haemodynamic role of NO was complicated by the possibility that L-arginine analogues may increase vascular sensitivity to catecholamines (Seidel & La Rochelle, 1987; Moncada *et al.*, 1991b; Vo *et al.*, 1991) and may therefore modify responses to noradrenaline and to physiological changes in vasomotor tone. It is not certain whether the sensitivity increases because catecholamines stimulate NO release or whether the change is a secondary consequence of NO deficiency or altered vascular tone (Vargas *et al.*, 1990; Chyu *et al.*, 1992). Tests to detect modulation of NO release have not as yet been performed before and after replacement of the potentially variable endogenous secretion by equivalent controlled administration of a nitrodilator.

This paper has three main objectives. Firstly, to describe and relate the changes in MCFP, R_{vr} and cardiac output evoked by the direct vascular actions of L-NAME. Secondly, to measure the reversal of these changes by sodium nitroprusside, in order to devise a procedure for replacement of endogenous NO by a nitrodilator. Thirdly, to discover whether this replacement therapy alters the haemodynamic response to noradrenaline. In order to isolate the direct cardiovascular actions of the drugs, reflexes were suppressed, aortic pressure was maintained by infusion of noradrenaline, and positive pressure ventilation controlled intrathoracic pressure and blood gas tensions. The results obtained provide a basis for investigation of the more complex interactions in intact animals.

Methods

Male cats (3.5–5.3 kg) were used under anaesthesia induced with chloroform and maintained with chloralose (70 mg kg^{-1}, i.a.). Preliminary surgery took 2 h; spontaneous respiratory movements and autonomic reflexes were then suppressed by continuous i.v. infusion of a blocking solution described below, and positive pressure ventilation was applied at 50 breaths min^{-1}. Before starting the infusion, the level of anaesthesia was tested by forelimb toe pinch, corneal stimulation and sudden noise or vibration; a supplementary dose of chloralose (35–47 mg kg^{-1}) was given if responses were not absent or weak. Thereafter, reliance was placed on the well established stability of chloralose anaesthesia, and the experiment was terminated by overdose of anaesthetic at a maximum of 5 h after starting the infusion. Previous experiments on spontaneously breathing cats had shown that the anaesthetic regime maintained stable surgical anaesthesia for at least this period. In three cats, the blocking infusion was stopped at the end of the experiment; spontaneous respiration returned in 30–60 min, and it was confirmed that full surgical anaesthesia persisted at 5 h after commencement of the infusion.

The trachea was cannulated for artificial ventilation. Double-lumen catheters (4F; Vygon) passed down the left femoral and left jugular veins served respectively for continuous infusion of drugs and for injection of thermal indicator and measurement of right atrial pressure (RAP). Methods used for measurement of cardiac output by thermal dilution and of aortic, portal venous and right atrial pressures have been described elsewhere (Bower & Ead, 1976; Barnes *et al.*, 1980; 1986), as has the technique for MCFP measurement by circulatory arrest (Bower & O'Donnell, 1991). From analysis of variance of data from four cats in Figure 3, coefficients of variation were 0.044 for cardiac output and 0.029 for MCFP.

Apparatus

Heart rate and pressure signals were recorded by a four-channel penwriter (Multitrace 4; Lectromed Ltd.) and were transmitted to a computer (M290; Olivetti Ltd, with AS-1F interface; Cambridge Electronic Design) which sampled the inputs at 1000 Hz and displayed traces of the four signals. For measurement of MCFP, the heart rate trace was displaced off screen, the pressure traces were set to equal zero positions and sensitivities, and the display was set to stop after one sweep on closure of a switch at the moment of circulatory arrest. On demand, the computer calculated mean values of the input signals over a preset period (normally 8 s) and stored them in a disc file. Oxygen tension (Pa_{O_2}), carbon dioxide tension (Pa_{CO_2}) and pH in samples of arterial blood were measured by means of a blood micro system analyser (BMS3 Mk2; Radiometer). Drugs were infused by means of peristaltic pumps (Minipuls 3; Gilson).

Drugs and chemicals

The following drugs were used in the experiments: chloroform (Fisons Ltd.), α-chloralose (B.D.H. Ltd.), vecuronium bromide (Organon Teknika Ltd.), [β-mercapto-β,β-cyclopentamethylenepropionyl[1],-O-Me-Tyr[2],Arg[8]]-vasopressin (Manning compound; Sigma), captopril (Sigma), noradrenaline bitartrate (Sigma), sodium nitroprusside (B.D.H. Ltd.), N^{ω}-nitro-L-arginine methyl ester hydrochloride (Sigma), heparin (Sigma). Nitroprusside was dissolved in 3% Na citrate solution and was protected from light, noradrenaline was dissolved in a solution of 5% glucose and 1% $Na_2S_2O_5$ at pH 3.6, and other drugs were dissolved in 0.9% NaCl solution. Contrast medium for fluoroscopy was 54% Na iothalamate injection B.P. (Conray 325; May & Baker Ltd.). Blood volume was expanded with 6% dextran 150 in 0.9% NaCl solution (Dextraven 150; Fisons Ltd.).

The blocking solution used to suppress spontaneous respiratory movements, cardiovascular reflexes and the effects of circulating angiotensin I and vasopressin was based on that described by Gardiner *et al.* (1990b), and it contained vecuronium (0.5 mg ml^{-1}), hexamethonium (10 mg ml^{-1}), captopril (2 mg ml^{-1}) and Manning compound (20 μg ml^{-1}). The solution was administered by continuous infusion (0.5 ml kg^{-1} + 0.5 ml kg^{-1} h^{-1}, i.v.), and aortic pressure was restored to the middle of its safe range by infusion of noradrenaline (6 nmol kg^{-1} min^{-1}, i.v.). Table 1 shows the cardiovascular effects of this procedure. The immediate consequence of the positive pressure ventilation was a rise in RAP. Aortic pressure and TPR were reduced by 18%. MCFP was not significantly changed, and cardiac output was maintained by a 13% fall in R_{vr} helped by a 15% increase in heart rate.

Experimental procedure

The cat lay on its back on a heated table, and all manometers were zeroed by reference to a Marriotte's bottle filled with saline to the level of half the height of the cat's sternum. After completion of the surgery, heparin was administered (5 mg kg^{-1}, i.v.), anaesthesia was assessed and controlled ventilation was instituted. When circulatory conditions were steady at 5–15 min after any change, a computer sample of the heart rate and vascular pressures and a cardiac output measurement was taken, immediately followed by a measurement of MCFP. The measurements were repeated after 2–3 min to provide at least two samples at each steady state of the experiment. The computer derived TPR from:

(aortic pressure – RAP)/(cardiac output) mmHg l^{-1} min kg,

R_{vr} from:

Table 1 Cardiovascular parameters in eighteen cats under chloralose anaesthesia before and after infusion of blocking solution and noradrenaline and establishment of positive pressure ventilation as described in Methods

	Before blockade	*After blockade*	*Mean change*
Aortic pressure (mmHg)	140 ± 3.6	114 ± 4.3	−26.1 ± 3.3***
Portal venous pressure (mmHg)	6.58 ± 0.28	7.60 ± 0.32	+1.02 ± 0.24***
Right atrial pressure (mmHg)	0.563 ± 0.19	1.91 ± 0.24	+1.35 ± 0.24***
Mean circulatory filling pressure (mmHg)	10.64 ± 0.49	10.5 ± 0.50	−0.131 ± 0.25
Heart rate (beats min^{-1})	202 ± 7.0	232 ± 5.9	+29.9 ± 6.3***
Cardiac output ($ml\ min^{-1}\ kg^{-1}$)	147 ± 4.3	143 ± 4.1	−3.86 ± 3.2
Left ventricular stroke work ($mJ\ beat^{-1}\ kg^{-1}$)	13.8 ± 0.64	9.56 ± 0.59	−4.20 ± 0.41***
Total peripheral resistance ($mmHg\ l^{-1}\ min\ kg$)	962 ± 37	786 ± 28	−176 ± 33***
Resistance to venous return ($mmHg\ l^{-1}\ min\ kg$)	69.3 ± 3.2	60.4 ± 2.6	−8.9 ± 2.5**

Values are mean ± s.e.mean.
Probabilities of changes: $^{**}P<0.01$; $^{***}P<0.001$ (paired t test).

$$(\text{MCFP} - \text{RAP})/(\text{cardiac output})\ \text{mmHg}\ l^{-1}\ \text{min kg},$$

and left ventricular stroke work from:

$$(\text{aortic pressure} \times \text{cardiac output})/(\text{heart rate} \times 0.0075)\ \text{mJ beat}^{-1}\ \text{kg}^{-1},$$

where cardiac output is in $1\ min^{-1}$. Dose-response relationships for nitroprusside and noradrenaline were obtained by varying infusion from a middle to a minimum rate, then to a maximum, and back to the middle rate.

Comparisons were made between experiment means based on at least two consecutive steady-state computer, cardiac output and MCFP samples at each stage, and estimates of variance for groups of experiments were derived by analysis of variance of the combined experiment means. Group means are quoted plus and minus the standard error of the mean unless otherwise stated; they were compared by a paired Student's t test. Mean linear regression coefficients for groups of experiments were derived from the combined sums of squares of errors calculated for each experiment, and were compared by t test (Snedecor & Cochran, 1967). Linearities of log dose-response relationships were tested by comparing the upper and lower segments of responses by paired and unpaired t tests and by t test of regression coefficients.

Results

The vascular response to N^{ω}-nitro-L-arginine methyl ester

Table 2 shows cardiovascular changes which followed administration of L-NAME ($37\ \mu mol\ kg^{-1}$, i.v.) in ten consecutive experiments, and Figure 1 shows the relationships between cardiac output, RAP and MCFP. Arterial blood parameters were: pH, 7.36 ± 0.01; Pa_{O_2}, 86.6 ± 3.5 mmHg; Pa_{CO_2}, 34.4 ± 1.4 mmHg; haematocrit, 40.6 ± 1.14%. When L-NAME was given, aortic pressure rose over 3–6 min to a transient peak in some cats or to a steady level in others. Measurements were taken when conditions were stable, after 10–20 min. In all cases aortic pressure and MCFP rose, and cardiac output fell by 29.7 ± 3.3%. The reduced output was associated with a rise in R_{vr} of 58.4 ± 4.5% (Figure 1) and the increased aortic pressure with a rise in TPR of 108 ± 7.2%. Changes in RAP, portal venous pressure, heart rate and left ventricular stroke work were usually small and inconsistent, but in one cat RAP rose by 4.5 mmHg and cardiac output and stroke work fell by 44% and 47% respectively. Four cats received second doses of L-NAME (37–370 $\mu mol\ kg^{-1}$) at times from 20 min to 170 min later; none showed any further significant increase in MCFP, TPR or R_{vr}.

Table 2 Cardiovascular parameters in ten cats before and after i.v. administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME, $37\ \mu mol\ kg^{-1}$)

	Before L-NAME	*After L-NAME*	*Mean change*
Aortic pressure (mmHg)	106 ± 7.7	153 ± 6.9	+47.5 ± 7.1***
Portal venous pressure (mmHg)	7.72 ± 0.51	7.88 ± 0.57	+0.153 ± 0.38
Right atrial pressure (mmHg)	1.70 ± 0.30	2.28 ± 0.63	+0.572 ± 0.46
Mean circulatory filling pressure (mmHg)	10.0 ± 0.58	11.6 ± 0.72	+1.56 ± 0.36**
Heart rate (beats min^{-1})	230 ± 5.9	229 ± 7.4	−0.626 ± 6.5
Cardiac output ($ml\ min^{-1}\ kg^{-1}$)	130 ± 5.4	91.8 ± 3.5	−38.6 ± 4.3***
Left ventricular stroke work ($mJ\ beat^{-1}\ kg^{-1}$)	8.28 ± 0.99	8.33 ± 0.62	+0.055 ± 0.82
Total peripheral resistance ($mmHg\ l^{-1}\ min\ kg$)	796 ± 45	1660 ± 73	+860 ± 57***
Resistance to venous return ($mmHg\ l^{-1}\ min\ kg$)	64 ± 3.3	101 ± 5.5	+37.4 ± 2.9***

Reflexes were suppressed as described in Methods.
Values are mean ± s.e.mean.
For changes evoked by L-NAME: $^{**}P<0.01$; $^{***}P<0.001$ (paired t test).

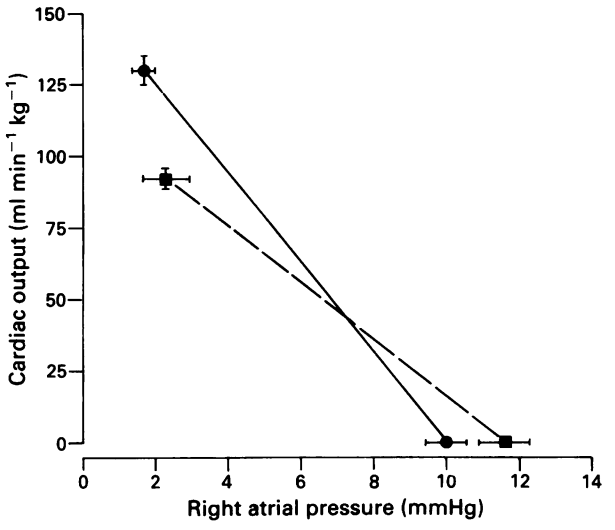


Figure 1 The relationship of cardiac output and right atrial pressure to mean circulatory filling pressure (MCFP) before (●) and after (■) i.v. administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 37 μmol kg^{-1}). Values are mean ± s.e.mean; lines join related output and MCFP values, and their slopes are inversely proportional to R_{vr}. Reflexes were suppressed as described in Methods.

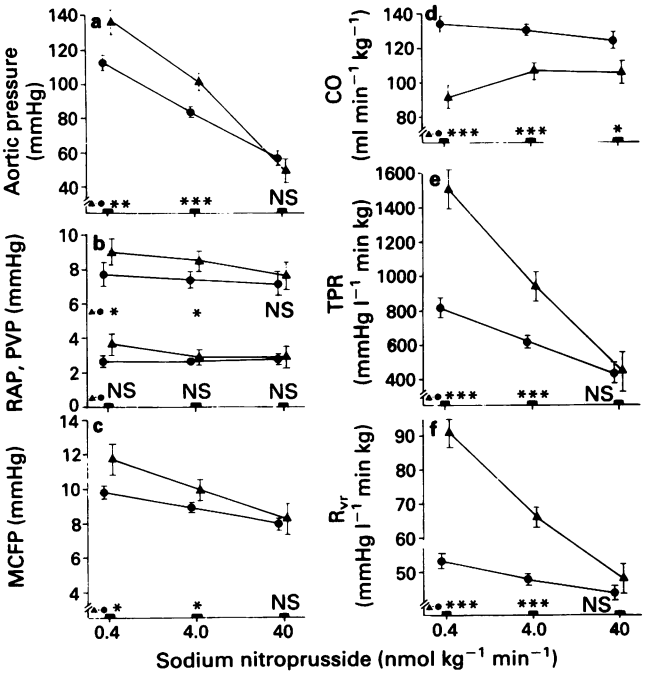


Figure 2 Cardiovascular parameters during i.v. infusion of sodium nitroprusside in four cats before (●) and after (▲) administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 37 μmol kg^{-1}): (a) aortic pressure; (b) portal venous pressure (PVP; upper lines) and right atrial pressure (RAP; lower lines); (c) mean circulatory filling pressure (MCFP); (d) cardiac output (CO); (e) total peripheral resistance (TPR); (f) resistance to venous return (R_{vr}). Reflexes were suppressed as described in Methods. Values are mean ± s.e.mean. For differences between pairs of points: $*P<0.05$; $**P<0.01$; $***P<0.001$ (paired t test).

The vascular response to sodium nitroprusside

The response to sodium nitroprusside infusion over the range 0.4–40 nmol kg^{-1} min^{-1} was explored in four cats before and after administration of L-NAME. Throughout this series, heart rate was 253 ± 3.9 beats min^{-1} and did not vary significantly. Other results are shown in Figure 2 and Table 3.

With increasing nitroprusside dose before L-NAME, aortic pressure, MCFP, TPR and R_{vr} fell progressively (Figure 2a, c,e,f). Variations in portal venous pressure and RAP were small and inconsistent (Figure 2b). Mean cardiac output was 130 ± 2.3 ml min^{-1} kg^{-1} and changed little (Figure 2d), but the mean regression coefficient indicated a significant fall (Table 3). The falls in MCFP, TPR and R_{vr} were linearly related to the logarithm of the nitroprusside dose (correlation coefficient $r>0.73$, $P<0.01$), and their mean regression coefficients were all significant (Table 3; $P<0.001$).

After L-NAME (37 μmol kg^{-1}), aortic pressure, MCFP, TPR and R_{vr} at the lower nitroprusside doses were higher and changed more with increasing infusion rate (Figure 2a, c,e,f). Portal venous pressure and RAP still showed little variation (Figure 2b). Mean cardiac output fell to 102 ± 3.6 ml min^{-1} kg^{-1} after L-NAME, and changes were small and variable (Figure 2d), but the mean regression coefficient indicated a rise in output which differed significantly from the fall observed previously (Table 3). The enhanced sensitivities of MCFP, TPR and R_{vr} to nitroprusside were reflected in their regression coefficients (Table 3), which were increased by 139 ± 34%, 176 ± 19% and 351 ± 39% respectively.

In the absence of nitroprusside before L-NAME, the mean value for TPR was 891 ± 87 mmHg l^{-1} min kg and for MCFP was 10.4 ± 0.67 mmHg. The nitroprusside infusion required after L-NAME to reduce TPR to its previous value was calculated from the regression data to be $5.8 \times 10^{\pm 0.41}$ nmol kg^{-1} min^{-1}. Comparison of nitroprusside doses calculated for each cat indicated that the dose required for restoration of TPR was $3.3 \times 10^{\pm 0.51}$ times greater than that for MCFP.

The vascular response to noradrenaline

The response to noradrenaline infusion over the range 2–18 nmol kg^{-1} min^{-1} was explored in four cats before and after administration of L-NAME and institution of replacement therapy (Figure 3). After L-NAME (37 μmol kg^{-1}),

Table 3 Linear regression coefficients of cardiovascular parameters on log dose of sodium nitroprusside in four cats before and after i.v. administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 37 μmol kg^{-1})

	Before L-NAME	*After L-NAME*
Cardiac output (ml min^{-1} kg^{-1} per log unit)	−4.79 ± 1.8	+7.05 ± 3.3**
Mean circulatory filling pressure (mmHg per log unit)	−0.788 ± 0.12	−1.88 ± 0.24***
Total peripheral resistance (mmHg l^{-1} min kg per log unit)	−193 ± 14	−532 ± 34***
Resistance to venous return (mmHg l^{-1} min kg per log unit)	−4.75 ± 0.92	−21.4 ± 1.6***

Reflexes were suppressed as described in Methods.
Values are mean ± s.e.mean.
For all coefficients, $P<0.05$; for changes evoked by L-NAME: $**P<0.01$; $***P<0.001$ (t test).

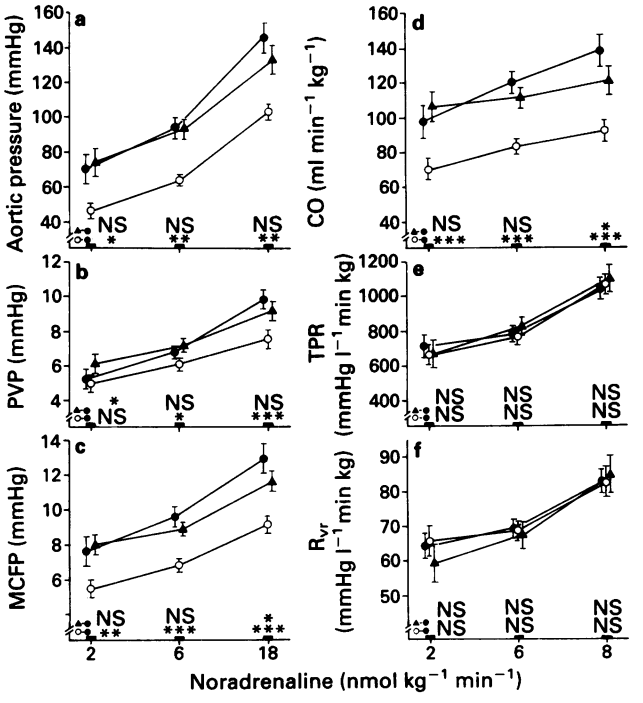


Figure 3 Cardiovascular parameters during i.v. infusion of noradrenaline in four cats: (●) before N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME); (○) during continuous infusion of sodium nitroprusside (4.4 nmol kg^{-1} min^{-1}) after administration of L-NAME (37 μmol kg^{-1}); (▲) during nitroprusside infusion after L-NAME and after administration of dextran-saline solution (6.9 ± 0.6 ml kg^{-1}). (a) Aortic pressure; (b) portal venous pressure (PVP); (c) mean circulatory filling pressure (MCFP); (d) cardiac ouput (CO); (e) total peripheral resistance (TPR); (f) resistance to venous return (R_{vr}). Reflexes were suppressed as described in Methods. Values are mean ± s.e.mean. For differences between pairs of points: $*P<0.05$; $**P<0.01$; $***P<0.001$ (paired t test).

sodium nitroprusside (4.4 nmol kg^{-1} min^{-1}) was infused continuously for the rest of the experiment and the series of tests was repeated. The circulating blood volume was then expanded with a volume of dextran-saline solution judged sufficient to restore MCFP to its previous value (5–7.5 ml kg^{-1}; mean 6.9 ml kg^{-1}), and the tests were repeated again.

With increasing noradrenaline before L-NAME, heart rate rose by 53.5 ± 11 beats min^{-1} (25.6 ± 5.3%) from 209 beats min^{-1}. All vascular pressures except RAP rose progressively, and the increases were associated with rises in cardiac output, TPR and R_{vr} (Figure 3). Mean RAP was 1.35 ± 0.16 mmHg and did not vary significantly.

After administration of L-NAME and commencement of nitroprusside infusion, heart rate values and changes were not significantly altered, but vascular pressures and cardiac output were depressed. At a noradrenaline dose of 6 nmol kg^{-1} min^{-1}, L-NAME plus nitroprusside reduced MCFP by 1.76 ± 0.24 mmHg from 8.69 ± 0.65 mmHg and cardiac output by 22.0 ± 1.9% from 108 ± 5.3 ml min^{-1} kg^{-1} ($P<0.01$; paired t test). All parameters except RAP varied with noradrenaline (Figure 3); mean RAP was 1.16 ± 0.21 mmHg and did not vary significantly. Values for TPR and R_{vr} did not differ significantly from those observed before L-NAME (Figure 3e,f).

After expansion of the circulating blood volume, heart rate values and changes were not significantly altered, but vascular pressures and cardiac output were restored to values close to those observed before L-NAME. All parameters except RAP varied with noradrenaline (Figure 3); mean RAP was 1.59 ± 0.12 mmHg and did not vary significantly. Changes in cardiac output were small, but the mean regression coefficient indicated a significant rise (Table 4). Values for TPR and R_{vr} did not differ significantly from those observed before L-NAME (Figure 3e,f).

Although MCFP and cardiac output values at 2–6 nmol kg^{-1} min^{-1} noradrenaline did not differ significantly from their corresponding values before L-NAME, at 18 nmol kg^{-1} min^{-1} noradrenaline both were lower than before (Figure 3c, d). Log dose-response relationships for heart rate, cardiac output, MCFP, TPR and R_{vr} before and after administration of L-NAME, nitroprusside and dextran-saline solution did not depart significantly from linearity, and changes were assessed by calculation of mean linear regression coefficients (Table 4). After treatment, sensitivity to noradrenaline was reduced by 31.8 ± 12% for MCFP and by 66.5 ± 18% for cardiac output. For heart rate, TPR and R_{vr}, the differences between the responses before and after treatment were not significant.

Discussion

The rise in MCFP of 1.5 mmHg evoked by L-NAME is modest but significant. For comparison, increments of up to 6 mmHg were elicited by strong reflex vasomotor stimulation in hypoxia (Bower & O'Donnell, 1991). Changes in MCFP are not proportional to their initial values and cannot be compared directly with resistance changes. However, if nitroprusside was infused at a rate sufficient to restore TPR to its level before L-NAME, MCFP was reduced by 1.8 mmHg below its previous value, and the circulating blood volume had to be expanded by 7 ml kg^{-1} to restore it. The estimated replacement dose of nitroprusside was about one third as great for MCFP as for TPR. These observations are consistent with reports that less NO is released by venous than by arterial endothelium.

The changes in TPR were comparable to those reported for the rat with intact and with suppressed reflexes (Gardiner

Table 4 Linear regression coefficients of cardiovascular parameters on log dose of noradrenaline in four cats before and after i.v. administration of N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 37 μmol kg^{-1}), sodium nitroprusside (4.4 nmol kg^{-1} min^{-1}) and dextran-saline (6.9 ± 0.6 ml kg^{-1})

	Before L-NAME	*After L-NAME*
Heart rate (beats min^{-1} per log unit)	+55.7 ± 3.8	+53.0 ± 7.7
Cardiac output (ml min^{-1} kg^{-1} per log unit)	+46.3 ± 7.1	+15.5 ± 4.7***
Mean circulatory filling pressure (mmHg per log unit)	+5.56 ± 0.50	+3.79 ± 0.42**
Total peripheral resistance (mmHg l^{-1} min kg per log unit)	+321 ± 42	+415 ± 43
Resistance to venous return (mmHg l^{-1} min kg per log unit)	+17.6 ± 2.3	+23.1 ± 2.5

Reflexes were suppressed as described in Methods.
Values are mean ± s.e.mean.
For all coefficients, $P<0.05$; for changes evoked by L-NAME: $**P<0.01$; $***P<0.001$ (t test).

et al., 1990a,b; Van Gelderen *et al.*, 1991). In the cat, L-NAME has been reported to increase resistance to perfusion of limb tissues by more than 90% (Ekelund & Mellander, 1990; Bellan *et al.*, 1991). McMahon *et al.* (1992) observed rises in aortic pressure of 37% in reserpine-treated cats and 27% in normal cats. Van Gelderen *et al.* (1991) reported that in the cat, unlike the rat, changes in aortic pressure, cardiac output and peripheral resistance evoked by L-NAME were small; they gave L-NAME in progressively increasing doses to cats with intact reflexes, so changes may have been obscured by homeostatic adaptations.

The proportionate rise in R_{vr} was half as great as that in TPR, which is substantially more than would be expected from the relative effects of L-NAME on venous and arterial resistances in skeletal muscle (Ekelund & Mellander, 1990). There may therefore have been a contribution to the rise in R_{vr} from redistribution of blood volume or flow in addition to any increase in resistance due to actual venoconstriction. The lack of change in portal venous pressure argues against pooling of blood in the splanchnic vessels by specific constriction of hepatic vessels. Available information indicates that arterial constriction is general (Gardiner *et al.*, 1990a), and would lead to volume redistribution from venous to arterial compartments with migration of MCFP locations towards the sites of arteriolar constriction, thus lengthening the path for venous return.

The increase in R_{vr} evoked by L-NAME dominated venous return and was largely responsible for the 30% depression of cardiac output (Figure 1). The rise in MCFP partly countered the effect of the rise in resistance, and in its absence output would have fallen by about 40%. L-NAME is known to induce constriction of coronary vessels (Woodman & Dusting, 1991; Jones & Brody, 1992; Ueeda *et al.*, 1992; Lamontagne *et al.*, 1992). Coronary vasoconstriction would be partly compensated by the rise in aortic pressure, and Sarnoff *et al.* (1960) reported that if coronary flow was adequate, variation in flow had little effect on cardiac contractility. In the present experiments cardiac contractility was not specifically investigated, but heart rate did not change significantly and there was little evidence that the heart impeded venous return after L-NAME administration. In most cats, RAP rose or fell by less than 0.5 mmHg and changes in left ventricular stroke work were small and inconsistent, as the fall in venous return approximately matched the rise in aortic pressure. In the one cat in which RAP rose by 4.5 mmHg and cardiac output and stroke work fell substantially, ventricular performance may have been impaired by NO deficiency. Gardiner *et al.* (1990a) noted that the fall in output observed after L-NAME in the rat with intact reflexes was not associated with a rise in RAP, despite a substantial fall in heart rate, and Widdop *et al.* (1992) reported that atropine abolished the fall in heart rate but gave no lasting improvement in cardiac output.

The depression of aortic pressure, MCFP, cardiac output and TPR observed during infusion of sodium nitroprusside is consistent with previous reports on the dog, cat and rat (Pouleur *et al.*, 1980; Ignarro *et al.*, 1981; Thomas *et al.*, 1988). The fall in cardiac output with no change in RAP indicates that the fall in MCFP here outweighed the benefit to venous return of the reduced R_{vr}. After administration of L-NAME, there was a marked increase in the sensitivity of resistance and MCFP to nitroprusside, which is consistent with up-regulation of soluble guanylate cyclase in the absence of NO (Shirasaki & Su, 1985; Moncada *et al.*, 1991b; Gardiner *et al.*, 1991). The sensitivity of resistance increased more than did that of MCFP, perhaps due to a greater basal release of NO in resistance vessels. A consequence for venous return was that the fall in R_{vr} now outweighed the depression of MCFP and cardiac output tended to rise slightly. Nevertheless, a nitroprusside infusion which restored TPR depressed MCFP below its previous value, so venous return did not recover fully until MCFP was restored by expansion of the circulating blood volume.

The MCFP increments evoked by noradrenaline were about half as great as those induced by similar doses of adrenaline in the dog under total spinal anaesthesia (Guyton *et al.*, 1958). Unlike adrenaline in the dog, noradrenaline also significantly increased R_{vr}, but the changes in MCFP were dominant and venous return rose by 41%. For a given rise in MCFP, the increase in R_{vr} was seven times greater after L-NAME than after noradrenaline, and the contrast between the effects of L-NAME and noradrenaline on venous return is due mainly to the difference in their relative actions on resistance and capacitance vessels. Cardiac stimulation by noradrenaline would have helped the heart to accept the augmented venous return without a rise in RAP, but it could not by itself have generated an output increase of the magnitude observed (Guyton *et al.*, 1958; Barnes *et al.*, 1980; 1986). Indeed, after replacement of NO by nitroprusside, the output increase evoked by noradrenaline was reduced by two thirds although the cardiac acceleration was unchanged (Table 4).

After NO replacement, MCFP responses to noradrenaline were depressed sufficiently to account for the smaller changes in venous return, and resistance responses were not significantly altered. The dose of L-NAME used was enough to suppress basal release of NO, as in the rat (Vargas *et al.*, 1991), and would be expected to reduce evoked release. A higher dose of L-NAME (370 μmol kg^{-1}) with replacement therapy was tested in one cat, but there was no evidence of increased noradrenaline sensitivity. Potentially, noradrenaline might release NO not only directly but also by increasing endothelial shear stress (Rubanyi *et al.*, 1986; Hutcheson & Griffith, 1991; Lamontagne *et al.*, 1992), and the aortic pressure and cardiac output increments evoked by the transition from low to high dose imply an increase of about 90%. The need to keep within safe levels of aortic pressure limited the range of noradrenaline doses used, compared to those possible *in vitro*. Higher local concentrations may perhaps be a more effective stimulus to NO release (Vo *et al.*, 1991), but local constriction reduces rather than increases shear stress unless flow is maintained by a rise in aortic pressure. The present results therefore imply that noradrenaline in doses consistent with physiological levels of aortic pressure has little effect on endothelial NO secretion either directly or through increased shear stress.

For replacement of endothelial NO, sodium nitroprusside provides a readily controllable source of NO (Feelisch & Noack, 1987; Bates *et al.*, 1991). At low RAP values, cardiac output varies with MCFP and is simpler to measure. A satisfactory replacement therapy appears to be to administer L-NAME, to infuse nitroprusside at a rate adjusted to restore TPR, and to expand circulating blood volume until cardiac output is restored as well. The responsiveness of capacitance vessels is then reduced by about one third, but in other respects the general haemodynamic conditions resemble those prevailing before L-NAME. Possible changes in cardiac output distribution remain to be studied. Subject to these limitations, the procedure is potentially useful for *in vivo* investigation of NO secretion. Preliminary tests in unblocked, chloralose-anaesthetized cats required nitroprusside doses similar to those reported here.

In conclusion, administration of L-NAME evoked a modest increase in MCFP opposed by a substantial increase in R_{vr} which accounted for the observed decrease in cardiac output. Infusion of nitroprusside reversed the increases, but the dose adequate to restore the resistances was excessive for MCFP and therefore left cardiac output depressed; MCFP and cardiac output could however be restored by expansion of the blood volume. Resistance responses to noradrenaline were not significantly changed and MCFP responses were slightly depressed by this replacement of endogenous NO.

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References

AISAKA, K., GROSS, S.S., GRIFFITH, O.W. & LEVI, R. (1989). N^G-methyl-arginine, an inhibitor of endothelium-derived nitric oxide synthesis. Is a potent pressor agent in the guinea pig: does nitric oxide regulate blood pressure in vivo? *Biochem. Biophys. Res. Commun.*, **160**, 881–886.

BARNES, R.J., BOWER, E.A. & RINK, T.J. (1980). Haemodynamic responses to stimulation of the cardiac autonomic nerves in the anaesthetized cat with closed chest. *J. Physiol.*, **299**, 55–73.

BARNES, R.J., BOWER, E.A. & RINK, T.J. (1986). Haemodynamic responses to stimulation of the splanchnic and cardiac sympathetic nerves in the anaesthetized cat. *J. Physiol.*, **378**, 417–436.

BATES, J.N., BAKER, M.T., GUERRA, R. Jr. & HARRISON, D.G. (1991). Nitric oxide generation from nitroprusside by vascular tissue. Evidence that reduction of the nitroprusside anion and cyanide loss are required. *Biochem. Pharmacol.*, **42**, S157–S165.

BELLAN, J.A., MINKES, R.K., MCNAMARA, D.B. & KADOWITZ, P.J. (1991). N^{ω}-nitro-L-arginine selectivity inhibits vasodilator responses to acetylcholine and bradykinin in cats. *Am. J. Physiol.*, **260**, H1025–H1029.

BOWER, E.A. & EAD, H.W. (1976). An extrapolating analogue computer for measurement of cardiac output by thermodilution. *J. Physiol.*, **263**, 108–110P.

BOWER, E.A. & O'DONNELL, C.P. (1991). Mean circulatory filling pressure during splanchnic nerve stimulation and whole-body hypoxia in the anaesthetized cat. *J. Physiol.*, **432**, 543–556.

CHYU, K.-Y., GUTH, P.H. & ROSS, G. (1992). Effect of N^{ω}-nitro-L-arginine methyl ester on arterial pressure and on vasodilator and vasoconstrictor responses: influence of initial vascular tone. *Eur. J. Pharmacol.*, **212**, 159–164.

DE MAY, L.G. & VANHOUTTE, P.M. (1982). Heterogeneous behaviour of canine arterial and venous wall. Importance of endothelium. *Circ. Res.*, **51**, 439–447.

EKELUND, U. & MELLANDER, S. (1990). Role of endothelium-derived nitric oxide in the regulation of tonus in large-bore arterial resistance vessels, arterioles and veins in cat skeletal muscle. *Acta Physiol. Scand.*, **140**, 301–311.

FEELISCH, M. & NOACK, E.A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.*, **139**, 19–30.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990a). 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. (1990b). Regional and cardiac haemodynamic responses to glyceryl trinitrate, acetyl choline, 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. (1991). 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.

GRUETTER, C.A. & LEMKE, S.M. (1986). Bradykinin-induced endothelium-dependent relaxation of bovine intrapulmonary artery and vein. *Eur. J. Pharmacol.*, **122**, 363–367.

GUYTON, A.C., ABERNATHY, B., LANGSTON, J.B. KAUFMANN, B.N. & FAIRCHILD, H.M. (1959). Relative importance of venous and arterial resistances in controlling venous return and cardiac output. *Am. J. Physiol.*, **196**, 1008–1014.

GUYTON, A.C., JONES, C.E. & COLEMAN, T.G. (1973). *Circulatory Physiology: Cardiac Output and its Regulation.* 2nd edn. Philadephia: W.B. Saunders.

GUYTON, A.C., LINDSAY, A.W., ABERNATHY, J.B. & LANGSTON, J.B. (1958). Mechanism of the increased venous return and cardiac output caused by epinephrine. *Am. J. Physiol.*, **192**, 126–130.

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.

IGNARRO, L.J. (1989). Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circ. Res.*, **65**, 1–21.

IGNARRO, L.J., BUGA, G.M. & CHAUDHURI, G. (1988). EDRF generation and release from perfused bovine pulmonary artery and vein. *Eur. J. Pharmacol.*, **149**, 79–88.

IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.J. & GRUETTER, C.A. (1981). Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.*, **218**, 739–749.

JONES, L.F. & BRODY, M.J. (1992). Coronary blood flow in rats is dependent on the release of vascular nitric oxide. *J. Pharmacol. Exp. Ther.*, **260**, 627–631.

KLABUNDE, R.E., RITGER, R.C. & HELGREN, M.C. (1991). Cardiovascular actions of inhibitors of endothelium-derived relaxing factor (nitric oxide) formation/release in anesthetized dogs. *Eur. J. Pharmacol.*, **199**, 51–59.

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.

MCMAHON, T.J., HOOD, J.S. & KADOWITZ, P.J. (1992). Pulmonary vasodilator response to vagal stimulation is blocked by N^{ω}-nitro-L-arginine methyl ester in the cat. *Circ. Res.*, **70**, 364–369.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991a). Nitric oxide: Physiology, pathophysiology, 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.W.S., 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.

POULEUR, H., COVELL, J.W. & ROSS, J. Jr. (1980). Effects of nitroprusside on venous return and central blood volume in the absence and presence of acute heart failure. *Circulation*, **61**, 328–337.

REES, D.D., PALMER, R.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, 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., ROMERO, J.C. & VANHOUTTE, P.M. (1986). Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.*, **250**, H1145–H1149.

SARNOFF, S.J., MITCHELL, J.H., GILMORE, J.P. & REMENSNYDER, J.P. (1960). Homeometric autoregulation in the heart. *Circ. Res.*, **8**, 1077–1091.

SEIDEL, C.L. & LA ROCHELLE, J. (1987). Venous and arterial endothelia: different dilator abilities in dog vessels. *Circ. Res.*, **60**, 626–630.

SHIRASAKI, Y. & SU, C. (1985). Endothelium removal augments vasodilatation by sodium nitroprusside and sodium nitrite. *Eur. J. Pharmacol.*, **114**, 93–96.

SNEDECOR, G.W. & COCHRAN, W.G. (1967). *Statistical Methods*, 6th edn. Ames, Iowa: Iowa State University Press.

THOMAS, G.R., THIEMERMANN, C., WALDER, C. & VANE, J.R. (1988). The effects of endothelium-dependent vasodilators on cardiac output and their distribution in the anaesthetized rat: a comparison with sodium nitroprusside. *Br. J. Pharmacol.*, **95**, 986–992.

UEEDA, M., SILVIA, S.K. & OLSSON, R.A. (1992). Nitric oxide modulates coronary autoregulation in the guinea pig. *Circ. Res.*, **70**, 1296–1303.

VAN GELDEREN, E.M., HEILIGERS, J.P.C. & SAXENA, P.R. (1991). Haemodynamic changes and acetylcholine-induced hypotensive responses after N^G-nitro-L-arginine methyl ester in rats and cats. *Br. J. Pharmacol.*, **103**, 1899–1904.

VARGAS, H.M., CUEVAS, J.M., IGNARRO, L.J. & CHAUDHURI, G. (1991). Comparison of the inhibitory potencies of N^G-methyl-, N^G-nitro- and N^G-amino-L-arginine on EDRF function in the rat: evidence for continuous basal EDRF release. *J. Pharmacol. Exp. Ther.*, **257**, 1208–1215.

VARGAS, H.M., IGNARRO, L.J. & CHAUDHURI, G. (1990). Physiological release of nitric oxide is dependent on the level of vascular tone. *Eur. J. Pharmacol.*, **190**, 393–397.

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.

WIDDOP, R.E., GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1992). The influence of atropine and atenolol on the cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats. *Br. J. Pharmacol.*, **105**, 653–656.

WOODMAN, O.L. & DUSTING, G.J. (1991). N^G-nitro L-arginine causes coronary vasoconstriction and inhibits endothelium-dependent vasodilatation in anaesthetized greyhounds. *Br. J. Pharmacol.*, **103**, 1407–1410.

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Relaxing actions of corticotropin-releasing factor on rat resistance arteries

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1 Although it well established that corticotropin-releasing factor (CRF) injected i.v. can cause hypotension and vasodilatation, there is no *in vitro* evidence that CRF acts as a vasodilator. We have therefore tested the hypothesis that the hypotensive effect of i.v. CRF is due to a direct vasodilator action by carrying out experiments *in vitro* on rat resistance arteries (i.d. 150–300 μm).

2 Initial *in vivo* experiments confirmed that CRF (1.5 nmol·kg^{-1}) injected i.v. caused hypotension in rats, this being partially antagonized by the CRF analogue CRF_{9-41}.

3 For the *in vitro* experiments, vessels were taken from the mesenteric, cerebral and femoral vascular beds, and mounted as ring preparations in an isometric myograph. The vessels were pre-contracted with one of 3 agonists (prostaglandin $F_{2\alpha}$, arginine vasopressin or noradrenaline) or with a high-potassium solution (K^+).

4 With maximal concentrations of the agonists, CRF caused relaxation of mesenteric and cerebral vessels with 10 nM, and near complete relaxation with 100 nM. Femoral vessels preconstricted with agonists and all vessels preconstricted with K^+ were less affected by CRF. In the mesenteric vessels, with sub-maximal levels of pre-constriction, CRF caused substantial relaxation at 1 nM and could cause complete relaxation at 10 nM.

5 The relaxant effect of CRF on contractions of mesenteric vessels was antagonized by 100 nM CRF_{9-41}. Neither tetraethyl ammonium (30 mM) nor glibenclamide (3 μM) antagonized the relaxant effect of CRF.

6 The relaxant effect of CRF on mesenteric small arteries was found to be unaffected by removal of the endothelium.

7 The results indicate that CRF causes an endothelial-independent vasodilatation of rat resistance arteries under *in vitro* conditions at concentrations which are consistent with this being an important cause of the hypotension observed with i.v. injection of CRF.

Keywords: Corticotropin-releasing factor (CRF); CRF_{9-41}; corticotropin-releasing hormone; resistance arteries; blood pressure; heart rate; vasodilatation

Introduction

Corticotropin-releasing factor (CRF), a 41-residue peptide, is secreted from the hypothalamus and conducted through a portal system to the anterior pituitary, where it causes the release of adrenocorticotropic hormone (ACTH) and other peptides derived from pro-opiomelanocortin (Vale *et al.*, 1981; Taylor & Fishman, 1991; Pralong *et al.*, 1991). CRF thus plays a key role in the hypothalamo-pituitary-adrenal axis which leads to the release of cortisol and catecholamines from the adrenal gland (Vale *et al.*, 1981; Owens & Nemeroff, 1991; Fisher, 1989). However, CRF is not only secreted from the hypothalamus: CRF can be released from the adrenal gland as a result of sympathetic stimulation (Edwards & Jones, 1988) or haemorrhage (Bruhn *et al.*, 1987), but the role of this extra-hypothalamic CRF is not known. Exogenous CRF has been found to have a number of effects: in calf, for example, CRF infused into the adrenal artery can cause the release of cortisol from the adrenal gland (Jones & Edwards, 1990), while i.v. infused CRF can inhibit thermal injury (Kiang & Wei, 1992). CRF has also been found to inhibit the protein leakage caused by substance P (Gao *et al.*, 1991). The cardiovascular effects of exogenous CRF depend on whether the drug is applied centrally or peripherally. Intracerebroventricular (i.c.v.) injection of CRF causes an increase in blood pressure and heart rate, a response dependent on an intact sympathetic nervous system (Brown & Fisher, 1983; Overton & Fisher, 1989), and may thus participate in the defence and stress reactions (Fisher & Brown, 1991). Intravenous (i.v.) injection in rats causes reduction in blood pressure, with a reflex rise in heart rate (Fisher *et al.*, 1983). Similar hypotensive actions of CRF have been seen in other species (Lenz, 1987; Kali *et al.*, 1983; Udelsman *et al.*, 1986), including man (Hermus *et al.*, 1987). The hypotensive action of CRF is caused by vasodilatation (Kiang & Wei, 1992), especially in the mesenteric bed (MacCannell *et al.*, 1986; Lenz, 1987; Gardiner *et al.*, 1988; Grosskreutz & Brody, 1988). The relaxation does not appear to be due to ACTH secretion (Pralong *et al.*, 1991), and it has been suggested that it is due to a direct action of CRF on the vascular smooth muscle (Corder *et al.*, 1992). This possibility has not, however, been tested on vascular preparations *in vitro*. We have therefore tested the hypothesis that CRF has a direct vasodilator action using rat resistance arteries (i.d. 150–300 μm) taken from the mesenteric, cerebral and femoral vascular beds, and mounted as rings in an isometric myograph (Mulvany & Halpern, 1977). The results showed that CRF can indeed have a direct vasodilator action, particularly for the mesenteric and cerebral resistance arteries. The specificity of the effect was tested by use of the CRF analogue, CRF_{9-41}, which has been shown to inhibit both the hypertensive effect of an i.c.v. CRF injection (Brown *et al.*, 1986) as well as the hypotensive effect of i.v. CRF injection (Corder *et al.*, 1992).

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Methods

Male Wistar rats (Møllegaard Breeding Centre, Ll Skensved, Denmark) weight ca. 320 g were used. All animals were housed in temperature and humidity-controlled quarters and provided with food and water *ad libitum*.

In vivo *experiments*

Rats were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.) and polypropylene catheters (PE50, Portex) were inserted in the right femoral artery and right femoral vein. Blood pressure and heart rate were recorded from the femoral artery using a BIOMONITOR (Messgerätewerk Zwönitz, former GDR) pressure transducer and cardiotachometer. Data were calculated and analyzed with a computer.

After the surgical manipulations, about 30 min was allowed to stabilize the blood pressure and heart rate baselines before commencing investigations. Intravenous (i.v.) injections of saline (vehicle) or peptides were administered in a volume of 0.2 ml. The rats were divided randomly into three groups which were subjected to one of the following protocols: (a) CRF (1.5 nmol kg^{-1}) 15 min after i.v. injection of vehicle; (b) CRF (1.5 nmol kg^{-1}) 15 min after pretreatment with CRF_{9-41} (15 nmol kg^{-1}); (c) single i.v. injection of vehicle.

In vitro *experiments*

Preparation Segments (ca. 2 mm long) of small arteries were taken from the mesenteric (Mulvany & Halpern, 1977), cerebral (Aalkjaer *et al.*, 1985), and femoral (Nilsson & Mulvany, 1981) vasculatures, one or two vessels of each type, as described previously. The segments were mounted as ring preparations on a double isometric myograph (Mulvany & Nyborg, 1980) and set to an optimal lumen diameter, l_1, equal to 90% of the diameter the vessels were estimated to have when relaxed and under a transmural pressure of 100 mmHg, based on the relaxed wall tension-internal circumference relation (Mulvany & Halpern, 1977). The optimal diameters were 144 μm–306 μm (mesenteric),. 135 μm–245 μm (femoral) and 160 μm–270 μm (cerebral). During dissection and mounting the vessels were kept in oxygenated physiological salt solution (PSS, see below) at 37°C.

After equilibration for 30 min in PSS, arteries were activated in turn with control, activating solution (10 μM noradrenaline (NA) in 125 mM K^+, see below), twice, 10 μM NA, 125 mM K^+, and again with control activating solution, 2 min per activation, 5 min washout between, to test the contractility of the preparations. If the final response to control activating solution corresponded to an effective pressure (equal to wall tension $\times 2 \times l_1^{-1}$) less than 100 mmHg, the vessel was discarded (Mulvany & Halpern, 1977). The vessels were then equilibrated in PSS for a further 10 min.

Protocol CRF concentration-relaxation curves were obtained as follows. Vessels were contracted with one of three agonists (prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), arginine vasopressin (AVP) or NA) or with K^+, in the concentrations indicated for 5 min, after which CRF was applied in a cumulative fashion (10^{-9} M, 10^{-8} M and 10^{-7} M), for a period of 5 min for each concentration. Unless otherwise indicated, responses are given as the wall tension at the end of each period expressed as a percentage of the wall tension just before the first addition of CRF. Vessels were then washed in PSS for 20 min, before a new CRF concentration-relaxation curve was obtained with a different agonist. The order in which the agonists were applied was random. Time control experiments showed that the responses of all vessels to all agonists remained constant to within about 10% between 5 min and 20 min after adding the agonist. The responses were unaffected by the vehicle (see below).

Experiments designed to investigate the possible inhibitory effects of drugs were performed as follows. CRF concentration-relaxation curves, determined as above, were obtained first under control conditions, and then in the presence of the drug indicated. The vessel was exposed to the drug for 5 min before the start of the determination.

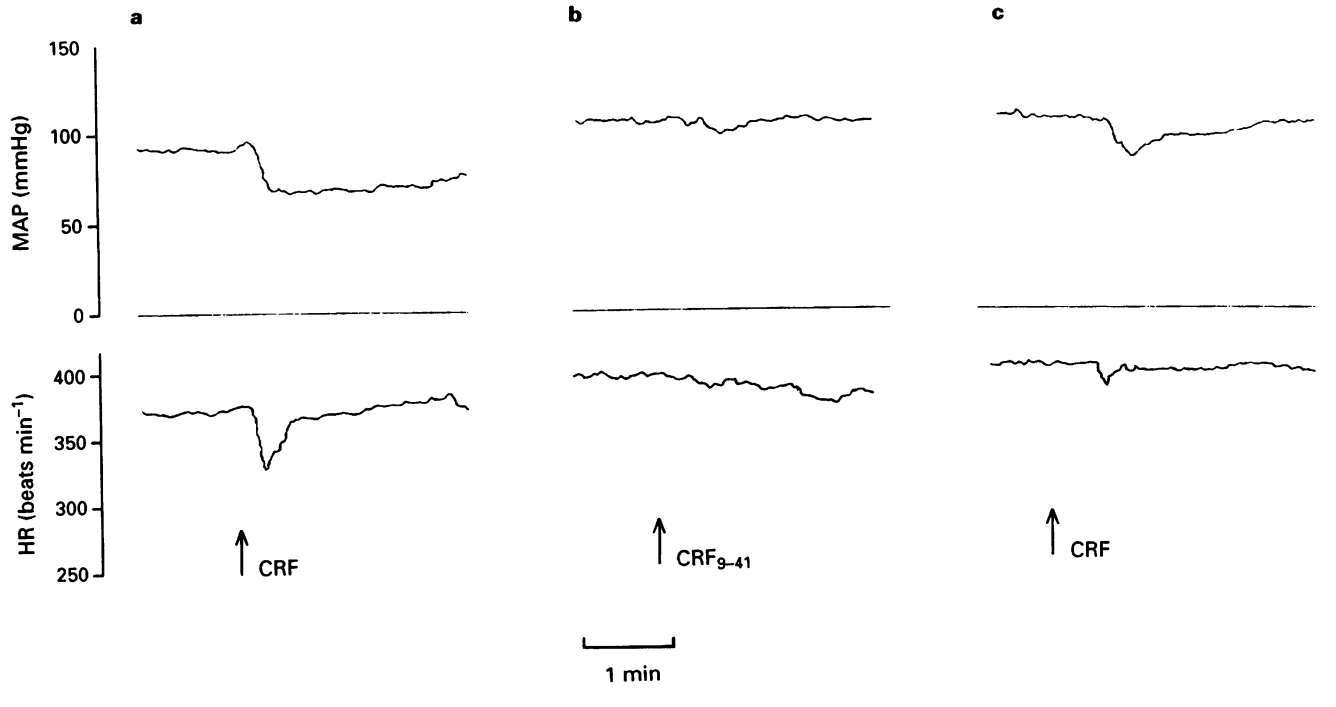


Figure 1 Measurements of mean blood pressure (MAP) and heart rate (HR) in anaesthetized Wistar rats. (a) Hypotensive action of i.v. injection of corticotropin releasing factor (CRF, 1.5 nmol kg^{-1}); (b) lack of effect of CRF_{9-41} on MAP and HR and later in same rat (c) partial antagonism of hypotensive effect of CRF when CRF_{9-41} had been injected 15 min previously. Note that hypotensive effect of CRF is maintained in (a), but is transient in (c).

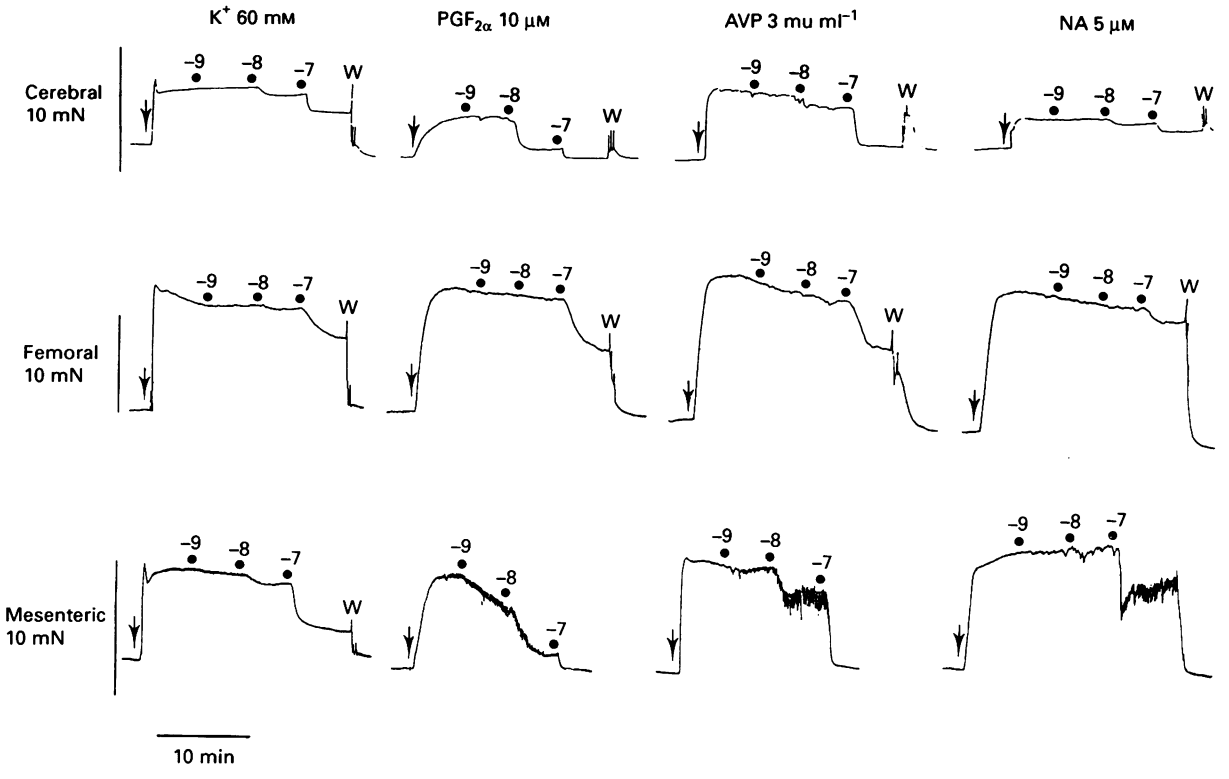


Figure 2 Traces showing relaxing effects of corticotropin releasing factor (CRF) on rat (from top) all cerebral, femoral and mesenteric small arteries precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 μM) arginine vasopressin (AVP, 3 mu m^{-1}), noradrenaline (NA, 5 μM) or K^+ (60 mM), as indicated. CRF concentrations are given as log concentrations (M). Arrows indicate addition of agonists. Washout with PSS indicated by 'W'.

The possible influence of the endothelium was tested in mesenteric small arteries by obtaining CRF concentration-relaxation curves first under control conditions, and then after the endothelium had been removed by rubbing the inner surface of the artery with a human hair. Functional evidence for endothelial removal was obtained by lack of relaxation of vessels to 10 μM acetylcholine after precontraction with 10 μM NA.

Solutions Physiological salt solution (PSS) contained (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, ethylene-diamenetetraacetic acid 0.026, glucose 5.5. x mM K^+, as for PSS but with the indicated concentration (x) of K^+, obtained by an equimolar exchange of KCl for NaCl. Other substances used were arginine vasopressin (AVP, Sandoz), glibenclamide (Sigma), NA-HCl (NA, Sigma), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Upjohn), tetraethylammonium (TEA, Merck). Corticotropin-releasing factor (human CRF either as trifluoroacetate (CRF-TFA) or HCl (CRF-HCl) salts) and the CRF analogue CRF_{9-41} were kindly prepared by Drs M. Beyermann and E. Krause at the Institute of Molecular Pharmacology, Berlin, Germany. Stock solutions of CRF (0.5 mM) were prepared by dissolving CRF in 50% acetonitrile and 50% water. The CRF-TFA was used in the *in vivo* experiments, and both CRF-TFA and CRF-HCl were used in the *in vitro* experiments. *In vitro* investigations showed that CRF-TFA and CRF-HCl had identical potency and efficacy in relaxing responses due to 3 mu ml^{-1} AVP ($n = 8$), and the two forms were therefore treated as identical.

Statistics Data are expressed as mean ± s.e.mean. The data from the *in vivo* experiments were analyzed by one-way analysis of variance, followed by Dunnett's multiple comparison test. In the *in vitro* experiments, differences were tested for significance by paired two-tailed Student's *t* test. Significance was accepted at $P<0.05$. Degrees of freedom were based on number of rats in the *in vivo* experiments and number of vessels in the *in vitro* experiments.

Results

In vivo *experiments*

Bolus injection (i.v.) of CRF (1.5 nmol kg^{-1}) caused a reduction of mean blood (MAP) pressure, but no change in heart rate (HR) apart from a short-lasting transient drop (Figure 1a). MAP fell within a few seconds from 107 ± 7 mmHg to 79 ± 5 mmHg, the corresponding heart rates being 402 ± 15 beats min^{-1} and 398 ± 16 beats min^{-1} (8 rats). The blood pressure reduction was sustained for about 5–6 min. When the CRF injection was performed in 8 other rats pretreated with CRF_{9-41}, the fall in blood pressure was attenuated (mean blood pressure fell from 114 ± 7 mmHg to 98 ± 7 mmHg), and the fall was transient (Figure 1c). HR was not changed (392 ± 13 beats min^{-1} and 386 ± 14 beats min^{-1}, respectively). CRF_{9-41} (15 nmol kg^{-1}) injection alone did not affect MAP or HR (Figure 1b), nor did injection of vehicle (data not shown).

In vitro *experiments*

CRF caused a concentration-dependent relaxation of rat mesenteric, cerebral and femoral small arteries preconstricted with 10 μM $PGF_{2\alpha}$, 3 mu ml^{-1} AVP, 5 μM NA or 60 mM K^+ (Figures 2 and 3). CRF was least potent with K^+ preconstriction for all vessel types. In the femoral small arteries, CRF also had little effect against the other preconstrictors, substantial relaxations being seen only with 100 nM CRF. In

the cerebral small arteries, CRF caused significant relaxations with 10 nM CRF and close to complete relaxation with 100 nM CRF for all preconstrictors, except K^+. In the mesenteric small arteries, CRF was most potent in vessels preconstricted with $PGF_{2\alpha}$, giving a 50% relaxation with 10 nM CRF. Complete relaxation with 100 nM CRF was seen in vessels preconstricted with either $PGF_{2\alpha}$ or AVP.

The dependence of the relaxant effect of CRF on the type of preconstrictor used was found in mesenteric small arteries also to depend on the level of activation (Figure 4). Thus for low levels of activation (0.15 mu ml^{-1} AVP, 1 μM NA, 25 mM K^+), significant relaxation was observed with 1 nM CRF, while 10 nM CRF gave 50% relaxation with K^+ and complete relaxation with AVP.

The relaxant effect of CRF on contractions of mesenteric small arteries induced by AVP was antagonized by 100 nM CRF_{9-41} (Figures 5 and 6). The dose-ratio for 100 nM CRF_{9-41}

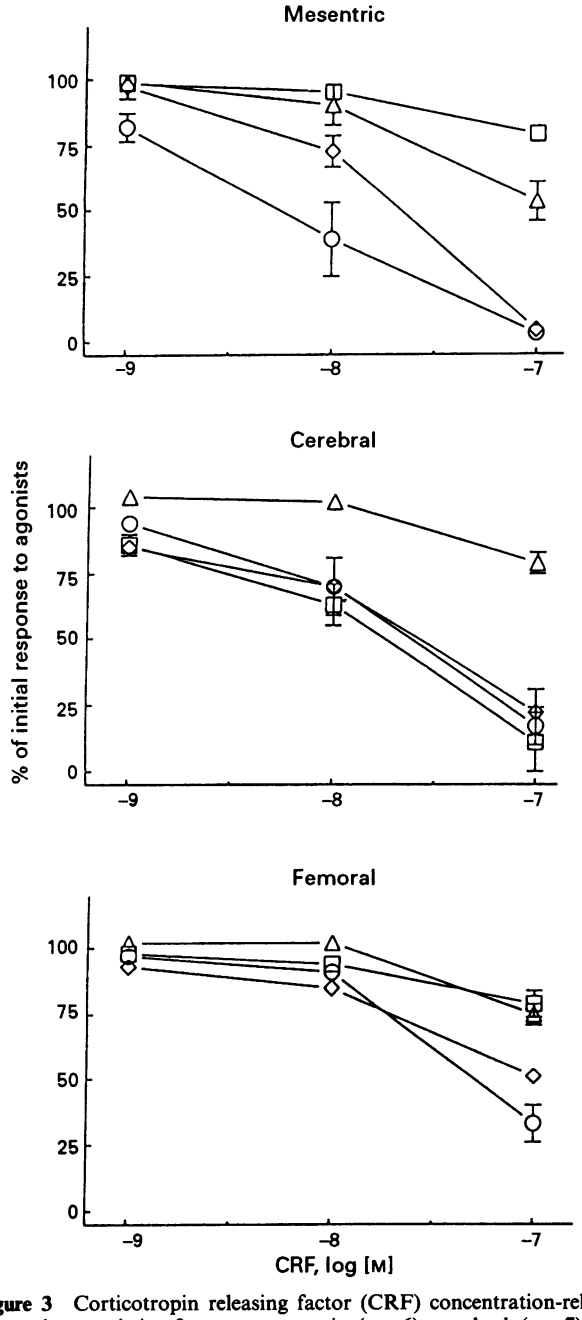


Figure 3 Corticotropin releasing factor (CRF) concentration-relaxation characteristics for rat mesenteric ($n = 6$), cerebral ($n = 7$) or femoral ($n = 6$) small arteries determined in experiments similar to those shown in Figure 2. Arteries activated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 μM) (○), arginine vasopressin (AVP, 3 mu ml^{-1}) (◇), noradrenaline (NA, 5 μM) (□) or K^+ 60 mM (△). Values show mean ± s.e.mean of steady state tensions, expressed as percentage of initial tensions in the absence of CRF. The initial tensions were as follows. $PGF_{2\alpha}$: 2.3 ± 0.1 Nm^{-1} (mesenteric), 0.8 ± 0.1 Nm^{-1} (cerebral), 2.4 ± 0.3 Nm^{-1} (femoral). AVP: 2.5 ± 0.2 Nm^{-1} (mesenteric), 1.0 ± 0.2 Nm^{-1} (cerebral), 2.8 ± 0.3 Nm^{-1} (femoral). NA: 2.8 ± 0.3 Nm^{-1} (mesenteric), 0.5 ± 0.1 Nm^{-1} (femoral), 2.7 ± 0.4 Nm^{-1} (femoral. K^+: 2.2 ± 0.2 Nm^{-1} (mesenteric), 1.0 ± 0.2 Nm^{-1} (cerebral), 2.2 ± 0.3 Nm^{-1} (femoral).

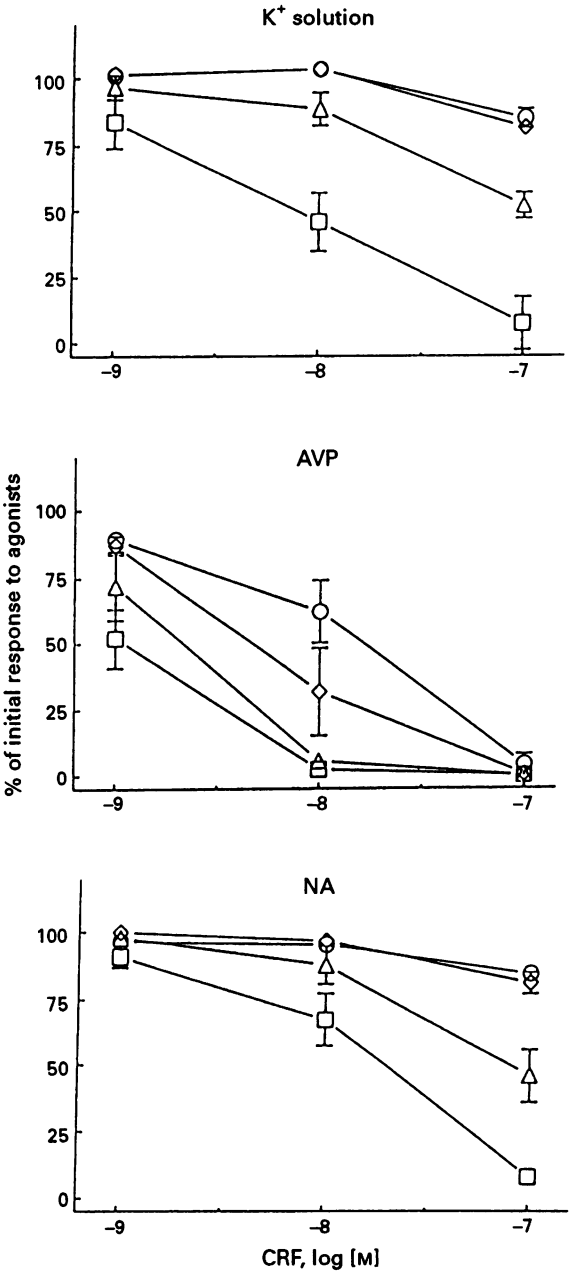


Figure 4 Corticotropin releasing factor (CRF) concentration-relaxation characteristics for rat mesenteric small arteries when activated with arginine vasopressin (AVP, $n = 6$), noradrenaline (NA, $n = 7$) or K^+ ($n = 6$). K^+: 25 (□); 40 (△); 80 (◇); 125 mM (○). AVP: 0.15 (□); 0.3 (△); 0.6 (◇); 3.0 mu ml^{-1} (○). NA: 1 (□); 2 (△); 5 (◇); 10 μM (○). Values show mean ± s.e.mean of steady state tensions, expressed as percentage of initial tensions in the absence of CRF. The initial tensions were as follows. AVP: 1.9 ± 0.3 Nm^{-1} (0.15 mu ml^{-1}), 2.3 ± 0.2 Nm^{-1} (0.3 mu ml^{-1}), 2.4 ± 0.2 Nm^{-1}(0.6 mu ml^{-1}), 2.5 ± 0.2 Nm^{-1} (3 mu ml^{-1}). NA: 1.8 ± 0.2 Nm^{-1} (1 μM), 2.2 ± 0.2 Nm^{-1} (2 μM), 2.5 ± 0.2 Nm^{-1} (5 μM), 2.8 ± 0.3 Nm^{-1} (10 μM). $K^{\pm}$: 0.4 ± 0.1 Nm^{-1} (25 mM), 1.0 ± 0.2 Nm^{-1} (40 mM), 2.5 ± 0.3 Nm^{-1} (80 mM), 2.6 ± 0.3 Nm^{-1} (125 mM).

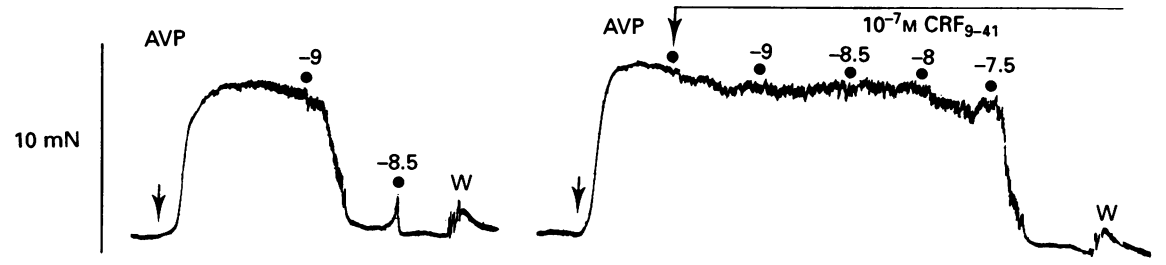


Figure 5 Traces showing inhibitory effects of CRF_{9-41} (100 nM) on the relaxing effects of corticotropin releasing factor (CRF) on rat mesenteric small arteries precontracted with arginine vasopressin (AVP, 0.3 mu ml^{-1}). CRF concentrations are given as log concentrations (M). Arrows indicate addition of agonists. Washout with PSS indicated by 'W'.

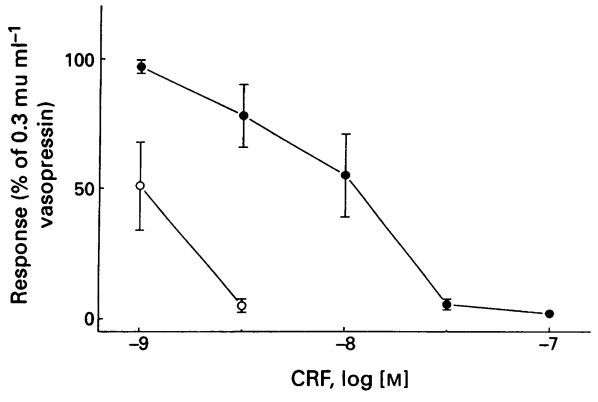


Figure 6 Effect of CRF_{9-41} (0.1 μM, ●) on the corticotropin releasing factor (CRF) concentration-relaxation characteristics of 7 rat mesenteric small arteries pre-contracted with 0.3 mu ml^{-1} AVP, determined as indicated in Figure 5. Control (○) curves show characteristics of the vessels concerned in the absence of CRF_{9-41}. Values show mean ± s.e.mean of steady state tensions, expressed as percentage of initial tensions in the absence of CRF. Initial tension was not significantly affected by CRF_{9-41}.

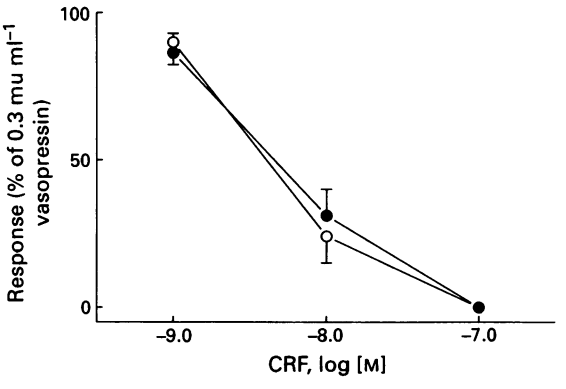


Figure 7 Lack of effect of removal of endothelium on the corticotropin releasing factor (CRF) concentration-relaxation characteristics of 6 rat mesenteric small arteries pre-contracted with 0.3 mu ml^{-1} arginine vasopressin: (●) intact vessels; (○) vessels after removal of the endothelium. Values show mean ± s.e.mean of steady state tensions, expressed as percentage of initial tensions in the absence of CRF. Initital tension was not significantly affected by removal of the endothelium.

Table 1 Effects of glibenclamide and tetraethyl ammonium (TEA) on the steady state wall tensions produced by corticotropin releasing factor (CRF) on rat mesenteric small arteries precontracted with 0.3 mu ml^{-1} (arginine vasopressin (AVP) (expressed as a percentage of precontraction levels)

	Number of vessels	[CRF] (nM) *1*	*10*	*100*
Glibenclamide, 3 μM	6	68 ± 6	12 ± 4	1 ± 1
Control	6	81 ± 6	27 ± 9	1 ± 1
TEA 30 mM	8	67 ± 10	27 ± 12	1 ± 1
Control	8	48 ± 14	14 ± 7	1 ± 1

Values show mean ± s.e.mean of steady state tensions, expressed as a percentage of initial tensions in the absence of CRF, either in the presence of the indicated drug or without drug (control). Glibenclamide reduced the initital tensions by 15% ($P<0.05$), but TEA did not affect the initial tensions significantly. No significant effects of glibenclamide or TEA on the effects of CRF were found.

was about 10. Neither TEA (30 mM) nor glibenclamide (3 μM) antagonized the relaxant effect of CRF (Table 1).

The relaxant effect of CRF on mesenteric small arteries was found to be unaffected by removal of the endothelium (Figure 7).

Discussion

The main result of this investigation is that CRF causes an endothelium-independent vasodilatation of rat resistance arteries under *in vitro* conditions. When precontracted with maximal concentrations of agonist drugs, the threshold for the relaxation of the mesenteric and cerebral resistance arteries was around 1 nM, but around 10 nM for the femoral resistance arteries. The vasodilator effect was inhibited by 100 nM CRF_{9-41}. The results support the hypothesis that the vasodilator action of i.v. CRF injection is mediated through a direct action on the vascular smooth muscle.

Although a number of peptides closely related to CRF, such as urotensin I (Gerritsen *et al.*, 1981; Bolt *et al.*, 1989), have been investigated *in vitro*, the *in vitro* actions of CRF on vascular smooth muscle have not been investigated previously. This investigation is therefore the first to demonstrate that CRF can have a direct vasodilator action. Moreover, the vessels which we have examined are small enough to contribute substantially to the peripheral resistance (Baumbach *et al.*, 1988; Christensen & Mulvany, 1992), and therefore the finding that they are relaxed by CRF is consistent with the reduction in peripheral resistance caused by i.v. CRF injection being due to a direct action on the vasculature (Fisher, 1989). Furthermore, our observation that CRF was more potent in the mesenteric resistance arteries than in the femoral resistance arteries is consistent with previous observations that i.v. CRF injection caused specific vasodilatation of the mesenteric and renal vascular beds compared to the

hindquarter vascular bed (Gardiner *et al.*, 1988; Grosskreutz & Brody, 1988). However, since CRF was also a potent dilator of the cerebral resistance arteries, it may be that CRF is a general vasodilator, the femoral vasculature being exceptional. Further experiments on other vascular beds, as well as different sizes of vessel, both more proximal and more distal, are required to analyze this. It may be noted that urotensin I also causes selective dilatation of rat mesenteric arteries (Bolt *et al.*, 1989), consistent with i.v. injection of this peptide causing selective dilatation of the mesenteric circulation compared to the hindquarters circulation (MacCannell & Lederis, 1983; Lederis *et al.*, 1983).

The CRF dose given in the present experiments (1.5 nmol kg^{-1}) is similar to that used by other investigators to obtain similar hypotensive effects (Gardiner *et al.*, 1988; Corder *et al.*, 1992); Corder *et al.* (1992) used 2 nmol kg^{-1} and measured a resulting plasma CRF concentration of 50 nM. In other experiments, steady infusions of CRF have been given (e.g. 0.5 nmol kg^{-1} min^{-1} which caused a 40% drop in mean blood pressure over 30 min; Pralong *et al.*, 1991). In the present experiments, assuming that CRF is distributed alone in the plasma (volume ca. 30 ml kg^{-1} per rat), the plasma concentration of CRF resulting from a bolus injection of 1.5 nmol kg^{-1} CRF would be ca. 50 nM. Although the assumptions behind this calculation are uncertain, the concentration estimated in this manner corresponds quite well to the concentrations giving relaxation in the present *in vitro* experiments. This is particularly true under conditions where the level of activation in the pre-constriction was not maximal (Figure 4), where significant relaxation was seen with 1 nM CRF with all agonists used, and up to full relaxation could be obtained with 10 nM CRF. It should, however, be noted that lower concentrations of CRF are able to stimulate ACTH secretion in cultured pituitary cells (Rivier *et al.*, 1984; Corder *et al.*, 1992), where under 1 nM CRF gives a half maximal stimulation. The potency of CRF as a vasodilator also appears to be less than urotensin I, where the IC_{50} concentrations of the peptide are less than 1 nM (Lederis *et al.*, 1983).

The mechanism by which CRF causes relaxation is not known. The finding that CRF_{9-41} inhibited the effect of CRF suggests that the effect is specific, this conclusion being supported by the demonstration of specific CRF binding sites in vascular tissue, such as the rabbit aorta (Dashwood *et al.*, 1987). Since we found that the dilator effect was not diminished by removal of the endothelium, the mechanism appears to be endothelial-independent, even though the work of Dashwood and colleagues (1987) indicated that the aortic endothelium contained CRF binding sites. Our finding that CRF was less potent against vessels precontracted with 125 mM potassium, compared to the potency against vessels precontracted with drugs, suggests that the relaxation could be mediated by hyperpolarization (which would be prevented in a high potassium solution), possibly though the activation of K-channels. However, since neither glibenclamide (an inhibitor of ATP-sensitive K-channels) nor TEA (a less specific inhibitor of K-channels) affected the CRF-induced relaxation, this possibility seems less likely. The vasodilator effect of CRF *in vitro* is in contrast to the *in vitro* inotropic effect of CRF on guinea-pig ventricular myocardium, where it was suggested that CRF induces slow inward calcium currents (Saitoh *et al.*, 1990).

The right-ward shift in the CRF concentration-relaxation relation (Figure 6) caused by CRF_{9-41} is similar to the right-shifts caused by this analogue in the dose-response relations regarding the hypotensive effect of i.v. CRF injection (Corder *et al.*, 1992). In primary cultures of rat anterior pituitary cells, 0.5–1 μM CRF_{9-41} caused a right-shift in the EC_{50} of the CRF concentration-ACTH release curve of about 1 log unit (Rivier *et al.*, 1984; Corder *et al.*, 1992), but these data are probably confounded by the intrinsic activity which was also found for CRF_{9-41} (Corder *et al.*, 1992). Corder and colleagues (Corder *et al.*, 1992) also found that the hypotensive action of i.v. injection of 2 nmol CRF was antagonized by prior i.v. injection of a total of 87.5 nmol CRF_{9-41}. The present experiments indicated that 100 nM CRF_{9-41} caused a right-shift in the CRF concentration-relaxation curve of about 1 log unit, which would suggest that the dissociation constant of the CRF binding site for CRF_{9-41} is of the order of 10 nM (dissociation constant = (antagonist concentration)/(dose-ratio − 1)), neglecting any possible intrinsic activity of CRF_{9-41}.

In contrast to the well-established central effects of CRF (Vale *et al.*, 1981; Fisher, 1989; Chrousos & Gold, 1992), the possible physiological or pathophysiological role of plasma CRF remains unclear. Physiological levels of plasma CRF are normally low (e.g. 5 pg ml^{-1} in calves (Edwards & Jones, 1988); under 10 pM in athletes (Hohtari *et al.*, 1992); under 10 pg ml^{-1} in dog (Bruhn *et al.*, 1987)). The plasma concentrations of CRF resulting from the increase in adrenal release of CRF as a result of haemorrhage (Bruhn *et al.*, 1987) have not been reported, but since the adrenal secretion reached only 185 pg min^{-1} the effects on plasma CRF will have been minimal. The only situation in which high levels of plasma CRF have been reported is during human pregnancy, where plasma CRF levels rise dramatically to around 1700 pg ml^{-1} (Campbell *et al.*, 1987) or 2215 pg ml^{-1} (Sasaki *et al.*, 1987) at term, and 4409 pg ml^{-1} (ca. 1 nM) in labour (Sasaki *et al.*, 1987). At these levels, the present data indicate that the direct vasodilator action of CRF could be relevant. The data do not exclude the possibility that lower levels of CRF might modulate the vasoactive actions of other hormones.

In summary, the results indicate that CRF causes an endothelial-independent vasodilatation of rat resistance arteries under *in vitro* conditions. The concentrations causing relaxation are consistent with this direct action of CRF on vascular smooth muscle being an important cause of the hypotension observed with i.v. injection of CRF.

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References

AALKJAER, C., DANIELSEN, H., JOHANNESEN, P., PEDERSEN, E.B., RASMUSSEN, A. & MULVANY, M.J. (1985). Abnormal vascular function and morphology in pre-eclampsia: a study of isolated resistance vessels. *Clin. Sci.*, **69**, 477–482.

BAUMBACH, G.L., DOBRIN, P.B., HART, M.N. & HEISTAD, D.D. (1988). Mechanics of cerebral arterioles in hypertensive rats. *Circ. Res.*, **62**, 56–64.

BOLT, G.R., ITOH, H., LEDERIS, K. & MACCANNELL, K.L. (1989). Differential antagonism of alpha-1 and alpha-2 adrenoceptor-mediated vasoconstrictor responses by a vasodilator peptide, urotensin I: comparison with nifedipine. *J. Pharmacol. Exp. Ther.*, **251**, 1147–1154.

BROWN, M.R. & FISHER, L.A. (1983). Central nervous system effects of corticotropin releasing factor in the dog. *Brain Res.*, **280**, 75–79.

BROWN, M.R., GRAY, T.S. & FISHER, L.A. (1986). Corticotropin-releasing factor receptor antagonist: effects on the autonomic nervous system and cardiovascular function. *Regul. Pept.*, **16**, 321–329.

BRUHN, T.O., ENGELAND, W.C., ANTHONY, E.L.P., GANN, D.S. & JACKSON, I.M.D. (1987). Corticotropin-releasing factor in the dog adrenal medulla is secreted in response to hemorrhage. *Endocrinology*, **120**, 25–33.

CAMPBELL, E.A., LINTON, E.A., WOLFE, C.D., SCRAGGS, P.R., JONES, M.T. & LOWRY, P.J. (1987). Plasma corticotropin-releasing hormone concentrations during pregnancy and parturition. *J. Clin. Endocrinol. Metab.*, **64**, 1054–1059.

CHRISTENSEN, K.L. & MULVANY, M.J. (1992). Mesenteric arcade arteries contribute substantially to vascular resistance in conscious rats. *J. Vasc. Res.*, (in press).

CHROUSOS, G.P. & GOLD, P.W. (1992). The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. *J. Am. Med. Ass.*, **267**, 1244–1252.

CORDER, R., TURNILL, D., LING, N. & GAILLARD, R.C. (1992). Attenuation of corticotropin releasing factor-induced hypotension in anesthetized rats with the CRF antagonist, α-helical CRF9-41; comparison with effect on ACTH release. *Peptides*, **13**, 1–6.

DASHWOOD, M.R., ANDREWS, H.E. & WEI, E.T. (1987). Binding of Tyr-corticotropin-releasing factor to rabbit aorta is reduced by removal of the endothelium. *Eur. J. Pharmacol.*, **135**, 111–112.

EDWARDS, A.V. & JONES, C.T. (1988). Secretion of corticotrophin releasing factor from the adrenal during splanchnic nerve stimulation in conscious calves. *J. Physiol.*, **400**, 89–100.

FISHER, L.A. (1989). Corticotropin-releasing factor: endocrine and autonomic integration of responses to stress. *Trends Pharmacol. Sci.*, **10**, 189–193.

FISHER, L.A. & BROWN, M.R. (1991). Central regulation of stress responses. *Clin. Endocrinol. Metab.*, **5**, 35–50.

FISHER, L.A., JESSEN, G. & BROWN, M.R. (1983). Corticotropin-releasing factor (CRF): mechanism to elevate mean arterial pressure and heart rate. *Regul. Pept.*, **5**, 153–161.

GAO, G.C., DASHWOOD, M.R. & WEI, E.T. (1991). Corticotrophin-releasing factor inhibition of substance P-induced vascular leakage in rats: possible sites of action. *Peptides*, **12**, 639–644.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1988). Regional haemodynamic effects of depressor neuropeptides in conscious, unrestrained, Long Evans and Brattleboro rats. *Br. J. Pharmacol.*, **95**, 197–208.

GERRITSEN, M.E., PARKS, T.P., PRINTZ, M.P. & LEDERIS, K. (1981). A proposed role for prostaglandins in the modulation of the relaxation response to urotensin I in isolated rat arteries. II. Nature of prostaglandin synthesis in and effects of urotensin I on release of PGE and 6-keto-PGF from isolated rat tail and mesenteric arteries. *Prostaglandins*, **22**, 873–892.

GROSSKREUTZ, C.L. & BRODY, M.J. (1988). Regional hemodynamic responses to central administration of corticotropin-releasing factor (CRF). *Brain Res.*, **442**, 363–367.

HERMUS, A.R.M.M., PIETERS, G.F.F.M., WILLEMSEN, J.J., ROSS, H.A., SMALS, A.G.H., BENRAAD, T.J. & KLOPPENBORG, P.W.C. (1987). Hypotensive effects of ovine and human corticotrophin-releasing factors in man. *Eur. J. Clin. Pharmacol.*, **31**, 531–534.

HOHTARI, H., ELOVAINIO, R., SALIMINEN, K. & LAATIKAINEN, T. (1992). Plasma corticotropin-releasing hormone, corticotropin, and endorphins at rest and during exercise in eumenorrheic and amenorrheic athletes. *Fert. Steril.*, **50**, 233–238.

JONES, C.T. & EDWARDS, A.V. (1990). Adrenal responses to corticotrophin-releasing factor in conscious hypophysectomized calves. *J. Physiol.*, **430**, 25–36.

KALIN, N.H., SHELTON, S., KRAEMER, G. & MCKINNEY, W. (1983). Corticotropin-releasing factor causes hypotension in rhesus monkeys. *Lancet*, **ii**, 1042.

KIANG, J.G. & WEI, E.T. (1992). Corticotropin-releasing factor inhibits thermal injury. *J. Pharmacol. Exp. Ther.*, **243**, 517–520.

LEDERIS, K., LETTER, A., MCMASTER, D., ICHIKAWA, T., MACCANNELL, K.L., KOBAYASHI, Y., RIVIER, J., RIVIER, C., VALE, W. & FRYER, J. (1983). Isolation, analysis of structure, synthesis, and biological actions of urotensin I neuropeptides. *Can. J. Biochem. Cell. Biol.*, **61**, 602–614.

LENZ, H.J. (1987). Extrapituitary effects of corticotropin-releasing factor. *Horm. Metabl. Res. Suppl.*, **16**, 17–23.

MACCANNELL, K.L. & LEDERIS, K. (1983). Mammalian pharmacology of the fish neuropeptide urotensin I. *Fed. Proc.*, **42**, 91–95.

MACCANNELL, K.L., NEWTON, C.A., LEDERIS, K., RIVIER, J. & TIFFANY, M. (1986). Use of selective mesenteric vasodilator peptides in experimental nonocclusive mesenteric ischemia in the dog. *Gastroenterology*, **90**, 669–676.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19–26.

MULVANY, M.J. & NYBORG, N.C.B. (1980). An increased calcium sensitivity of mesenteric resistance vessels in young and adult spontaneously hypertensive rats. *Br. J. Pharmacol.*, **71**, 585–596.

NILSSON, H. & MULVANY, M.J. (1981). Prolonged exposure to ouabain eliminates the greater noradrenaline-dependent calcium sensitivity of resistance vessels in spontaneously hypertensive rats. *Hypertension*, **3**, 691–697.

OVERTON, J.M. & FISHER, L.A. (1989). Central nervous system actions of corticotropin-releasing factor on cardiovascular function in the absence of locomotor activity. *Regul. Pept.*, **25**, 315–324.

OWENS, M.J. & NEMEROFF, C.B. (1991). Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol. Rev.*, **43**, 425–474.

PRALONG, F.P., CORDER, R. & GAILLARD, R.C. (1991). Responses of the rat pituitary-adrenal axis to hypotensive infusions of corticotropin-releasing factor, vasoactive intestinal peptide and other depressor agents. *Regul. Pept.*, **32**, 217–226.

RIVIER, J., RIVIER, C. & VALE, W. (1984). Synthetic competitive antagonists of corticotropin-releasing factor: effect on ACTH secretion in the rat. *Science*, **224**, 889–891.

SAITOH, M., HASEGAWA, J. & MASHIBA, H. (1990). Effect of corticotropin-releasing factor on the electrical and mechanical activities of the guinea-pig ventricular myocardium. *Gen. Pharmacol.*, **21**, 337–342.

SASAKI, A., SHINKAWA, O., MARGIORIS, A.N., LIOTTA, A.S., SATO, S., MURAKAMI, O., GO, M., SHIMIZU, Y., HANEW, K. & YOSHINGA, K. (1987). Immunoreactive corticotropin-releasing hormone in human plasma during pregnancy, labor, and delivery. *J. Clin. Endocrinol. Metab.*, **64**, 224–229.

TAYLOR, A.L. & FISHMAN, L.M. (1991). Corticotropin-releasing hormone. *New. Engl. J. Med.*, **319**, 213–222.

UDELSMAN, R., GALLUCCI, W.T., BACHER, J., LORIAUX, D.L. & CHROUSOS, G.P. (1986). Functional corticotropin-releasing factor receptors in the anesthetized cynomolgus monkey. *Peptides*, **7**, 465–471.

VALE, W., SPIESS, J., RIVIER, C. & RIVIER, J. (1981). Characterization of a 41-residue ovine hypothalmic peptide that stimulates secretion of corticotropin and β-endorphin. *Science*, **213**, 1394–1397.

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Effects of high calcium diet on arterial smooth muscle function and electrolyte balance in mineralocorticoid-salt hypertensive rats

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1 The effects of a high calcium diet (2.5%) on blood pressure, electrolyte balance, plasma and tissue atrial natriuretic peptide (ANP), cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), and arterial smooth muscle responses were studied in one-kidney deoxycorticosterone (DOC)-NaCl hypertensive Wistar rats.

2 Calcium supplementation for 8 weeks markedly attenuated the development of DOC-NaCl hypertension and the associated cardiac hypertrophy, and prevented the DOC-NaCl-induced sodium-volume retention as judged by reduced plasma Na^+, and decreased plasma and ventricular ANP concentrations in high calcium-fed DOC-NaCl rats. However, calcium supplementation did not affect the DOC-NaCl-induced rise in platelet $[Ca^{2+}]_i$.

3 Smooth muscle contractions of isolated mesenteric arterial rings in response to depolarization by K^+ (20–30 mM) were enhanced in DOC-NaCl-treated rats, this enhancement being abolished by concurrent oral calcium loading. The Ca^{2+} entry blocker nifedipine (10 nM) inhibited the contractions induced by K^+ (30–125 mM) more effectively in DOC-NaCl rats than in controls, while the inhibition in calcium-loaded DOC-NaCl rats was significantly greater than in controls only with 30 mM K^+.

4 The contractions of mesenteric arterial rings induced by omission of K^+ from the organ baths were used to evaluate cell membrane permeability to ions. In chemically denervated rings the onset of the gradual rise in contractile force in K^+-free medium occurred earlier, and the rate of the contraction was faster in DOC-NaCl-treated rats than in controls and high calcium-fed DOC-NaCl rats. Smooth muscle relaxation induced by 0.5 mM K^+ upon K^+-free contractions was clearly slower in DOC-NaCl rats than in controls and calcium-supplemented DOC-NaCl rats.

5 The functions of arterial smooth muscle Na^+, Ca^{2+} exchange and Ca^{2+}-ATPase were evaluated by the aortic contractions elicited by low Na^+ medium, and the subsequent relaxation responses induced by Ca^{2+}-free solution (in the presence of 5 mM caffeine, 1 μM nifedipine and 10 μM phentolamine). The rate of aortic low Na^+ contractions (evaluating Ca^{2+} influx via Na^+, Ca^{2+} exchange), as well as that of subsequent relaxations was slower in DOC-NaCl-treated rats than in controls, whether the relaxation was induced in normal (144.0 mM) or low (1.2 mM) organ bath Na^+ concentration (reflecting Ca^{2+} extrusion by both Ca^{2+}-ATPase and Na^+, Ca^{2+} exchange, and by Ca^{2+}-ATPase alone, respectively). However, in calcium-supplemented DOC-NaCl rats the aortic responses did not differ from control. The difference between the relaxation rate in normal and low Na^+ concentration in each aortic ring, representing the contribution of Na^+, Ca^{2+} exchange in these relaxations, was comparable in all groups.

6 In conclusion, calcium supplementation clearly attenuated the development of hypertension, cardiac hypertrophy, and sodium retention induced by the DOC-NaCl treatment. However, the associated rise in platelet $[Ca^{2+}]_i$ was not prevented, suggesting that in this form of experimental hypertension increased dietary calcium does not lower blood pressure by reducing $[Ca^{2+}]_i$. The results from vascular responses *in vitro* suggest that in arterial smooth muscle the DOC-NaCl treatment increased contractile sensitivity to depolarization, voltage-dependent Ca^{2+} entry and cell membrane permeability to ions, and attenuated relaxation responses and vascular Na^+, K^+-ATPase function. The results further suggest reduced ability of the cell membrane to transport Ca^{2+} (possibly via Ca^{2+}-ATPase) in DOC-NaCl hypertension. The high calcium diet opposed these alterations. The present results thus provide evidence that the antihypertensive effect of a high calcium diet in mineralocorticoid-salt hypertension is mediated by its beneficial effects on systemic sodium balance and arterial smooth muscle function.

Keywords: Arterial smooth muscle; atrial natriuretic peptide; blood pressure; dietary calcium; electrolyte balance; intracellular free calcium; mineralocorticoid-salt hypertension

Introduction

Increased dietary calcium lowers blood pressure (BP) in hypertensive patients (McCarron & Morris, 1985; Lasaridis *et al.*, 1989) and experimental animals (Resnick *et al.*, 1986; Pörsti *et al.*, 1992). However, the mechanisms underlying the antihypertensive effect of high calcium intake are yet undefined. Oral calcium loading may correct altered cellular calcium metabolism in hypertension, since in spontaneously hypertensive rats (SHR) a high calcium diet has been reported to reduce intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Young *et al.*, 1988; Furspan *et al.*, 1989; Pörsti *et al.*, 1992), a primary determinant of arterial contractions (Stull *et al.*, 1991). The reduction of abnormally elevated $[Ca^{2+}]_i$ could lead to an improvement in different cell functions, which has indeed been described in arterial smooth muscle from high calcium-fed SHR (Pörsti, 1992; Pörsti *et*

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al., 1992). However, in mineralocorticoid-salt hypertension the effects of increased dietary calcium on vascular smooth muscle function are largely unexplored. They would be of interest, since calcium supplementation has been reported to decrease arterial peripheral resistance in this form of experimental hypertension (DiPette *et al.*, 1989).

Oral calcium loading has been found to promote natriuresis (Ayachi, 1979). In addition, the BP reduction induced by calcium supplementation appears to be augmented in low renin type of human essential hypertension (Lasaridis *et al.*, 1989), in sodium-volume-dependent (i.e. low renin) experimental hypertension (Resnick *et al.*, 1986), and in SHR on high sodium intake (McCarron *et al.*, 1985). We have previously shown that a high calcium diet effectively counteracts the effects of mineralocorticoid treatment in SHR (Pörsti *et al.*, 1990; 1991) by enhancing natriuresis with an accompanying decrease in plasma atrial natriuretic peptide (ANP) (Pörsti *et al.*, 1991). Thus, it is evident that oral calcium loading also has effects on sodium metabolism.

The present study investigated the effects of a high calcium diet on BP, electrolyte balance, plasma and tissue ANP, $[Ca^{2+}]_i$, and arterial smooth muscle function in one-kidney deoxycorticosterone (DOC)-NaCl hypertensive Wistar rats. This mineralocorticoid-salt treatment considerably alters the systemic and cellular electrolyte balance (Friedman & Tanaka, 1987; Morton *et al.*, 1990), and can thus be expected to disturb cellular ion fluxes across cell membrane. Furthermore, it has been suggested that calcium supplementation may decrease membrane permeability to ions (Bohr *et al.*, 1988). Therefore, the study of arterial smooth muscle responses was designed to explore effects of DOC-NaCl treatment and high calcium diet on cell membrane function.

Methods

Animals and experimental design

Male Wistar rats were housed two animals to a cage in an experimental animal laboratory room at 22°C with a 12 h light-dark cycle, and provided standard chow (containing 1.1% calcium, 0.7% sodium chloride, 0.2% magnesium; Ewos, Södertälje, Sweden) and drinking fluid (tap water) *ad libitum*. At the age of 8 weeks the left kidney was removed from all rats under ether anaesthesia. Postoperative pain was relieved with buprenorphine (Temgesic, 0.3 mg kg^{-1} twice a day, s.c.) during the first 3 days, and 2 weeks were allowed for recovery. Then the 10-week-old rats were divided into four groups of equal mean systolic BPs, which were measured from the conscious rats by the tail cuff method at 28°C (Model 129 Blood Pressure Meter: IITC Inc., Woodland Hills, Ca., U.S.A.). One group was injected weekly with DOC (Percorten M, 25 mg kg^{-1} $week^{-1}$, s.c.) and 0.7% NaCl was added to the drinking water, while the chow remained unaltered (Group DOC, $n = 13$). For the second group the DOC-NaCl treatment was combined with dietary calcium supplementation by increasing the calcium content of the chow to 2.5% (Group Ca-DOC, $n = 13$), the increment being supplied as calcium carbonate. Two additional groups were injected weekly with equal volumes of saline instead of DOC, kept on normal drinking fluid, and fed with the high calcium (Group Ca-Wistar, $n = 8$) or standard chow (Group Wistar, $n = 13$).

BP was regularly measured during the 8-week study. In study weeks 1 and 8, urine was collected for 24 h in metabolic cages, where the rats had free access to food and drinking fluid. Fluid and food consumptions were determined by weighing the bottles and chow pellets. Urine volumes were measured and samples stored at − 20°C until assayed. At the end of the study the 18-week-old rats were decapitated and exsanguinated, and blood samples were collected into chilled heparinized tubes for plasma electrolyte and platelet $[Ca^{2+}]_i$ determinations, and chilled tubes on ice containing 2.7 mM EDTA for plasma ANP assays. Blood samples for electrolyte and ANP determinations were centrifuged, and plasma stored at − 20°C until analysis; $[Ca^{2+}]_i$ measurements were carried out immediately after blood collection. After exsanguination, the hearts, mesenteric arteries and aortae were immediately excised. The experimental design of the study was approved by the Animal Experimentation Committee of the University of Tampere, Finland.

Electrolyte measurements and platelet intracellular free calcium determination

Plasma and urine concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} were measured by atomic absorption spectrophotometer (AAS; Spectr AA-30, Varian, Techtron Ltd., Victoria, Australia). For the Ca^{2+} measurements, 5 mM $LaCl_3$ was used as ionization suppressant. Samples of abdominal aortae were cleaned of adherent connective tissue, dried overnight at 100°C and weighed. Then they were ashed in a graphite oven (Naber Industrieofenbau, Bremen, Germany) for 2 h at 800°C, and the inorganic salts dissolved in 20% nitric acid for the measurement of electrolytes by AAS. Aortic tissue electrolyte contents are expressed as a concentration per g tissue dry weight. Platelet $[Ca^{2+}]_i$ was measured with a fluorescent indicator quin-2 as previously described (Pörsti *et al.*, 1992).

Atrial natriuretic peptide determinations

Left and right auricles of the hearts were removed to be used for atrial ANP measurements, and the remaining atrial tissue carefully dissected from the ventricular tissue. The ventricles were cut into superior (15–20% of total weight) and inferior parts, the latter being used for ventricular ANP determinations to avoid atrial tissue contamination. The cardiac samples were blotted dry, weighed and stored at − 70°C until assayed. ANP was extracted from plasma as previously described (Ruskoaho *et al.*, 1989), and from the cardiac tissues by the HCl method (Ruskoaho & Leppäluoto, 1988). For the radioimmunoassay (RIA) the atrial and ventricular extracts were diluted 3×10^5 and 1.2×10^3 fold, respectively, with the RIA buffer. The tissue extracts and plasma samples were then incubated in duplicates of 100 µl with 100 µl of the specific rabbit ANP antiserum in the final dilution of 1:25000. ANP was determined from plasma and tissue samples by RIA as described by Ruskoaho *et al.* (1989). Tissue ANP is expressed as concentration per mg tissue wet weight.

Arterial responses in vitro

Two standard sections (3 mm in length) of the mesenteric artery, beginning 1.5 cm distally from the mesenteric artery aorta junction, and one standard section (4 mm) of the thoracic aorta just above the diaphragm were cut from each rat. The vascular endothelium was removed from all preparations by gently rubbing with a scuffed injection needle. The rings were then placed between hooks and mounted in an organ bath chamber. The mesenteric arterial rings were bathed in physiological salt solution (PSS) (adjusted to pH 7.4 with HCl) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, KCl 4.7, $CaCl_2$ 1.6, KH_2PO_4 1.2 and $MgSO_4$ 1.2. The aortic rings were bathed in a modified PSS (adjusted to pH 7.4 with Tris/HCl) containing (mM): NaCl 142.8, glucose 11.1, HEPES 10.0, KCl 4.7, $CaCl_2$ 1.6, HaH_2PO_4 1.2 and $MgSO_4$ 1.2 (Ashida *et al.*, 1989). All rings were aerated with 95% O_2: 5% CO_2, and rinsed with fresh solutions every 20 min, during which time the pH in the baths remained stable. The rings were equilibrated for 1 h at 37°C with a resting tension of 1.5 and 2.0 g for mesenteric arteries and aortas, respectively. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph (FT03 transducers & Model 7 E Polygraph; Grass Instrument Co., Quincy, Ma., U.S.A.).

The absence of endothelium in vascular preparations was confirmed by a lack of relaxation response to 1 μM acetylcholine in 1 μM noradrenaline (NA)-precontracted arterial rings.

Mesenteric arterial preparation 1: After a 30 min stabilization period the more proximal section cut from each mesenteric artery was used to determine cumulative concentration-response curves for high concentrations of K^+. When maximal contractions after 125 mM K^+ had fully developed, the rings were rinsed with normal PSS and the subsequent relaxation was observed until resting tension was restored. After another 30 min the K^+-induced contractions were again generated in the presence of the Ca^{2+} entry blocker nifedipine (10 nM). In solutions containing high concentrations of K^+ (20–125 mM), NaCl of the normal PSS was replaced with KCl on an equimolar basis.

Mesenteric arterial preparation 2: The more distal ring of each mesenteric artery was used to study the contractile responses evoked by omission of K^+ from the organ baths and subsequent relaxations induced by re-addition of K^+. The K^+-free buffer solution (pH 7.4) was prepared by substituting KCl and KH_2PO_4 of the normal PSS with NaCl and NaH_2PO_4, respectively, on an equimolar basis. After the rings had reached a steady contraction plateau in K^+-free solution, 0.5 mM K^+ was re-added and the subsequent relaxation registered. In an additional study with Wistar rats under experimental design identical to that described above, we further investigated the mechanisms of contractions induced by the K^+-free solution. In order to eliminate the influence of endogenously released NA on these contractions, endothelium-denuded mesenteric arterial rings from these animals were subjected to chemical adrenergic denervation by the method described by Aprigliano & Hermsmeyer (1976). The rings were denervated *in vitro* by exposing them to a buffer-free solution containing 1.2 mM 6-hydroxydopamine, and vigorously gassing them with nitrogen for 15 min, followed by a 2 h recovery period in normal PSS. Thereafter, the above described K^+-free contraction procedure was performed.

Aortic preparation: The aortic rings were used to examine indirectly the vascular Na^+, Ca^{2+} exchange mechanism and Ca^{2+}-ATPase activity, using basically the procedure described by Ashida & Blaustein (1987). The preparations were first exposed to 5 mM caffeine, which induced rapid, spontaneously fading contractions. After 10 min at resting tension the modified PSS (Na^+ 144.0 mM) was changed to low Na^+ (1.2 mM) medium, and the gradually developing contractions were observed for 10 min. Thereafter the medium was replaced with Ca^{2+}-free solution containing normal Na^+ (144.0 mM), and the resultant relaxation was registered. After another 10 min at resting tension in the modified PSS the aortic rings were again contracted by low Na^+ medium, upon which relaxation was elicited by Ca^{2+}-free solution with low Na^+ concentration (1.2 mM). The low Na^+ solutions (pH 7.4) were prepared by equimolar substition of NaCl in the modified PSS with N-methyl-D-glucamine. In Ca^{2+}-free solutions $CaCl_2$ was replaced with $MgCl_2$ on an equimolar basis. All aortic contractile and relaxation responses were performed in the presence of 5 mM caffeine, 1 μM nifedipine and 10 μM phentolamine to inhibit intracellular Ca^{2+} sequestration, voltage-dependent Ca^{2+} entry, and effects of endogenously liberated NA on vascular responses, respectively.

Materials

The following compounds were used: acetoxymethyl ester of quin-2 (Aldrich Chemical Co., Milwaukee, Wi., U.S.A.), deoxycorticosterone trimethylacetate (Ciba-Geigy Ltd., Basel, Switzerland), noradrenaline hydrogentartrate (Fluka Chemie AG, Buchs SC, Switzerland), caffeine (E. Merck AG, Darmstadt, Germany), nifedipine (Orion Pharmaceutical Co., Espoo, Finland), buprenorphine hydrochloride (Reckitt & Colman, Hull, U.K.), and acetylcholine chloride, 6-hydroxydopamine hydrobromide, N-methyl-D-glucamine, phentolamine hydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.)

Analysis of results

The arterial contractile and relaxation responses were related to tissue dry weight, which was obtained after drying the arterial preparations overnight at 100°C. The effect of nifedipine on K^+-induced contractions was calculated by comparing the contractions in the presence of nifedipine to those without it. After re-addition of K^+ upon K^+-free contractions the reduction in smooth muscle contractile force during 1 min was considered the maximal relaxation rate, which was related to tissue dry weight. K^+ relaxation was additionally related to the pre-existing contraction induced by the K^+-free medium.

Statistical analysis was carried out by one-way analysis of variance (ANOVA) supported by Bonferroni confidence intervals using BMDP Statistical Software (Los Angeles, Ca., U.S.A.). $P<0.05$ was considered statistically significant. All results are expressed as mean ± s.e.mean.

Results

Blood pressure, heart and body weights, and plasma and tissue ANP

The mean systolic BP was stable and comparable in the Wistar and Ca-Wistar groups during the 8-week study. The DOC-NaCl treatment-induced steady rise in BP was markedly attenuated in the Ca-DOC group, whose final BP level remained about 35 mmHg lower than that of the DOC group. The final systolic BPs in the experimental groups were: Wistar 147 ± 4, Ca-Wistar 147 ± 5, DOC 201 ± 6, and Ca-DOC 165 ± 3 mmHg (Figure 1). The heart weights in the Wistar and Ca-Wistar groups were similar. The cardiac hypertrophy induced by the DOC-NaCl treatment was significantly decreasd by concurrent oral calcium loading (Table 1). The body weights in the Wistar, DOC and Ca-

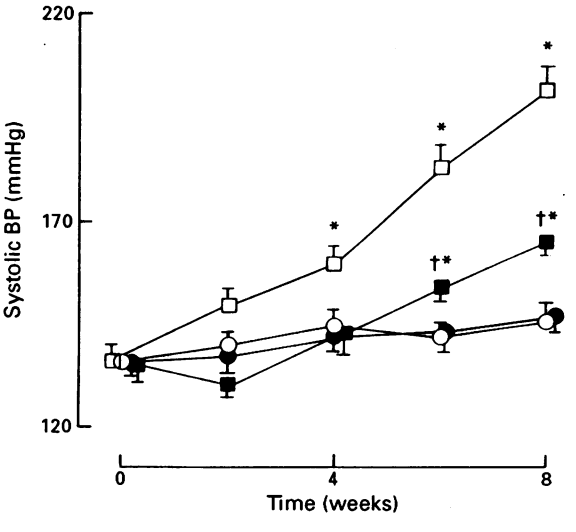


Figure 1 Systolic blood pressure (BP) in control (○, $n=13$), calcium-supplemented (●, $n=8$), deoxycorticosterone (DOC)-NaCl-treated (□, $n=13$), and calcium-supplemented DOC-NaCl-treated (■, $n=13$) Wistar rats during the 8-week study. Symbols indicate means with s.e.mean. *$P<0.05$ when compared with control rats; †$P<0.05$ when compared with DOC-NaCl-treated rats (Bonferroni test).

Table 1 Experimental group data at the end of the 8-week study

Variable	*Wistar*	*Ca-Wistar*	*DOC*	*Ca-DOC*
Body weight (g)	435 ± 9	395 ± 12*	441 ± 13	429 ± 11
Heart weight (mg)				
Right auricle	19 ± 1	19 ± 1	23 ± 2	21 ± 1
Left auricle	22 ± 2	20 ± 2	37 ± 2*	28 ± 1*†
Ventricles	868 ± 26	838 ± 37	1281 ± 41*	1049 ± 41*†
Heart:body weight ratio (mg g^{-1})	2.4 ± 0.1	2.5 ± 0.1	3.5 ± 0.1*	2.9 ± 0.1*†
Plasma electrolytes (mM)				
Na^+	121.7 ± 2.8	119.4 ± 3.3	140.6 ± 3.0*	127.9 ± 2.2†
K^+	4.19 ± 0.12	3.95 ± 0.12	4.35 ± 0.17	4.06 ± 0.12
Ca^{2+}	2.25 ± 0.04	2.54 ± 0.03*	2.44 ± 0.02*	2.38 ± 0.04*
Mg^{2+}	1.17 ± 0.03	1.09 ± 0.02*	0.96 ± 0.03*	0.89 ± 0.02*
Aortic tissue				
Na^+ (μmol g^{-1})	337 ± 22	343 ± 28	399 ± 22	347 ± 13
K^+ (μmol g^{-1})	155 ± 11	156 ± 13	154 ± 6	189 ± 12†
Na^+:K^+ ratio	2.2 ± 0.2	2.2 ± 0.2	2.6 ± 0.2	1.9 ± 0.1†
Platelet intracellular free Ca^{2+} (nM)	108 ± 4	108 ± 5	131 ± 8*	129 ± 7*

Values are mean ± s.e.mean. Ca = calcium-supplemented Wistar rats, DOC = deoxycorticosterone-NaCl-treated Wistar rats, $n = 13$ for Wistar, DOC and Ca-DOC groups, $n = 8$ for Ca-Wistar group.
*$P < 0.05$ compared with Wistar group; †$P < 0.05$ compared with DOC group (Bonferroni test).

DOC groups were comparable, but were slightly lower in the Ca-Wistar group (Table 1).

The concentrations of ANP in plasma and cardiac tissues were comparable in the Wistar and Ca-Wistar groups. The plasma ANP in the DOC group, but not in the Ca-DOC group, was significantly higher than in the Wistar group. The clear increase in ventricular ANP induced by the DOC-NaCl treatment was attenuated by calcium supplementation. The concentrations of ANP in the left atrium were decreased in both DOC and Ca-DOC groups, and in the right atrium in the Ca-DOC group (Figure 2).

Plasma and tissue electrolytes, and platelet intracellular free calcium

The DOC-NaCl treatment elevated the plasma Na^+ concentration, and the high calcium diet inhibited the elevation in the Ca-DOC group, while it did not affect plasma Na^+ in the Ca-Wistar group. Plasma K^+ levels were similar in all study groups. Both oral calcium supplementation and the DOC-NaCl treatment, and their combination, increased Ca^{2+} and decreased Mg^{2+} concentrations in plasma (Table 1). Tissue Na^+ and K^+ contents, and the Na^+:K^+ ratios in the aorta were comparable in the Wistar, Ca-Wistar and DOC groups, although tissue Na^+ tended to be increased in the DOC group. In the Ca-DOC group aortic tissue K^+ content was higher and the Na^+:K^+ ratio lower than in the DOC group (Table 1). Platelet $[Ca^{2+}]_i$, measured with quin-2, was equal in the Wistar and Ca-Wistar groups and significantly lower than in the DOC and Ca-DOC groups. Thus, the oral calcium loading did not affect the DOC-NaCl-induced rise in $[Ca^{2+}]_i$ (Table 1).

Food, fluid and electrolyte intake, and urinary electrolyte excretion

Chow consumption was comparable between the Wistar and Ca-Wistar rats, and also between the DOC and Ca-DOC rats, the consumption in the two latter being more pronounced than in the two former groups at the end of the study. Thus, the Ca-Wistar and Ca-DOC groups gained approximately 2.5 fold more Ca^{2+} than the corresponding groups on standard diet. Fluid consumption was greater in both DOC-NaCl-treated groups than in the other groups throughout the study. However, during week 8 the fluid intake (and thus Na^+ gain) in the Ca-DOC rats was attenuated to only about 60% of that in the DOC rats, Na^+ intake being increased 9.6 and 5.7 fold in the DOC and Ca-DOC groups, respectively, when compared with the Wistar group. The K^+ and Mg^{2+} gains were comparable in the

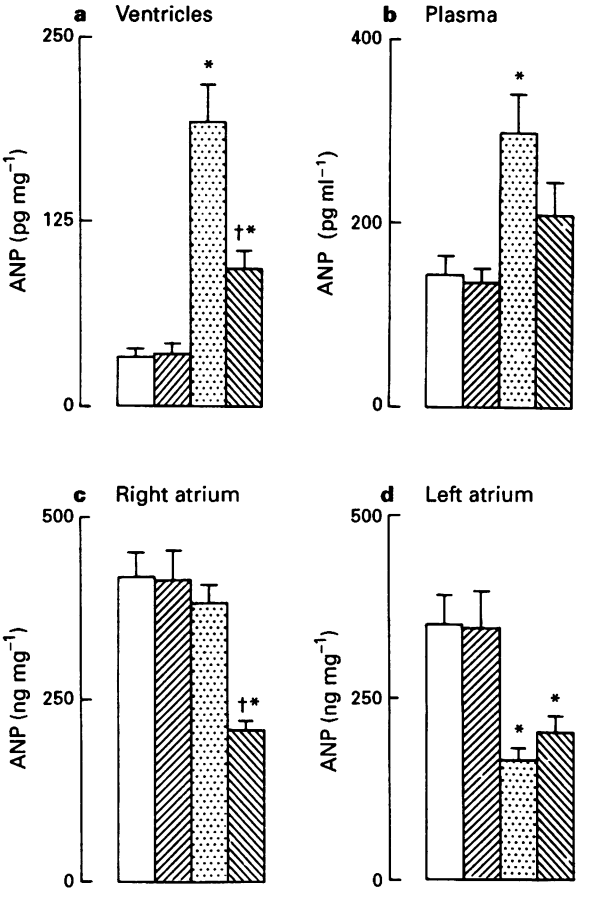


Figure 2 Atrial natriuretic peptide (ANP) concentrations in ventricular tissue (a), plasma (b), and in right (c) and left atrial tissues (d) of Wistar rats. Groups are control (□, $n = 12$), calcium-supplemented (▨, $n = 8$), deoxycorticosterone (DOC)-NaCl-treated (▩, $n = 11$), and calcium-supplemented DOC-NaCl-treated (▧, $n = 11$) Wistar rats. Columns indicate means with s.e.mean. *$P < 0.05$ when compared with control rats; †$P < 0.05$ when compared with DOC-NaCl-treated rats (Bonferroni test).

Wistar and Ca-Wistar groups, and increased in both DOC-NaCl-treated groups at the end of the study (Table 2). The urinary excretion of Na^+ was unaltered in the Ca-Wistar group, and increased in both DOC-NaCl-treated groups. Despite the lower Na^+ intake in the Ca-DOC than in the DOC group at the end of the study, Na^+ excretion did not differ significantly between these groups. K^+ excretion was proportional to the intake in all groups. Both oral calcium supplementation and DOC-NaCl treatment independently elevated urinary excretions of Ca^{2+} and Mg^{2+} to a level higher than would be expected on the basis of intakes. In the Ca-DOC group, Ca^{2+} excretion was further enhanced, whereas that of Mg^{2+} was reduced when compared to the DOC group (Table 2).

Arterial responses in vitro

The smooth muscle contractions in response to depolarization by K^+ (20–125 mM) in mesenteric arterial rings were similar in the Wistar, Ca-Wistar and Ca-DOC groups, but significantly enhanced in the DOC group with lower K^+ concentrations (20–30 mM) (Figure 3). Nifedipine (10 nM) was clearly more effective in inhibiting the K^+-evoked contractions in the DOC group than in the Wistar and Ca-Wistar groups. In the Ca-DOC group the effect of nifedipine was significantly greater than in the Wistar group only with 30 mM K^+. Thus the K^+ response in the presence of nifedipine in the Ca-DOC group was shifted towards that in the Wistar controls when compared with the DOC group (Figure 3). The washout time after maximal 125 mM K^+-induced contractions was comparable in the Wistar, Ca-Wistar and Ca-DOC groups, and shorter than in the DOC group (Table 3).

The contractions elicited by omission of K^+ from the organ baths in endothelium-denuded mesenteric arterial rings with intact adrenergic nerve-endings (representative tracing of the response shown in Figure 4) were similar in the Wistar and Ca-Wistar groups. In the DOC group the K^+-free response started earlier but the contractile rate and maximal force attained were lower than in the Wistar group. The dietary calcium supplementation during the DOC-NaCl treatment shifted the curve notably towards control (Figure 5 and Table 3). In an additional study, where the calcium supplementation again attenuated the DOC-NaCl-induced rise in BP (by 27 mmHg), the K^+-free contractions in endothelium-denuded mesenteric arterial rings were elicited after *in vitro* adrenergic denervation with 6-hydroxydopamine. When compared with the results obtained in rings with intact nerve-endings, the denervation markedly decreased the maximal K^+-free contractile force, and prolonged the time from K^+ omission to the onset of contraction and that required to reach the maximal contraction in control and calcium-supplemented Wistar rats. In DOC-NaCl-treated rats this attenuation of the K^+-free contractile rate was much smaller than in controls. In contrast to rings with intact nerve-endings, the denervated rings of DOC-NaCl-treated rats developed significantly greater maximal contractile force in shorter time, and thus a faster rate of K^+-free contraction, than those of control rats. The concurrent calcium supplementation again shifted the response towards that in controls (Table 4). The relaxations induced by 0.5 mM K^+ upon full K^+-free contractions were similar in the Wistar and Ca-Wistar groups and faster than in the DOC group, in which complete relaxation was not achieved. In the Ca-DOC group the K^+ relaxation was also slower than in the Wistar group, but nevertheless the response returned to the baseline and was clearly faster than in the DOC group (Figure 5 and Table 3).

The 5 mM caffeine-induced aortic contractions were enhanced by the DOC-NaCl treatment, but were unaffected by oral calcium loading (Table 3). The contractile force development in response to exposure to low Na^+ medium in the presence of 5 mM caffeine, 1 μM nifedipine and 10 μM phentolamine, the response thus reflecting Ca^{2+} entry via Na^+,

Table 2 Metabolic data (per 24 h) from experimental groups during study weeks 1 and 8

Variable	*Wistar*	*Ca-Wistar*	*DOC*	*Ca-DOC*
Week 1				
Chow intake (g)	26 ± 1	26 ± 1	23 ± 4	29 ± 1
Fluid intake (ml)	37 ± 1	34 ± 2	75 ± 9*	67 ± 8*
Urine volume (ml)	17 ± 1	15 ± 1	43 ± 7*	35 ± 5*
Electrolyte intake (mmol)				
Na^+	3.4 ± 0.1	3.4 ± 0.1	12.9 ± 1.6*	12.4 ± 1.1*
K^+	5.3 ± 0.2	5.3 ± 0.2	4.8 ± 0.8	5.9 ± 0.2
Ca^{2+}	7.1 ± 0.2	16.2 ± 0.6*	6.4 ± 1.1	17.8 ± 0.6*†
Mg^{2+}	2.1 ± 0.1	2.1 ± 0.1	1.9 ± 0.3	2.4 ± 0.1
Urinary excretion (mmol)				
Na^+	3.3 ± 0.2	3.1 ± 0.1	14.8 ± 1.3*	12.8 ± 1.3*
K^+	3.9 ± 0.2	4.0 ± 0.2	4.1 ± 0.4	4.2 ± 0.2
Ca^{2+}	0.09 ± 0.02	0.24 ± 0.03*	0.20 ± 0.03*	0.43 ± 0.07*†
Mg^{2+}	0.36 ± 0.06	0.73 ± 0.09*	0.90 ± 0.13*	0.86 ± 0.10*
Week 8				
Chow intake (g)	13 ± 2	15 ± 1	25 ± 2*	22 ± 2*
Fluid intake (ml)	23 ± 2	20 ± 1	100 ± 17*	53 ± 4*†
Urine volume (ml)	15 ± 2	12 ± 1	80 ± 12*	41 ± 4*†
Electrolyte intake (mmol)				
Na^+	1.7 ± 0.2	2.0 ± 0.1	16.3 ± 2.4*	9.7 ± 0.7*†
K^+	2.7 ± 0.3	3.2 ± 0.2	5.1 ± 0.4*	4.5 ± 0.3*
Ca^{2+}	3.6 ± 0.5	9.7 ± 0.5*	6.8 ± 0.5*	13.6 ± 1.0*†
Mg^{2+}	1.1 ± 0.1	1.3 ± 0.1	2.0 ± 0.2*	1.8 ± 0.1*
Urinary excretion (mmol)				
Na^+	2.5 ± 0.2	2.4 ± 0.2	17.0 ± 2.6*	12.2 ± 0.9*
K^+	3.5 ± 0.3	3.5 ± 0.2	5.4 ± 0.4*	5.0 ± 0.4*
Ca^{2+}	0.09 ± 0.01	0.31 ± 0.05*	0.40 ± 0.05*	0.69 ± 0.08*†
Mg^{2+}	0.21 ± 0.02	0.50 ± 0.03*	0.84 ± 0.13*	0.53 ± 0.03*†

Values are mean ± s.e.mean. Ca = calcium-supplemented Wistar rats, DOC = deoxycorticosterone-NaCl-treated Wistar rats, $n = 13$ for Wistar, DOC and Ca-DOC groups, $n = 8$ for Ca-Wistar group.
*$P < 0.05$ compared with Wistar group; †$P < 0.05$ compared with DOC group (Bonferroni test).

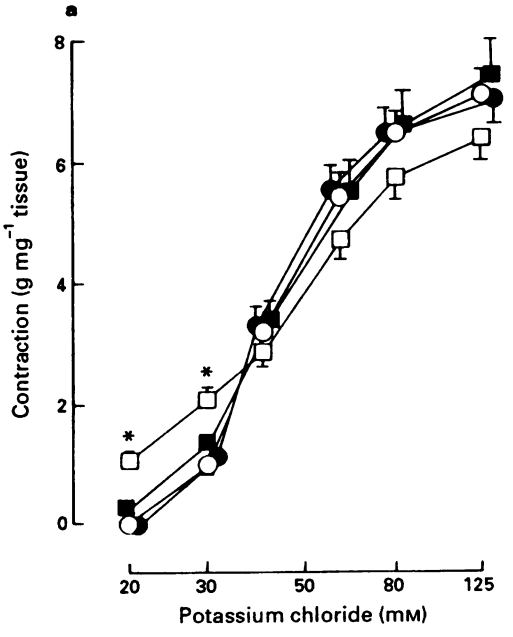


Figure 3 Concentration-response curves of Wistar rat endothelium-denuded mesenteric arterial rings to potassium chloride (a), and the effect of 10 nM nifedipine on these contractions (b). Groups are control (○, $n = 11$), calcium-supplemented (●, $n = 8$), deoxycorticosterone (DOC)-NaCl-treated (□, $n = 10$), and calcium-supplemented DOC-NaCl-treated (■, $n = 10$) Wistar rats. Symbols indicate means with s.e.mean. *$P < 0.05$ when compared with control rats (Bonferroni test).

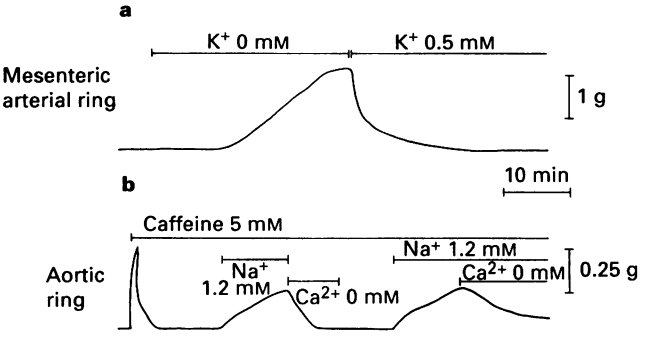


Figure 4 Representative tracings of the contractile response of an endothelium-denuded mesenteric arterial ring induced by K^+-free solution and the relaxation after the re-addition of 0.5 mM K^+ to the organ bath (a); and the contractile responses of an endothelium-denuded aortic ring induced by 5 mM caffeine, and by reduction of organ bath Na^+ concentration from 144.0 to 1.2 mM (Na^+ replaced by N-methyl-D-glucamine), and plots of subsequent aortic relaxations elicited by Ca^{2+}-free solution in normal (144.0 mM) and low (1.2 mM) organ bath Na^+ concentration. All aortic contractile and relaxation responses were performed in the presence of 5 mM caffeine, 1 μM nifedipine and 10 μM phentolamine (b).

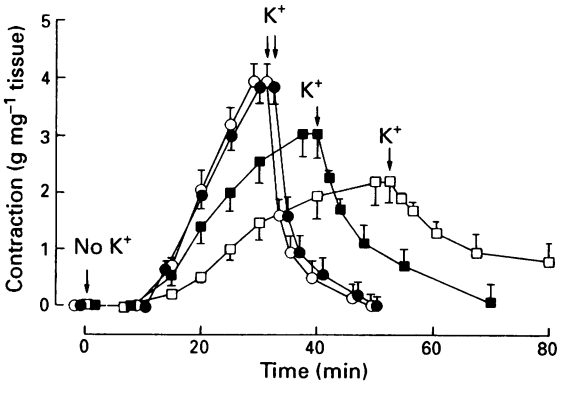


Figure 5 Contractile responses induced by K^+-free solution and subsequent relaxations in response to re-addition of 0.5 mM K^+ in Wistar rat endothelium-denuded mesenteric arterial rings with intact adrenergic nerve-endings. Groups are control (○, $n = 11$), calcium-supplemented (●, $n = 8$), deoxycorticosterone (DOC)-NaCl-treated (□, $n = 10$), and calcium-supplemented DOC-NaCl-treated (■, $n = 10$) Wistar rats. Symbols indicate means with s.e.mean. The onset of K^+-free contractions occurred earlier, and the maximal force was lower and attained later in DOC-NaCl-treated than control rats ($P < 0.05$). The concurrent calcium supplementation clearly diminished these differences. Subsequent K^+ relaxations were slower in DOC-NaCl-treated rats than in control and calcium-supplemented DOC-NaCl-treated rats ($P < 0.05$). Statistical analysis was performed by Bonferroni tests.

Ca^{2+} exchange, was similar in aortic rings from the Wistar, Ca-Wistar and Ca-DOC groups in two successive responses (representative tracing in Figure 4). In the DOC group the low Na^+ medium-induced contractions were slower than in the other groups. The rates of relaxation evoked by Ca^{2+}-free medium in a normal organ bath Na^+ concentration (144.0 mM) upon low Na^+ contractions (the relaxation evaluating Ca^{2+} extrusion by both Na^+, Ca^{2+} exchange and Ca^{2+}-ATPase) were also equal in the Wistar, Ca-Wistar and Ca-DOC groups, and attenuated in the DOC group. When relaxations were induced by Ca^{2+}-free medium in a low organ bath Na^+ concentration (1.2 mM), the responses (evaluating Ca^{2+} extrusion by Ca^{2+}-ATPase alone) were slower than those in the normal Na^+ concentration, and again slowest in the DOC group and similar in the other groups (Figure 6 and Table 3). However, the difference between the relaxation rate in normal and low organ bath Na^+ in each aortic preparation (representing the contribution of Na^+, Ca^{2+} exchange in these relaxations) was comparable in all four groups (Table 3).

Discussion

The present study shows a marked antihypertensive effect of increased dietary calcium in one-kidney DOC-NaCl hyperten-

Table 3 Parameters of arterial smooth muscle responses at the end of the 8-week study

Variable	*Wistar*	*Ca-Wistar*	*DOC*	*Ca-DOC*
Mesenteric arterial ring[a]				
Washout time after 125 mM KCl (min)	8.2 ± 0.9	7.4 ± 1.1	20.4 ± 3.0*	11.0 ± 2.3†
K^+-free contraction				
Onset of contraction (min)	9.1 ± 0.7	10.2 ± 0.7	6.8 ± 0.6*	8.0 ± 0.3
Time to maximum (min)	28.8 ± 1.5	31.5 ± 2.3	51.8 ± 3.8*	36.7 ± 3.8†
Maximal force (g mg^{-1} tissue)	4.0 ± 0.3	3.9 ± 0.3	2.3 ± 0.4*	3.1 ± 0.6
K^+ relaxation				
Time to baseline (min)	18.0 ± 2.8	16.7 ± 2.1	[b]	31.8 ± 4.5*
30 min relaxation (% of K^+-free contraction)	100 ± 0	100 ± 0	65 ± 8*	96 ± 7†
Maximal rate (g mg^{-1} tissue min^{-1})	1.18 ± 0.13	1.24 ± 0.17	0.14 ± 0.04*	0.42 ± 0.07*†
Aortic ring				
Caffeine (5 mM) contraction (mg mg^{-1} tissue)	266 ± 28	255 ± 18	410 ± 48*	417 ± 23*
Mean low Na^+ medium-induced contractile rate (mg mg^{-1} tissue min^{-1})				
1st response	11.8 ± 0.7	12.5 ± 1.0	7.4 ± 0.6*	12.0 ± 1.4†
2nd response	13.1 ± 1.6	14.0 ± 1.0	8.3 ± 0.7*	14.1 ± 1.7†
Mean relaxation rate after low Na^+ contraction (mg mg^{-1} tissue min^{-1})				
1st response (normal Na^+, Ca^{2+}-free)	26.1 ± 2.4	26.5 ± 2.4	18.0 ± 1.3*	26.0 ± 2.7†
2nd response[c] (low Na^+, Ca^{2+}-free)	8.1 ± 1.5	8.6 ± 1.0	4.1 ± 0.4*	7.2 ± 0.5†
Difference between 1st and 2nd response	17.6 ± 2.2	16.6 ± 1.5	14.2 ± 1.2	18.8 ± 2.6
2nd response: relaxation in 30 min (mg mg^{-1} tissue)	104 ± 16	104 ± 18	58 ± 6*	101 ± 7†

Values are mean ± s.e.mean. Ca = calcium-supplemented Wistar rats, DOC = deoxycorticosterone-NaCl-treated Wistar rats, $n = 9-11$ for Wistar, DOC and Ca-DOC groups, $n = 8$ for Ca-Wistar group.
*$P < 0.05$ compared with Wistar group; †$P < 0.05$ compared with DOC group (Bonferroni test).
[a]Rings with intact adrenergic nerve-endings; [b]total relaxation not achieved; [c]calculated from the first 10 min of the 2nd relaxation.

Table 4 Parameters of contractile responses induced by K^+-free solution in chemically denervated mesenteric arterial rings

Variable	*Wistar*	*Ca-Wistar*	*DOC*	*Ca-DOC*
Onset of contraction (min)	40.2 ± 9.8	44.0 ± 9.6	3.8 ± 1.2*	8.0 ± 1.7*†
Time to maximum (min)	98.1 ± 8.5	106.2 ± 4.9	72.3 ± 3.1*	81.7 ± 1.9*†
Maximal force (g mg^{-1} tissue)	1.6 ± 0.2	1.7 ± 0.2	2.2 ± 0.1*	1.9 ± 0.1

Values are mean ± s.e.mean. Ca = calcium-supplemented Wistar rats, DOC = deoxycorticosterone-NaCl-treated Wistar rats, $n = 6$ for all groups. Adrenergic denervation of the rings was performed *in vitro* with 1.2 mM 6-hydroxydopamine.
*$P < 0.05$ compared with Wistar group; †$P < 0.05$ compared with DOC group (Bonferroni test).

sive rats. Earlier investigations have also reported considerable BP reductions by supplementary calcium in sodium-volume-dependent forms of experimental hypertension (Resnick *et al.*, 1986; Yang *et al.*, 1989; Pamnani *et al.*, 1990), whereas in genetic hypertension the effect is usually only moderate (Ayachi, 1979; Pörsti, 1992; Pörsti *et al.*, 1992). This suggests that the mechanisms of action of oral calcium loading may particularly involve the correction of those abnormalities characteristic of sodium-volume-dependent hypertension (e.g. in systemic and cellular electrolyte metabolism, and membrane permeability).

In the present work, the reduction in cardiac afterload achieved by calcium supplementation was confirmed by a clear attenuation of both DOC-NaCl-induced cardiac hypertrophy and rise in ventricular ANP concentrations. The hypertension-induced ventricular hypertrophy is associated with increased synthesis, storage and release of ANP (Lattion *et al.*, 1986; Ruskoaho *et al.*, 1989; Itoh *et al.*, 1991). Thus, the attenuated rise in ventricular ANP and coincident reduction in plasma ANP levels in the Ca-DOC group indicates diminished ventricular ANP synthesis and release when compared with the DOC group. The ANP concentrations in the left atria were reduced in both DOC-NaCl-treated groups. This probably reflects increased atrial ANP release in response to increased cardiac load (Sugimoto *et al.*, 1986) because the rate of ANP synthesis in the atria of hypertrophied rat heart is known to remain fairly constant (Kinnunen *et al.*, 1991). Fujimara *et al.* (1989) reported that acute hypercalcemia is a potent stimulus for ANP release from rat hearts. Since the right atrial ANP concentrations were reduced in the Ca-DOC group but unaffected by either high calcium diet or DOC-NaCl treatment alone, the combination of local hypercalcemia in the right atrium as a result of increased calcium delivery from the intestine to venous blood, and the DOC-NaCl-induced volume load apparently augmented ANP release from the right atria of Ca-DOC rats.

The DOC-NaCl treatment caused clear sodium retention in the present study as judged by the elevated plasma N^+ concentration (and a similar tendency in arterial tissue Na^+ content) in the DOC group. Plasma and ventricular ANP concentrations were also increased in the DOC group, reflecting an attempt by the heart to reduce the volume load (Lang *et al.*, 1987). Since plasma Na^+ and ANP levels in the Ca-DOC group were comparable to those in the Wistar controls, the sodium-volume retention was attenuated by oral calcium loading. This can partially be explained by lower

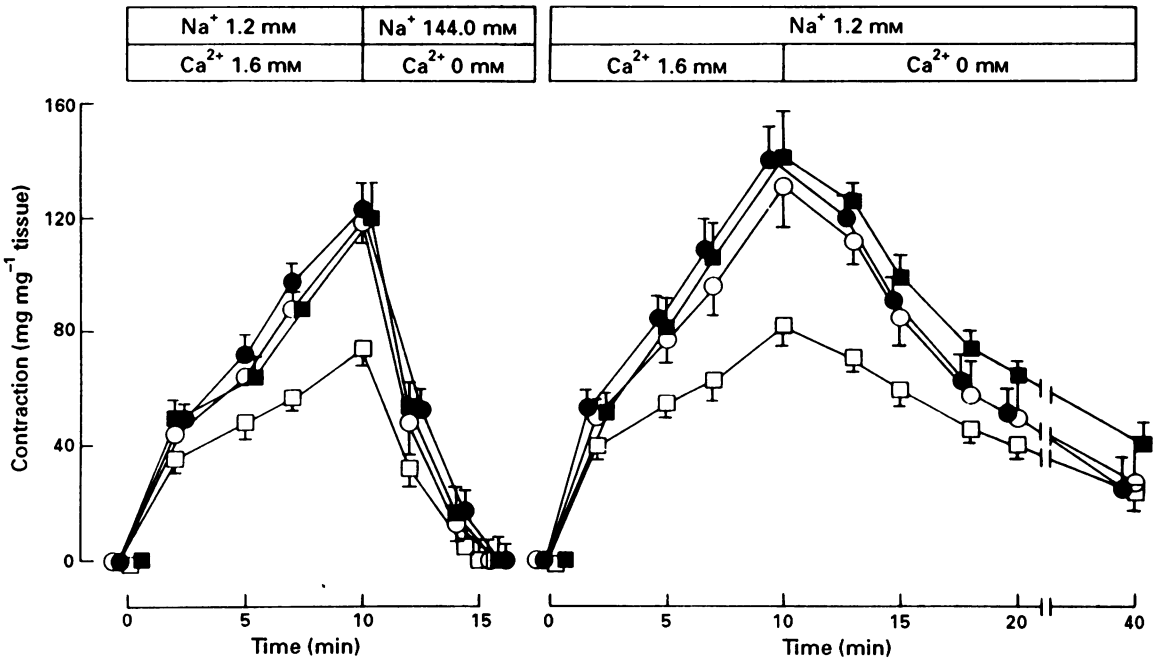


Figure 6 Contractile responses of Wistar rat endothelium-denuded aortic rings induced by reduction of organ bath Na^+ concentration from 144.0 to 1.2 mM (Na^+ replaced by N-methyl-D-glucamine), and subsequent aortic relaxations elicited by Ca^{2+}-free solution in normal (144.0 mM) and low (1.2 mM) organ bath Na^+ concentration. All responses were performed in the presence of 5 mM caffeine, 1 μM nifedipine and 10 μM phentolamine. Groups are control (○, $n = 10$), calcium-supplemented (●, $n = 8$), deoxycorticosterone (DOC)-NaCl-treated (□, $n = 10$), and calcium-supplemented DOC-NaCl-treated (■, $n = 9$) Wistar rats. Symbols indicate means with s.e.mean. The low Na^+ contractions and both subsequent Ca^{2+}-free solution-induced relaxations were slower in DOC-NaCl-treated than control rats ($P < 0.05$, Bonferroni test), while calcium-supplemented DOC-NaCl-treated rats did not differ from control rats.

sodium intake in the Ca-DOC than the DOC group. Lower sodium intake consequently decreased urinary Na^+ excretion in the Ca-DOC group, which nevertheless did not significantly differ from that in the DOC group. This suggests that additional dietary calcium enabled the Ca-DOC rats to maintain effective natriuresis at considerably lower BP levels when compared with the DOC rats. Since the pressure natriuresis was probably reduced along with the reduction of BP, the results point to the natriuretic effect of calcium supplementation previously demonstrated both in experimental animals (Ayachi, 1979; Pörsti *et al.*, 1991) and in man (Lasaridis *et al.*, 1989; Saito *et al.*, 1989). In the calcium-supplemented groups, urinary excretion of Ca^{2+} was increased more than would be expected on the basis of calcium intake, suggesting a relative enhancement of intestinal Ca^{2+} absorption during high calcium diet, a phenomenon which may be attributed to increased paracellular diffusion of Ca^{2+} in the gut (Wasserman & Fuller, 1989). Previously mineralocorticoid-salt treatment has been shown to induce hypercalciuria (Zikos *et al.*, 1986), a result also observed in the present study. Additional dietary calcium also increased the loss of Mg^{2+} into urine. This has been regarded as an adverse side-effect potentially leading to Mg^{2+} deficiency and thus impeding the beneficial effects of oral calcium loading (Evans *et al.*, 1990; Wuorela *et al.*, 1992). Accordingly, plasma Mg^{2+} levels were decreased in the calcium-supplemented groups, but no signs of compromised well-being of the animals were observed during the study, probably due to a relatively high Mg^{2+} content of the chow. Interestingly, despite its own Mg^{2+} wasting effect calcium supplementation seemed to attenuate urinary Mg^{2+} loss in the Ca-DOC group.

The concentration of cytosolic free Ca^{2+} is a primary factor determining arterial contractions (Stull *et al.*, 1991). Previously, increased dietary calcium has been found to lower both BP and $[Ca^{2+}]_i$ in SHR (Furspan *et al.*, 1989; Pörsti *et al.*, 1992; Wuorela *et al.*, 1992), giving rise to the conception that the reduction of $[Ca^{2+}]_i$ is an important antihypertensive mechanism of calcium supplementation in genetic hypertension. In the present study, platelet basal $[Ca^{2+}]_i$ was clearly higher in the DOC than the Wistar group, and was unaffected by oral calcium loading. These results are consistent with the finding that caffeine-induced aortic smooth muscle contractions, generated by Ca^{2+} release from intracellular stores (Karaki & Weiss, 1988), were also enhanced by the DOC-NaCl treatment and unaffected by the high calcium diet. Thus, the inability of calcium supplementation to decrease $[Ca^{2+}]_i$ despite a clear antihypertensive effect suggests that reduction of basal $[Ca^{2+}]_i$ is not a crucial BP-lowering mechanism of action by supplementary calcium in this type of sodium-volume-dependent hypertension.

The arterial smooth muscle contractions induced by K^+-free medium have commonly been used as a tool for studying cell membrane permeability to ions (Bohr *et al.*, 1988). The K^+-free solution inhibits vascular Na^+, K^+-ATPase and leads to Na^+ leak to the cells, which causes depolarization increasing Ca^{2+} entry through voltage-dependent channels (Mulvany, 1985). The depolarization of vascular adrenergic nerve-endings releases endogenous NA, which stimulates α-adrenoceptors and contributes to the contractile response (Vanhoutte & Lorenz, 1984). Additionally, passive Na^+ influx during Na^+, K^+-ATPase inhibition decreases the membrane Na^+ gradient and can thereby reduce Ca^{2+} extrusion by the Na^+, Ca^{2+} exchange mechanism and lead to increased intracellular Ca^{2+} (Ashida & Blaustein, 1987). However, since Na^+, Ca^{2+} exchange did not noticeably affect mesenteric arterial tone in our experimental conditions, voltage-dependent Ca^{2+} entry and endogenous release of NA are the main mechanisms of K^+-free contractions in rat mesenteric arteries (Arvola *et al.*, 1992). In the present study, the onset of K^+-free contractions occurred earlier in the DOC group than in the Wistar controls. Adrenergic denervation with

6-hydroxydopamine attenuated the K^+-free contractions less effectively in the DOC-NaCl-treated than in control rats, suggesting that these contractions are more dependent on Na^+ leak and Ca^{2+} influx to the smooth muscle cells in DOC-NaCl hypertensive than in normotensive rats, in which endogenous NA liberation is the predominant contraction mechanism. Similar finding has previously been shown in SHR (Arvola *et al.*, 1992). Furthermore, after adrenergic denervation the rate of contractile force development as well as the maximal force induced by the K^+-free solution were greater in the DOC-NaCl-treated rats than Wistar controls. The concurrent calcium supplementation partially abolished these differences. Since DOC-NaCl hypertension is known to be characterized by increased permeability to Na^+ and other substances in vascular smooth muscle (Friedman & Tanaka, 1987), the premature onset of the K^+-free contractions and the faster rate of the contractions in denervated rings in the DOC group suggest increased cell membrane permeability to ions in DOC-NaCl hypertension, which phenomenon dietary calcium supplementation appeared to reduce.

The relaxation responses following re-addition of K^+ upon K^+-free contractions indirectly evaluate vascular Na^+, K^+-ATPase activity (Hermsmeyer & Harder, 1986). In support of this, ouabain almost completely inhibits these relaxations in rat mesenteric arterial preparations (Arvola *et al.*, 1992). Na^+, K^+-ATPase has been reported to be both decreased (Songu-Mize *et al.*, 1982) and increased (Magliola *et al.*, 1986; Yang *et al.*, 1989) in DOC-NaCl hypertension. In the present study, K^+ relaxation was markedly attenuated in mesenteric arteries from the DOC group, while the response was clearly improved in the Ca-DOC group. The results support the concept of impaired vascular Na^+, K^+-ATPase function in chronic DOC-NaCl hypertension and its partial restoration during high calcium diet, a similar process being shown in calcium-supplemented SHR (Pörsti *et al.*, 1992). This indirect evidence of enhanced Na^+, K^+-ATPase by oral calcium is further supported by the reduced Na^+:K^+ ratio in aortic tissue from the Ca-DOC rats when compared with the DOC group. Since the membrane repolarization initiating the relaxation after re-addition of K^+ is probably very rapid, while complete K^+ relaxation takes several minutes, the rate of K^+ relaxation most likely also reflects general relaxation mechanisms (e.g. contractile protein dephosphorylation, Ca^{2+} sequestration and extrusion), which appear to be impaired in DOC-NaCl hypertension and enhanced by calcium supplementation. This interpretation is in agreement with the greatly prolonged washout time after maximal 125 mM KCl-induced contractions in the DOC group, but not in the Ca-DOC group.

Smooth muscle contractions induced by depolarizing the cell membrane with high concentrations of K^+ depend mainly upon Ca^{2+} influx through voltage-dependent channels, a minor proportion of the response resulting from the release of stored Ca^{2+} (Karaki & Weiss, 1988). Additionally, the release of endogenous NA during depolarization participates in these contractile responses (Fouda *et al.*, 1991). In the present study, the K^+-induced (20–125 mM) mesenteric arterial contractions were enhanced in the DOC group when using lower concentrations of K^+ (20–30 mM), and calcium supplementation abolished this enhancement. The concentration-response curves for K^+ in the presence of the dihydropyridine Ca^{2+} entry blocker, nifedipine, even more clearly differentiated the Wistar and DOC groups, nifedipine more effectively inhibiting the contractions in the DOC group. However, the inhibition by nifedipine appeared to be reduced in the Ca-DOC group. These results suggest that enhanced contractions and increased voltage-dependent Ca^{2+} entry in response to depolarization occur in DOC-NaCl hypertensive arterial smooth muscle, and that oral calcium can partially inhibit the development of these alterations. Since the effects of endogenous NA release were not eliminated in the present experiments, different adrenergic neuronal influences may have affected the results. However, the increased inhibition of these contractions by nifedipine, acting rather selectively on voltage-dependent Ca^{2+} channels, and the minor contribution of sympathetic nerve terminals on the K^+-free contractions in the DOC group suggest that increased receptor-operated Ca^{2+} entry due to enhanced NA release is not a likely explanation for the enhanced high K^+-induced contractions of the DOC rats.

The low Na^+ medium-induced vascular contractions in the presence of nifedipine, phentolamine and caffeine are considered to be due to Ca^{2+} entry via the Na^+, Ca^{2+} exchange, the dominant mechanism elevating smooth muscle $[Ca^{2+}]_i$ in these experimental conditions (Ashida & Blaustein, 1987). Nifedipine and phentolamine inhibit voltage-dependent Ca^{2+} entry activated by depolarization and receptor-operated Ca^{2+} entry stimulated by endogenous NA, respectively, and caffeine minimizes the cytosolic sequestration of influxed Ca^{2+}. The rate of low Na^+ contractions in endothelium-denuded aortic preparations was slower in the DOC than in the Ca-DOC group, which in turn did not deviate from the Wistar group. Therefore, if these contractions reflect Ca^{2+} entry into aortic smooth muscle via the Na^+, Ca^{2+} exchange, this appears to be diminished in DOC-NaCl hypertension and to be normalized by concurrent oral calcium loading. However, an alternative explanation for the attenuated low Na^+ contractions of the DOC rats may be more pronounced inhibition of aortic responses by nifedipine, since it more effectively inhibited the K^+-induced mesenteric arterial contractions in the DOC group than in the Wistar and Ca-DOC groups. Previously Ashida *et al.* (1989) have reported increased Na^+, Ca^{2+} exchange in SHR aortaes when compared with normotensive controls. Thus genetic and DOC-NaCl-induced forms of experimental hypertension appear to differ from each other in this respect. Different endothelial influences may however have affected the results, since in contrast to the present investigation the vascular endothelium was not removed in the above-mentioned study. The presence of intact endothelium may considerably depress slowly developing vascular contractions, particularly in arteries from normotensive animals (Arvola *et al.*, 1992). The role of Na^+, Ca^{2+} exchange in vascular smooth muscle function is still unclear (Matlib, 1991). This mechanism may be operative in large arteries (Ashida & Blaustein, 1987), but in smaller vessels its importance appears to be marginal (Mulvany *et al.*, 1984). Consistent with this, we have detected no contractile force development in mesenteric arterial preparations subjected to low Na^+ medium in the conditions described above (unpublished observations).

The rate of relaxation following removal of organ bath Ca^{2+} upon low Na^+ contractions reflects the ability of smooth muscle cells to pump out cytoplasmic Ca^{2+} by two potential mechanisms: Ca^{2+}-ATPase and passive Na^+, Ca^{2+} exchange (Ashida & Blaustein, 1987). The latter can mediate either Ca^{2+} entry or extrusion depending on the prevailing Na^+ and Ca^{2+} gradients across the cell membrane. At normal organ bath Na^+, the Ca^{2+}-free medium-induced relaxation response represents the combined effect of the two above mechanisms. However, at low Na^+ concentration, the relaxation is mediated by Ca^{2+}-ATPase alone, since in the absence of an inward Na^+ gradient the Na^+, Ca^{2+} exchange cannot translocate Ca^{2+} out of the cell. In the present study, the Ca^{2+}-free medium-induced relaxation responses upon low Na^+ contractions were slowest in aortic smooth muscle in the DOC group whether the organ bath Na^+ concentration was normal or low, whereas no significant differences were found between the Ca-DOC and Wistar groups. The difference between the relaxation rate in normal and low organ bath Na^+ concentration in each aortic preparation, evaluating the contribution of the Na^+, Ca^{2+} exchange to the relaxation response, was comparable in all study groups. Thus, the role of Na^+, Ca^{2+} exchange in aortic relaxation seems to be unaltered in DOC-NaCl hypertension. Instead, the slow relaxation rate induced by Ca^{2+}-free solution in aortae from DOC-NaCl-treated rats is suggestive of impaired ability of

the cell membrane to extrude Ca^{2+} via the active Ca^{2+}-ATPase, the function of which appears to be enhanced by concurrent calcium supplementation. Correspondingly, Wuorela *et al.* (1992) have previously reported that a high calcium diet activates erythrocyte Ca^{2+}-ATPase in SHR. However, since the decreased magnitude of the low Na^+ contractions in the DOC group suggests that less Ca^{2+} had entered into aortic smooth muscle cells of the DOC rats when compared with the other groups, the reduced rate of aortic relaxation in the DOC group may also be due to a decrease in the driving force for Ca^{2+} extrusion.

In conclusion, calcium supplementation clearly attenuated the development of hypertension, cardiac hypertrophy and sodium retention induced by the DOC-NaCl treatment. However, the associated rise in platelet basal $[Ca^{2+}]_i$ was not prevented, suggesting that in this form of experimental hypertension increased dietary calcium does not lower blood pressure by reducing $[Ca^{2+}]_i$. The results from vascular responses *in vitro* suggest that in arterial smooth muscle the DOC-NaCl treatment increased contractile sensitivity to depolarization, voltage-dependent Ca^{2+} entry, and cell membrane permeability to ions, and attenuated arterial relaxation and vascular Na^+, K^+-ATPase function. The results further suggest reduced ability of the cell membrane to transport Ca^{2+} (possibly via Ca^{2+}-ATPase) in DOC hypertension. The high calcium diet opposed these alterations. The present results thus provide evidence that the antihypertensive effect of a high calcium diet in mineralocorticoid-salt hypertension is mediated by its beneficial effects on systemic sodium balance and arterial smooth muscle function.

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References

APRIGLIANO, O. & HERMSMEYER, K. (1976). *In vitro* denervation of the portal vein and caudal artery of the rat. *J. Pharmacol. Exp. Ther.*, **94**, 325–334.

ARVOLA, P., PÖRSTI, I., VUORINEN, P., PEKKI, A. & VAPAATALO, H. (1992). Contractions induced by potassium-free solution and potassium relaxation in vascular smooth muscle of hypertensive and normotensive rats. *Br. J. Pharmacol.*, **106**, 157–165.

ASHIDA, T. & BLAUSTEIN, M.P. (1987). Regulation of cell calcium and contractility in mammalian arterial smooth muscle: the role of sodium-calcium exchange. *J. Physiol.*, **392**, 617–635.

ASHIDA, T., KURAMOCHI, M. & OMAE, T. (1989). Increased sodium-calcium exchange in arterial smooth muscle of spontaneously hypertensive rats. *Hypertension*, **13**, 890–895.

AYACHI, S. (1979). Increased dietary calcium lowers blood pressure in the spontaneously hypertensive rat. *Metabolism*, **28**, 1234–1238.

BOHR, D.F., BRUNER, C.A., LAMB, F.S. & WEBB, R.C. (1988). Physiology of vascular smooth muscle in relation to hypertension. *Acta Physiol. Scand.*, **133**, (suppl. 571), 15–24.

DiPETTE, D.J., GREILICH, P.E., KERR, N.E., GRAHAM, G.A. & HOLLAND, O.B. (1989). Systemic and regional hemodynamic effects of dietary calcium supplementation in mineralocorticoid hypertension. *Hypertension*, **13**, 77–82.

EVANS, G.H., WEAVER, C.M., HARRINGTON, D.D. & BABBS, C.F. (1990). Association of magnesium deficiency with the blood pressure-lowering effect of calcium. *J. Hypertens.*, **8**, 327–337.

FOUDA, A.-K., KAUFMANN, A., THORIN, E., HENRION, D., CAPDEVILLE-ATKINSON, C. & ATKINSON, J. (1991). The role of endogenous norepinephrine release in potassium-evoked vasoconstriction of the rat tail artery. *Eur. J. Pharmacol.*, **205**, 63–72.

FRIEDMAN, S.M. & TANAKA, M. (1987). Increased sodium permeability and transport as primary events in the hypertensive response to deoxycorticosterone acetate (DOCA) in the rat. *J. Hypertens.*, **5**, 341–345.

FUJIMURA, A., LOWRY, R.W. & KEM, D.C. (1989). Calcium infusion increases plasma atrial natriuretic factor in spontaneously hypertensive rats. *Hypertension*, **14**, 98–103.

FURSPAN, P.B., RINALDI, G.J., HOFFMAN, K. & BOHR, D.F. (1989). Dietary calcium and cell membrane abnormality in genetic hypertension. *Hypertension*, **13**, 727–730.

HERMSMEYER, K. & HARDER, D. (1986). Membrane ATPase mechanism of K^+-return relaxation in arterial muscles of stroke-prone SHR and WKY. *Am. J. Physiol.*, **250**, C557–C562.

ITOH, H., NAKAO, K., MUKOYAMA, M., ARAI, H., YAMADA, T., SAITO, Y., SHIONO, S., HOSODA, K., SHIRAKAMI, G., SUGA, S. & IMURA, H. (1991). Pathophysiological role of augmented atrial natriuretic polypeptide gene expression in DOCA-salt hypertension. *Am. J. Hypertens.*, **4**, 39–44.

KARAKI, H. & WEISS, G.B. (1988). Calcium release in smooth muscle. *Life Sci.*, **42**, 111–122.

KINNUNEN, P., TASKINEN, T., JÄRVINEN, M. & RUSKOAHO, H. (1991). Effect of phorphol ester on the release of atrial natriuretic peptide from the hypertrophied rat myocardium. *Br. J. Pharmacol.*, **102**, 453–461.

LANG, R.E., UNGER, T. & GANTEN, D. (1987). Atrial natriuretic peptide: a new factor in blood pressure control. *J. Hypertens.*, **5**, 255–271.

LASARIDIS, A.N., KAISIS, C.N., ZANANIRI, K.I., SYRGANIS, C.D. & TOURKANTONIS, A.A. (1989). Increased natriuretic ability and hypotensive effect during short-term high calcium intake in essential hypertension. *Nephron.*, **51**, 517–523.

LATTION, A.-L., MICHEL, J.-B., ARNAULD, E., CORVOL, P. & SOUBRIER, F. (1986). Myocardial recruitment during ANF mRNA increase with volume overload in the rat. *Am. J. Physiol.*, **251**, H890–H896.

MAGLIOLA, L., McMAHON, E.G. & JONES, A.W. (1986). Alterations in active Na-K transport during mineralocorticoid-salt hypertension in the rat. *Am. J. Physiol.*, **250**, C540–C546.

MATLIB, M.A. (1991). Role of sarcolemmal membrane sodium-calcium exchange in vascular smooth muscle tension. In *Sodium-Calcium Exchange*. ed. Blaustein, M.P., DiPolo, R. & Reeves, J.P. Vol. 639, pp. 531–542. New York: The New York Academy of Sciences.

McCARRON, D.A., LUCAS, P.A., SCHNEIDEMAN, R.J., LACOUR, B. & DRÜEKE, T. (1985). Blood pressure development in the spontaneously hypertensive rat after concurrent manipulations of dietary Ca^{2+} and Na^+. Relation to intestinal Ca^{2+} fluxes. *J. Clin. Invest*,. **76**, 1147–1154.

McCARRON, D.A. & MORRIS, C.D. (1985). Blood pressure response to oral calcium in persons with mild to moderate hypertension. A randomized, double-blind, placebo-controlled, crossover trial. *Ann. Intern. Med.*, **103**, (6 Pt. 1), 825–831.

MORTON, J.J., KENYON, C.J. & BEATTIE, E.C. (1990). Hormone and electrolyte changes in post-deoxycorticosterone salt hypertension in rats. *J. Hypertens.*, **8**, 1021–1026.

MULVANY, M.J. (1985). Changes in sodium pump activity and vascular contraction. *J. Hypertens.*, **3**, 429–436.

MULVANY, M.J., AALKJÆR, C. & PETERSEN, T.T. (1984). Intracellular sodium, membrane potential, and contractility of rat mesenteric small arteries. *Circ. Res.*, **54**, 740–749.

PAMNANI, M.B., CHEN, S., BRYANT, H.J., SCHOOLEY, J.F. & HADDY, F.J. (1990). Effect of increased dietary calcium on the development of reduced renal mass saline hypertension in rats. *J. Am. Coll. Nutr.*, **9**, 35–43.

PÖRSTI, I. (1992). Arterial smooth muscle contractions in spontaneously hypertensive rats on a high calcium diet. *J. Hypertens.*, **10**, 255–263.

PÖRSTI, I., ARVOLA, P., WUORELA, H., ILKKA, M., SÄYNÄVÄLAMMI, P., HUHTALA, H., METSÄ-KETELÄ, T. & VAPAATALO, H. (1990). Effects of a high calcium diet and deoxycorticosterone on vascular smooth muscle responses in spontaneously hypertensive rats. *J. Hypertens*, **8**, 835–841.

PÖRSTI, I., ARVOLA, P., WUORELA, H. & VAPAATALO, H. (1992). High calcium diet augments vascular potassium relaxation in hypertensive rats. *Hypertension*, **19**, 85–92.

PÖRSTI, I., WUORELA, H., ARVOLA, P., MÄMMI, P., NURMI, A.-K., KOISTINAHO, J., LAIPPALA, P. & VAPAATALO, H. (1991). Effects of calcium supplementation and deoxycorticosterone on plasma atrial natriuretic peptide and electrolyte excretion in spontaneously hypertensive rats. *Acta Physiol. Scand.*, **141**, 343–350.

RESNICK, L.M., SOSA, R.E., CORBETT, M.L., GERTNER, J.M., SEALEY, J.E. & LARAGH, J.H. (1986). Effect of dietary calcium on sodium volume vs. renin-dependent forms of experimental hypertension. *Trans. Assoc. Am. Physicians*, **99**, 172–179.

RUSKOAHO, H. & LEPPÄLUOTO, J. (1988). Immunoreactive atrial natriuretic peptide in ventricles, atria, hypothalmus, and plasma of genetically hypertensive rats. *Circ. Res.*, **62,** 384–394.

RUSKOAHO, H., KINNUNEN, P., TASKINEN, T., VUOLTEENAHO, O., LEPPÄLUOTO, J. & TAKALA, T.E.S. (1989). Regulation of ventricular atrial natriuretic peptide release in hypertrophied rat myocardium. Effect of exercise. *Circulation*, **80,** 390–400.

SAITO, K., SANO, H., FURUTA, Y. & FUKUZAKI, H. (1989). Effect of oral calcium on blood pressure response in salt-loaded borderline hypertensive patients. *Hypertension*, **13,** 219–226.

SONGU-MIZE, E., BEALER, S.L. & CALDWELL, R.W. (1982). Effect of AV3V lesions on development of DOCA-salt hypertension and vascular Na^+-pump activity. *Hypertension*, **4,** 575–580.

STULL, J.T., GALLAGHER, P.J., HERRING, B.P. & KAMM, K.E. (1991). Vascular smooth muscle contractile elements. Cellular regulation. *Hypertension*, **17,** 723–732.

SUGIMOTO, T., ISHII, M., HIRATA, Y., MATSUOKA, H., SUGIMOTO, T., MIYATA, A., TOSHIMORI, T., MASUDA, H., KANGAWA, K. & MATSUO, H. (1986). Increased release of atrial natriuretic polypeptides in rats with DOCA-salt hypertension. *Life Sci.*, **38,** 1351–1358.

VANHOUTTE, P.M. & LORENZ, R.R. (1984). Na^+,K^+-ATPase inhibitors and the adrenergic neuroeffector interaction in the blood vessel wall. *J. Cardiovasc. Pharmacol.*, **6,** (suppl. 1), S88–S94.

WASSERMAN, R.H. & FULLMER, C.S. (1989). On the molecular mechanism of intestinal calcium transport. *Adv. Exp. Med. Biol.*, **249,** 45–65.

WUORELA, H., ARVOLA, P., PÖRSTI, I., SILTALOPPI, E., SÄYNÄVÄLAMMI, P. & VAPAATALO, H. (1992). The effect of high calcium intake on Ca^{2+} ATPase and the tissue Na:K ratio in spontaneously hypertensive rats. *Naunyn-Schmidebergs Arch. Pharmacol.*, **345,** 117–122.

WUORELA, H., PÖRSTI, I., ARVOLA, P., MÄKYNEN, H. & VAPAATALO, H. (1992). Three levels of dietary calcium – effects on blood pressure and electrolyte balance in spontaneously hypertensive rats. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **346,** 542–549.

YANG, M.C.M., LEE, M.Y., KUO, J.S. & PANG, P.K.T. (1989). Effect of calcium diet on the vascular reactivity of DOCA-salt hypertensive rats. *Clin. Exp. Hypertens.-[A],* **11,** 501–519.

YOUNG, E.W., BUKOSKI, R.D. & MCCARRON, D.A. (1988). Calcium metabolism in experimental hypertension. *Proc. Soc. Exp. Biol. Med.*, **187,** 123–141.

ZIKOS, D., LANGMAN, C., GAFTER, U., DELAHAYE, B. & LAU, K. (1986). Chronic DOCA treatment increases Ca absorption: role of hypercalciuria and vitamine D. *Am. J. Physiol.*, **251,** E279–E284.

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Effect of dexamethasone on neutrophil accumulation and oedema formation in rabbit skin: an investigation of site of action

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1 The anti-inflammatory actions of dexamethasone on vascular and leukocyte responses in rabbit skin were investigated.

2 Neutrophil accumulation and oedema formation were simultaneously measured as the local accumulation of i.v. administered ^{111}In-labelled neutrophils and ^{125}I-labelled albumin. Systemically administered dexamethasone (3 mg kg^{-1}) inhibited neutrophil accumulation induced by i.d. zymosan activated plasma (ZAP), N-formyl-methionyl-leucyl-phenylalanine (FMLP) and leukotriene B_4 (LTB_4) when co-injected with prostaglandin E_2 (PGE_2). Dexamethasone also inhibited oedema formation elicited by these stimuli and the responses induced by i.d. platelet activating factor (PAF) + PGE_2 and bradykinin (BK) + PGE_2.

3 Intradermal dexamethasone (2×10^{-10} mol per site) but not indomethacin (10^{-8} mol per site) inhibited oedema formation induced by i.d. ZAP + PGE_2 and BK + PGE_2. This inhibitory effect of dexamethasone was significant only with pretreatment periods of 4 h, shorter pretreatment periods resulting in greatly reduced effects. Intradermal dexamethasone had no effect on neutrophil accumulation induced by ZAP + PGE_2.

4 Intradermal dexamethasone (2×10^{-10} mol per site) had no effect on increase in blood flow induced by PGE_2 as measured by ^{133}Xenon clearance.

5 The accumulation of neutrophils isolated from donor rabbits pretreated with i.v. saline or dexamethasone (3 mg kg^{-1}) was investigated in untreated recipient rabbits. The accumulation of neutrophils, induced by ZAP + PGE_2, FMLP + PGE_2 and LTB_4 + PGE_2, from dexamethasone-pretreated donors was significantly smaller than the accumulation of neutrophils from saline-pretreated donors.

6 The results of this study suggest that dexamethasone can have a direct effect on vascular endothelial cells resulting in an inhibition of oedema formation.

7 Neutrophil accumulation can be inhibited by an effect of dexamethasone on the neutrophil itself or on the vascular endothelium. These results indicate that at least part of the inhibitory effect is on the circulating neutrophil induced by dexamethasone or a dexamethasone-induced product.

Keywords: Dexamethasone; inflammation; vascular permeability; neutrophil accumulation; oedema; rabbit skin

Introduction

The glucocorticosteroids are potent anti-inflammatory drugs, widely used clinically against inflammatory disease states and able to suppress inflammatory signs and symptoms in many animal models. No single mechanistic explanation could suffice to account for all the biological effects of glucocorticosteroids. Numerous anti-inflammatory actions have been attributed to these drugs, including the suppression of inflammatory mediator generation, as reviewed by Williams & Yarwood (1990). The inhibitory effect of steroids on the generation of pro-inflammatory arachidonic acid metabolites such as prostaglandins, leukotrienes and platelet-activating factor (PAF) has been most extensively investigated (reviewed in Flower, 1988). This effect of glucocorticosteroids is believed to be mediated by the steroid-induced family of proteins known as lipocortins (Flower, 1988) or annexins (Crumpton & Dedman, 1992) which inhibit phospholipase A_2, the enzyme activity responsible for release of arachidonic acid (Blackwell *et al.*, 1980; Hirata *et al.*, 1980; Cloix *et al.*, 1983; Di Rosa *et al.*, 1984). Although the exact mechanism whereby these proteins inhibit phospholipase A_2 awaits resolution there is growing evidence supporting lipocortin 1 (annexin 1) as the candidate mediating steroid inhibition of eicosanoid synthesis. Human recombinant lipocortin 1 has been shown to inhibit the formation of arachidonate metabolites *in vitro* (Cirino *et al.*, 1987; Cirino & Flower, 1987a,b) and to mimic the suppressive action of glucocorticosteroids *in vivo* in models where lipid mediators play a major role (Cirino *et al.*, 1989).

In addition to suppression of mediator generation, glucocorticosteroids also inhibit inflammatory reactions *in vivo* in models unlikely to be mediated by eicosanoid synthesis. Tsurufuji *et al.* (1979) have shown that dexamethasone can inhibit oedema formation induced by chemical mediators including bradykinin (BK) and 5-hydroxytryptamine (5-HT) in a model of mouse foot pad oedema. The oedema responses were not affected by the cyclo-oxygenase inhibitor, indomethacin, suggesting that they are independent of local prostaglandin production and thus the inhibitory effect of dexamethasone is unlikely to be due to phospholipase A_2 inhibition. However, the anti-inflammatory effect of dexamethasone was completely blocked by either actinomycin D or cycloheximide, indicating a requirement for the synthesis of an endogenous anti-inflammatory protein (Tsurufuji *et al.*, 1979). Mediators such as BK and 5-HT increase microvascular permeability by a direct action on endothelial cells, causing cell contraction associated with opening of inter-endothelial cell junctions (Majno *et al.*, 1969). It is therefore suggested that dexamethasone or a steroid-induced protein(s) may inhibit oedema formation by a direct effect on the

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endothelium, rendering it less responsive to the action of mediators of increased microvascular permeability such as 5-HT (Tsurufuji *et al.*, 1979). These observations have been supported by other authors using different animal species and an array of inflammatory mediators. Glucocorticosteroids inhibit histamine-induced oedema in rat skin and mouse ear (Church & Miller, 1978) and plasma protein leakage induced by exogenous histamine, BK, PAF and leukotriene C_4 (LTC_4) in the hamster cheek pouch (Bjork *et al.*, 1985; Svensjo & Roempke, 1985).

Chemotactic mediators such as the complement fragment C5a, the arachidonate lipoxygenase product, LTB_4 and the bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) induce oedema formation in a neutrophil-dependent manner (Wedmore & Williams, 1981). Glucocorticosteroids have been shown to inhibit neutrophil-dependent oedema induced by exogenous LTB_4, FMLP and the complement fragment C5a in rabbit skin (Foster & McCormick, 1985; Griffiths & Blackham, 1988; Yarwood *et al.*, 1988; 1989; Peers & Flower, 1991). Foster & McCormick (1985) also showed that dexamethasone inhibited the local accumulation of neutrophils in response to LTB_4.

In this study we have investigated further the eicosanoid independent effects of steroids on vascular responses in the rabbit microcirculation. For this purpose we have studied the effects of dexamethasone on oedema response in rabbit skin induced by i.d. neutrophil-dependent and direct-acting mediators. In addition we have studied the effect of dexamethasone on mediator-induced accumulation of ^{111}In-labelled neutrophils in rabbit skin. By using protocols where we have treated either the neutrophil donor rabbit or the recipient rabbit with dexamethasone we have investigated the possible sites of action of steroids in inhibiting plasma protein leakage and neutrophil accumulation *in vivo*.

Methods

Animals

Male, specific pathogen-free New Zealand White rabbits (2–3 kg) were purchased from Froxfield Farm, Froxfield, Hampshire and Hacking and Churchill, Huntingdon, Cambridgeshire.

Measurement of blood flow

Blood flow was measured in the clipped dorsal skin of rabbits by use of a multiple site ^{133}Xe clearance technique (Williams, 1979). The effect of dexamethasone on blood flow induced by PGE_2 was assessed. The vasodilator was mixed with a solution of ^{133}Xe in saline (5–10 μCi/injection) and rapidly injected i.d. (0.1 ml volumes) at 10^{-11} and 3×10^{-10} mol per site, with ^{133}Xe in saline as control. Intradermal injections were given in random-block order according to a predetermined balanced site plan with 6 replicates for each treatment per animal. After 15 min animals were killed with an overdose of i.v. sodium pentobarbitone, the dorsal skin excised and injected sites removed with a 17 mm diameter punch. Skin samples and 0.1 ml aliquots of injection fluids, under paraffin oil in sealed tubes, were counted immediately in an automatic gamma-counter (LKB Wallac 1260 Multigamma II). The change in local blood flow was calculated as the percentage increase above saline controls from the equation: 100 (In^{133}Xe count of saline-injected skin – In^{133}Xe count of agent-injected skin)/(In^{133}Xe count of 0.1 ml injection fluid – In^{133}Xe count of saline-injected skin). The effect of dexamethasone on blood flow was assessed by pretreating skin sites locally with dexamethasone (80 ng per site, equivalent to 2×10^{-10} mol per site) or saline as a control for 4 h prior to re-injection of the sites with doses of PGE_2 or saline mixed with ^{133}Xe as described above.

Preparation of ^{111}In-labelled neutrophils

Rabbit neutrophils were isolated and ^{111}In-labelled according to the procedure previously described (Rampart & Williams, 1988; Nourshargh *et al.*, 1989). Briefly, donor rabbits were anaesthetized with i.v. Sagatal (30 mg kg^{-1}) and bled via a carotid cannula into acid citrate dextrose. Neutrophils (>90% pure) were separated at room temperature by centrifugation over a two layer discontinuous percoll plasma gradient (50% and 70%), after an initial red blood cell sedimentation with 3% hydroxyethyl starch. Neutrophils (approximately 3.5×10^7 cells in 1–2 ml volume) were incubated with $^{111}InCl_3$ (50–100 μCi in 10–15 μl) chelated to 2-mercaptopyridine-N-oxide (40 μg ml^{-1}) for 15 min at room temperature. Labelled leucocytes were then washed twice and finally resuspended in autologous cell free citrated plasma.

Measurement of ^{111}In-neutrophil accumulation and plasma protein exudation in rabbit skin

Rabbits were anaesthetized with i.v. Sagatal (30 mg kg^{-1}) with further maintenance doses given as necessary. Neutrophil infiltration and oedema formation in the rabbit back skin were measured simultaneously as the local accumulation of i.v. injected ^{111}In-neutrophils and ^{125}I-labelled human serum albumin (5 μCi kg^{-1}, mixed with 2 ml of a 2.5% Evans blue dye solution) as previously described (Rampart & Williams, 1988; Nourshargh *et al.*, 1989). Agents under investigation were injected intradermally in 0.1 ml volumes into the clipped dorsal skin according to a balanced site injection plan each treatment having six replicates. All mediators were coinjected with a fixed potentiating dose of PGE_2 (3×10^{-10} mol per site) or AA (3×10^{-9} mol per site) where indicated. After 30 min, a blood sample was taken by cardiac puncture into heparin (10 u ml^{-1} final concentration), animals were killed by an overdose of anaesthetic and the dorsal skin was removed. Injection sites were excised with a 17 mm diameter punch and skin, blood and plasma samples were counted in a gamma counter with automatic spill over and cross-talk correction. Plasma exudation was expressed as μl of plasma/skin site by dividing the skin sample ^{125}I counts by the ^{125}I counts in 1 μl of plasma. Cell accumulation was expressed as the number of ^{111}In-labelled neutrophils per site by dividing skin sample ^{111}In-counts by ^{111}In-counts per neutrophil. All numbers were corrected for 5×10^7 ^{111}In-neutrophils injected into each recipient animal.

In experiments where the effect of local dexamethasone was investigated, skin sites were pretreated i.d. with dexamethasone (2×10^{-10} mol per site) or indomethacin (10^{-8} mol per site) for the times indicated before the intravenous administration of ^{111}In-cells and ^{125}I-albumin. Test agents were then injected i.d. into the pretreated sites. Control sites were pretreated intradermally with saline. The accumulation of labels in skin sites was then measured over a 30 min *in vivo* test period as described above.

The experiments shown in Figures 1 and 2 were designed to evaluate the effect of systemic treatment of recipient rabbits with dexamethasone. Washed labelled neutrophils were divided equally for i.v. injection into two recipient animals, one test and one control rabbit. Test recipient animals were treated systemically with dexamethasone (3 mg kg^{-1}, i.v.) 4 h before i.v. injection of labelled cells and [^{125}I]-albumin. Control animals received an equal volume of saline.

The experiments shown in Figures 6 and 7 were designed to investigate the effect of dexamethasone specifically on the neutrophil *in vivo*. Pairs of neutrophil donor rabbits were treated i.v. with dexamethasone (3 mg kg^{-1}) or an equal volume of saline; 4 h later blood from each pair was collected, neutrophils isolated and ^{111}In-labelled in parallel as described above. ^{111}In-labelled cells from treated and control donor animals were then injected i.v. into two untreated recipient animals, together with [^{125}I]-albumin. Cell numbers were adjusted if necessary so that each pair of recipient

animals received the same number of labelled neutrophils. Neutrophil accumulation and oedema formation were measured in skin sites over 30 min as before. The procedures for each pair of saline and dexamethasone-treated rabbits were carried out in parallel.

Materials

BK, FMLP, prostaglandin E_2 (PGE_2), arachidonic acid (AA), zymosan, indomethacin and 2-mercaptopyridine-N-oxide were purchased from Sigma Chemical Co, Poole, Dorset. PAF was from Bachem, Saffron Walden, Essex. Evans blue dye was from British Drug Houses, Poole, Dorset. Sagatal (pentobarbitone sodium, 60 mg ml^{-1}) was from May and Baker, Dagenham, Essex. Percoll was from Pharmacia Fine Chemicals, Uppsala, Sweden. Hespan (6% hydroxy-ethyl starch in 0.9% NaCl) was from American Hospital Supply, Didcot, Oxfordshire. Steriflex (Sterile, pyrogen free isotonic saline solution) was from The Boots Co. Plc, Nottingham. $^{111}InCl_3$ (2 mCi in 0.2 ml sterile, pyrogen-free 0.04 N hydrochloric acid), ^{125}I-labelled human serum albumin (20 mg albumin per ml of sterile isotonic saline, 50 $\mu Ci\ ml^{-1}$) and $^{133}Xenon$ (10 mCi in 3 ml sterile pyrogen-free saline) were purchased from Amersham International Plc, Amersham, Buckinghamshire. Dexamethasone phosphate (8 mg dexamethasone phosphate in 2 ml ampoules) was from David Bull Laboratories, Warwick. We are grateful to Dr S. Foster of Imperial Chemical Industries, Macclesfield, Cheshire for the generous gift of leukotriene B_4 (LTB_4).

Zymosan-activated plasma (ZAP) as a source of C5a des Arg was prepared by incubating heparinized (10 u ml^{-1}) rabbit plasma with zymosan (1 mg ml^{-1}) for 30 min at 37°C. Zymosan was removed by centrifugation (2 × 10 min, 2500 *g*) and activated plasma stored in aliqots at − 25°C. The C5a des Arg content of activated plasma was approximately 5×10^{-7} M as measured by radioimmunoassay (Jose *et al.*, 1983).

Stock solutions were prepared as follows and kept at − 25°C: PGE_2 and BK 1 mg ml^{-1} in ethanol; arachidonic acid 10 mg ml^{-1} in ethanol; FMLP 0.219 mg ml^{-1} in ethanol; PAF 1 mg ml^{-1} in chloroform/methanol (4:1); LTB_4 0.1 mg ml^{-1} in methanol. For arachidonic acid an intermediate dilution (0.1 mg ml^{-1}) was made in 1.4% sodium bicarbonate solution. Indomethacin (3 mg ml^{-1}) was made up each day in 1.4% sodium bicarbonate solution. ZAP was used undiluted (100%) or diluted 1:10 (10%) in heparinized rabbit plasma. All working solutions were freshly prepared on the day of the experiment in sterile pyrogen-free 0.9% saline.

Statistical analysis

Results are presented as mean values ± s.e.mean for the number of rabbits indicated; one datum unit being the mean of six replicates in each rabbit. Statistical significance of results was evaluated by 2 way analysis of variance. A P value <0.05 was considered statistically significant. Calculations were performed using data after subtraction of the background count for each animal (i.e. i.d. saline).

Results

Effect of i.v. dexamethasone on ^{111}In-labelled neutrophil accumulation and oedema formation in rabbit skin

Figure 1 shows that over a 30 min period bradykinin (10^{-10} mol per site) and FMLP (5×10^{-11} mol per site) injected alone induced a significant but small increase in plasma leakage when compared with control saline injected sites. As previously documented, coinjection of the vasodilator substance PGE_2 (3×10^{-10} mol per site) resulted in a

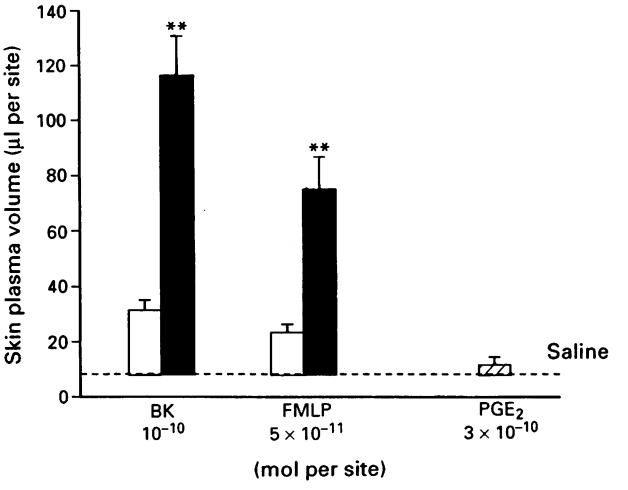


Figure 1 Oedema formation in rabbit skin induced by i.d. injection of bradykinin (BK, 10^{-10} mol per site) and N-formyl-methionyl-leucyl-phenylalanine (FMLP, 5×10^{-11} mol per site) measured over 30 min, showing synergism with prostaglandin E_2 (PGE_2). Open columns represent responses in the absence, and solid columns represent responses in the presence of a vasodilator dose of prostaglandin E_2 (3×10^{-10} mol per site). The hatched column represents the response to PGE_2 alone. The dashed line represents the response to i.d. saline. Each column represents the mean ± s.e.mean from $n = 6$ rabbits. Significant difference between the responses in the presence and absence of PGE_2: $^{**}P<0.01$.

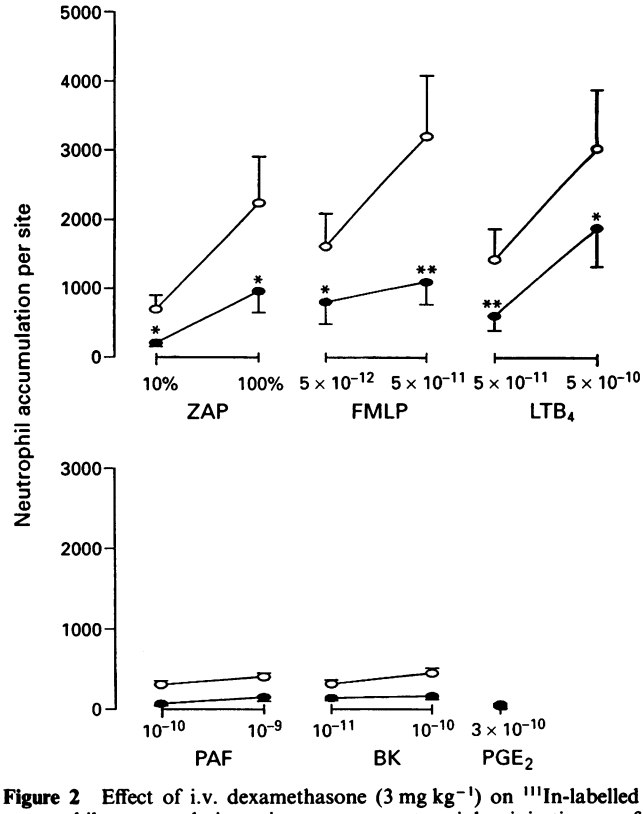


Figure 2 Effect of i.v. dexamethasone (3 mg kg^{-1}) on ^{111}In-labelled neutrophil accumulation in response to i.d. injections of inflammatory mediators. Concentrations are given as mol per site for N-formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene B_4 (LTB_4), platelet activating factor (PAF) and bradykinin (BK). Zymosan activated plasma (ZAP) was used undiluted (100%) or as a 1:10 dilution in heparinized rabbit plasma (10%). All stimuli were coinjected with a fixed potentiating dose of prostaglandin E_2 (PGE_2, 3×10^{-10} mol per site). ^{111}In-neutrophil accumulation was measured in recipient animals given i.v. dexamethasone (●) or saline (○) 4 h prior to i.v. ^{111}In-neutrophils and i.d. mediators. Responses are corrected for the low levels in saline-injected sites. Each point represents the mean ± s.e.mean for $n = 9$ pairs of rabbits. Significant difference from control: $^{*}P<0.05$, and $^{**}P<0.01$.

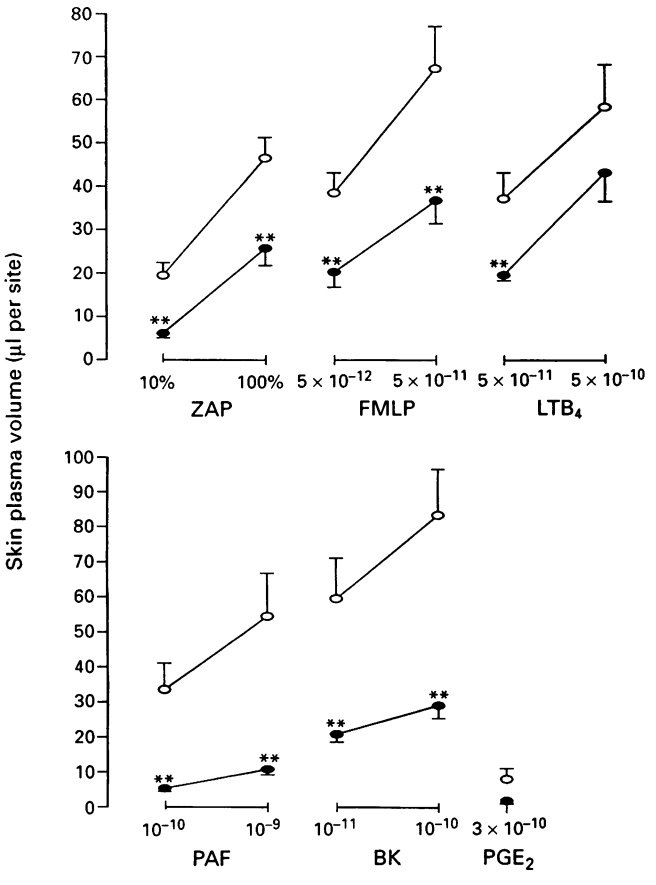


Figure 3 Effect of i.v. dexamethasone (3 mg kg^{-1}) on plasma protein extravasation in response to inflammatory mediators: oedema responses measured in the same recipient animals as in Figure 2. Concentrations are given as mol per site for N-formyl-methionyl-leucyl-phenylalanine (FMLP), leukotriene B_4 (LTB_4), PAF and bradykinin (BK). Zymosan activated plasma (ZAP) was used undiluted (100%) or as a 1:10 dilution in heparanized rabbit plasma (10%). Oedema formation was measured in dexamethasone pretreated recipient animals (●) and recipient animals pretreated with an equal volume of saline (○). Responses are corrected for the low levels in saline-injected sites. Each point represents the mean ± s.e.mean for $n = 9$ pairs of rabbits. Significant difference from control: $^{**}P < 0.01$.

large potentiation of oedema responses when compared with those induced by the same doses of bradykinin and FMLP (Figure 1) (Williams & Morley, 1973; Williams & Peck, 1977; Wedmore & Williams, 1981). PGE_2 alone (3×10^{-10} mol per site) did not induce a significant oedema response (Figure 1). Vasodilator prostaglandins have also been documented to potentiate ^{111}In-neutrophil accumulation in response to i.d. chemoattractants in rabbit skin (Movat *et al.*, 1984). Given that the glucocorticosteroids are known to inhibit the generation of mediators including prostaglandins (reviewed in Flower, 1988), all experiments were designed so that mediators were coinjected with a maximally potentiating dose of the vasodilator, PGE_2. This ensured that the effect of any endogenous vasodilator prostaglandin generation in skin sites was overridden; thus eliminating the possibility of a glucocorticosteroid effect being due to inhibition of prostaglandin synthesis.

For the experiments shown in Figures 2 and 3 recipient animals were treated i.v. with either dexamethasone (3 mg kg^{-1}) or saline (1 mg kg^{-1}) 4 h before i.v. administration of ^{111}In-labelled neutrophils, ^{125}I-labelled albumin and i.d. inflammatory mediators mixed with PGE_2. The accumulation of labels was measured over 30 min. Figure 2 shows that in control recipient animals there was a dose-related increase in ^{111}In-neutrophil accumulation in response to the three chemattractants, ZAP + PGE_2, FMLP + PGE_2 and LTB_4 + PGE_2. Systemic pretreatment of recipient animals with dexamethasone caused a significantly reduced accumulation of ^{111}In-neutrophils in response to all doses of chemoattractants tested. This inhibitory effect of dexamethasone was not due to a difference in the number of ^{111}In-neutrophils circulating in the rabbits as there was no statistical difference between the percentage of labelled leucocytes circulating in control and dexamethasone-treated recipient rabbits at the end of the *in vivo* test period (data not shown). The neutrophil-independent mediators PAF and BK did not, as expected, induce a significant accumulation of ^{111}In-neutrophils in either group of recipient animals (Figure 2).

The simultaneous measurement of plasma protein leakage illustrated in Figure 3 shows that oedema responses to both neutrophil-dependent (except 5×10^{-10} mol per site LTB_4) and neutrophil-independent mediators were significantly suppressed in dexamethasone-pretreated recipient animals. Intradermal injection of PGE_2 alone did not induce significant neutrophil accumulation (Figure 2) or oedema formation (Figure 3).

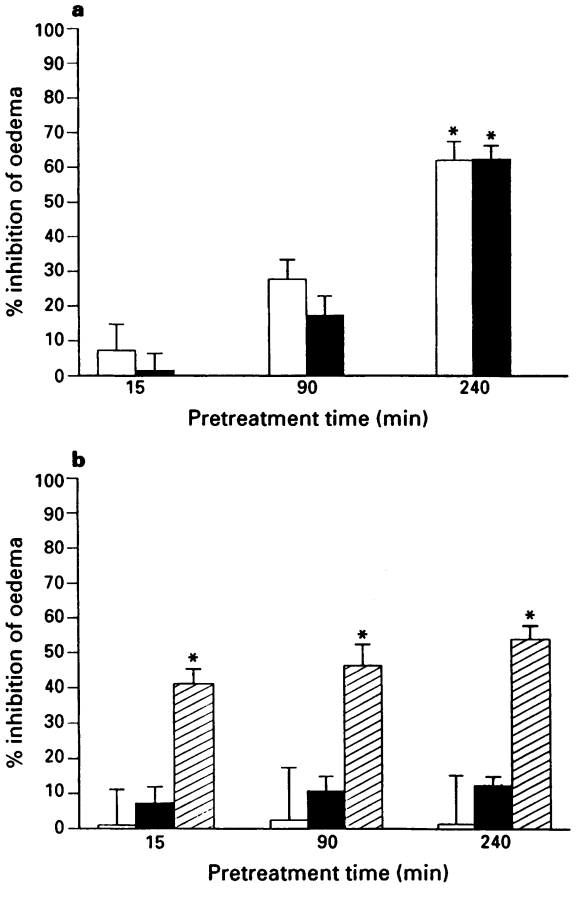


Figure 4 Effect of local i.d. pretreatment with dexamethasone (a) and indomethacin (b) on oedema formation induced by i.d. bradykinin (BK) + prostaglandin E_2 (PGE_2) (open columns), zymosan activated plasma (ZAP) + PGE_2 (solid columns) and BK + arachidonic acid (AA, hatched columns in b). Skin sites were pretreated locally with either dexamethasone (2×10^{-10} mol per site), indomethacin (10^{-8} mol per site) or saline for the times indicated prior to i.v. ^{125}I-albumin and ^{111}In-neutrophils. Pretreated sites were then reinjected with mixtures of mediators under test and the response allowed to proceed for 30 min as described in Methods. Concentrations of mediators used were as follows; BK (10^{-10} mol per site), ZAP (100%), PGE_2 (3×10^{-10} mol per site), AA (3×10^{-9} mol per site). For each time point, responses in drug pretreated sites were calculated as a percentage of those obtained in saline pretreated sites in the same rabbit. Calculations were performed using data after subtraction of the relevant background response for each animal. Responses are mean ± s.e.mean for $n = 4-7$ rabbits. Significant inhibition, $^{*}P < 0.05$.

Effect of local dexamethasone on oedema formation and ^{111}In-neutrophil accumulation in rabbit skin

As shown in Figure 4a, local pretreatment of skin sites with dexamethasone (2×10^{-10} mol per site) caused a time-dependent inhibition of oedema formation induced by both bradykinin (a direct-acting mediator of increased microvascular permeability) and ZAP used as a source of C5a des Arg (a neutrophil-dependent mediator) when coinjected with PGE_2: i.e. after 240 min dexamethasone pretreatment the inhibition was 62.0 ± 5.4% and 62.4 ± 3.8% for BK + PGE_2 and ZAP + PGE_2 respectively. This inhibition was considerably greater than that observed with 15 min and 90 min pretreatments which did not achieve significance. In contrast, pretreatment of skin sites with the cyclo-oxygenase inhibitor, indomethacin (10^{-8} mol per site) had no significant effect on oedema responses to bradykinin or ZAP when coinjected with PGE_2 (Figure 4b). However, indomethacin pretreatment did inhibit the potentiation of oedema produced by coinjection of BK with the prostaglandin precursor arachidonic acid and was thus evidently blocking cyclo-oxygenase at all pretreatment times tested (Figure 4b). The effect of local indomethacin was remarkably persistent; inhibition was not significantly different for the 15 min and 240 min pretreatment times. Like its metabolite PGE_2, arachidonic acid was a poor inducer of plasma protein leakage when injected alone (data not shown).

In some animals ^{111}In-neutrophil accumulation was simultaneously monitored. In contrast to the effect on neutrophil-dependent oedema formation, local dexamethasone pretreatment did not significantly inhibit ^{111}In-neutrophil accumulation induced by ZAP + PGE_2, even after 240 min pretreatment. Similarly, indomethacin had no significant effect on cell accumulation at any pretreatment time. Responses to ZAP + PGE_2 expressed as number of ^{111}In-neutrophils per site were as follows: in sites pretreated for 15 min with saline 1799 ± 359, with dexamethasone 1724 ± 336, with indomethacin 2116 ± 488; in skin sites pretreated for 240 min with saline 1804 ± 384, with dexamethasone 1451 ± 363, with indomethacin 2258 ± 372 (results are mean ± s.e.mean for $n = 4$ rabbits; all data subtracted for saline background levels).

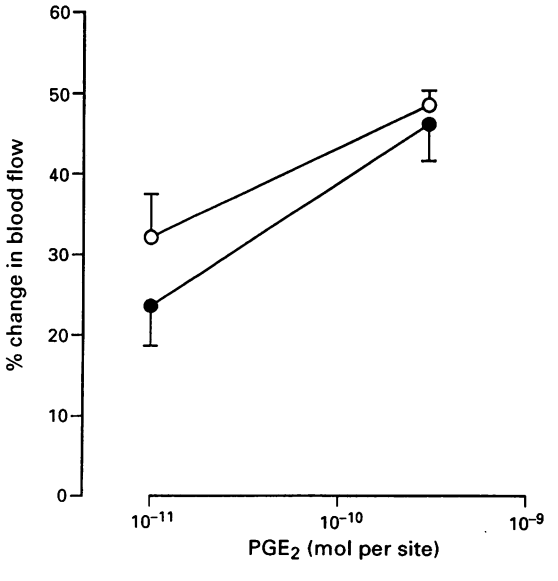


Figure 5 Effect of local pretreatment with dexamethasone (2×10^{-10} mol per site for 4 h) on blood flow induced by prostaglandin E_2 (PGE_2) in rabbit skin. Sites were pretreated locally with dexamethasone (2×10^{-10} mol per site, ●) or saline (○) for 4 h prior to reinjection with doses of PGE_2 shown (mol per site) mixed with ^{133}Xe. The washout of ^{133}Xe was measured over 15 min. The increase in blood flow was calculated as a percentage of saline-injected sites. Results are expressed as the mean of $n = 5$ experiments ± s.e.mean.

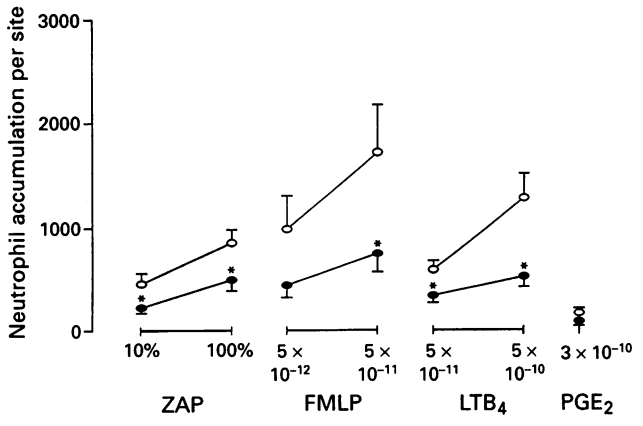


Figure 6 Accumulation of neutrophils isolated from saline (○) or dexamethasone (3 mg kg^{-1}, ●)-pretreated donor rabbits in untreated recipient animals. ^{111}In-neutrophil accumulation in recipient rabbits was induced by i.d. injections of doses of stimuli shown as mol per site for N-formyl-methionyl-leucyl-phenylalanine (FMLP) and leukotriene B_4 (LTB_4) and 100% and 10% zymosan activated plasma (ZAP), all mixed with a fixed potentiating dose of prostaglandin E_2 (PGE_2) (3×10^{-10} mol per site). Responses are corrected for the low levels in saline injected sites. Each point represents the mean ± s.e.mean for $n = 9$ pairs of rabbits. Significant difference from control, $^*P < 0.05$.

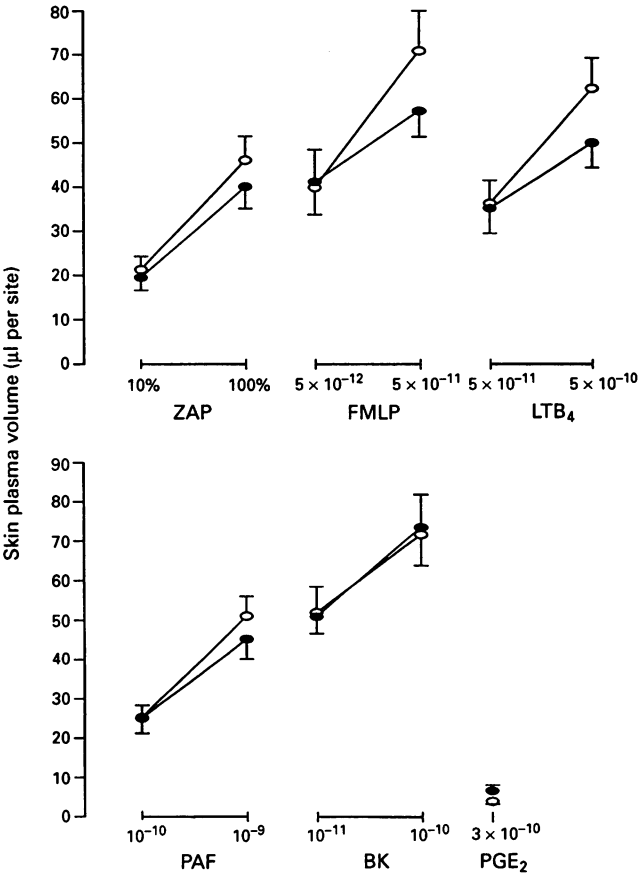


Figure 7 Oedema formation in untreated rabbits receiving labelled-neutrophils isolated from saline (○) or dexamethasone (3 mg kg^{-1}, ●)-pretreated donor rabbits. Oedema responses were measured in the same recipient animals as in Figure 6. Responses are corrected for the low levels in saline-injected sites. Each point represents the mean ± s.e.mean for $n = 9$ pairs of rabbits.

Effect of local dexamethasone on blood flow in rabbit skin

Figure 5 shows the results of experiments designed to determine whether the effects of locally administered dexamethasone on oedema formation (Figure 4) was due to local vasoconstriction. In these experiments we investigated the effect of 4 h pretreatment with dexamethasone (2×10^{-10} mol per site) or saline on vasodilatation induced by PGE_2. Dexamethasone had no significant effect on the dose-related increase in blood flow induced by prostaglandin E_2 (Figure 5). Further, dexamethasone had no significant effect on blood flow in saline-injected sites.

Effect of pretreating neutrophil donor rabbits with dexamethasone

The experiments shown in Figures 6 and 7 were designed to investigate a possible effect of steroid, or a steroid-induced product on the neutrophil *in vivo*. Figure 6 shows that ^{111}In-neutrophils from control, saline-treated donor animals accumulated in recipients in response to the three neutrophil-dependent mediators ZAP, FMLP and LTB_4 when co-injected with PGE_2. Treatment of neutrophil donor animals with dexamethasone significantly inhibited the accumulation of ^{111}In-neutrophils induced by all (except 5×10^{-12} mol per site FMLP) doses of chemoattractants in recipients. At the end of the 30 min accumulation period the percentage of circulating ^{111}In-neutrophils did not differ significantly between the two groups and therefore this did not account for the differences in cell accumulation observed.

Figure 7 shows oedema responses measured in the same recipient animals as in Figure 6. There was no significant difference in responses to direct-acting or neutrophil-dependent mediators between the two groups of recipient rabbits. This is presumably because the ^{111}In-labelled neutrophils account for approximately 3% of the total circulating neutrophils in recipients and therefore do not make a major functional contribution to oedema formation.

Discussion

Glucocorticosteroids are potent anti-inflammatory drugs capable of inhibiting oedema formation and neutrophil accumulation *in vivo*. The precise mechanism by which steroids induce these inhibitory activities is unknown although the possible sites of action are (1) the generation of inflammatory mediators, (2) the microvascular endothelial cell or (3) the neutrophil. In the present study, we have investigated the site of action of dexamethasone in inhibiting mediator-induced oedema formation and neutrophil accumulation in rabbit skin *in vivo*. The results suggest glucocorticosteroids can suppress oedema formation by an endothelial-associated effect and neutrophil accumulation by an effect on the neutrophil itself as well as a possible effect on the endothelium.

Increased microvascular permeability can be induced in a specific and complex manner by a host of inflammatory mediators. These mediators can be divided into two broad categories, depending on whether their effects are mediated by neutrophils (Wedmore & Williams, 1981). In this study we have shown that systemically administered dexamethasone inhibited neutrophil-dependent and independent oedema formation induced by exogenously administered inflammatory mediators. These results are in agreement with previous reports (Foster & McCormick, 1985; Griffiths & Blackham, 1988; Peers & Flower, 1991). In addition, we have shown that dexamethasone when administered i.v. inhibited neutrophil accumulation induced by C5a des Arg, FMLP and LTB_4. These findings clearly demonstrate the anti-inflammatory properties of dexamethasone but fail to indicate its mechanism of action. In this regard, it has been shown that the anti-inflammatory effects of glucocorticosteroids are triggered by a mechanism requiring receptors and synthesis of new RNA and protein (Tsurufuji *et al.*, 1979; Peers & Flower, 1991). To investigate further its site of action we studied the effect of locally administered dexamethasone on inflammatory responses and investigated the *in vivo* responsiveness of neutrophils isolated from dexamethasone-treated donor animals.

Locally administered dexamethasone inhibited oedema formation induced by neutrophil-dependent and direct-acting inflammatory mediators whilst having no effect on neutrophil accumulation. These results indicate that dexamethasone when applied locally can inhibit oedema formation by acting on endothelial cells whereas it has to be administered systemically in order to affect neutrophils. Studies where locally applied dexamethasone inhibited leucocyte accumulation most probably reflect an inhibition of mediator generation (Tsurufuji *et al.*, 1984). In our studies the use of preformed chemoattractants eliminates the occurrence of such a mechanism of action. The inhibitory effect of dexamethasone on oedema formation was time-dependent; inhibition was seen with a 4 h pretreatment but no significant effect was seen at 15 or 90 min. Previous studies have shown that the effect of glucocorticosteroids on oedema formation is protein synthesis-dependent suggesting that dexamethasone can induce the synthesis of a protein that inhibits microvascular responses by an action on the endothelium (Tsurufuji *et al.*, 1979; Peers & Flower, 1991). The identity of this inhibitory protein is yet to be determined. Lipocortin 1 is thought not to be involved as, although it has anti-inflammatory properties in some *in vivo* models (Cirino *et al.*, 1989; Errasfa & Russo-Marie, 1989; Perretti & Flower, 1992), it is not effective against preformed mediators in oedema assays and evidence that an inhibitory protein exists which is distinct from lipocortin has been obtained in rats (Di Rosa *et al.*, 1985). The anti-inflammatory protein activity has been detected in peritoneal lavage fluid from glucocorticosteroid-treated rats (Di Rosa *et al.*, 1985). This activity, which inhibited dextran-induced oedema in the rat paw ('vasocortin'), by chromatography eluted at approximately 100 kD whilst the phospholipase-inhibitory activity ('lipocortin') appeared at 40 kD (Carnuccio *et al.*, 1987). The inhibitory effect of local dexamethasone on oedema formation may be mediated by this or other newly synthetized proteins. However, the precise mechanisms underlying the effect of glucocorticosteroid-induced proteins on the endothelium remain to be determined.

Topically applied glucocorticosteroids have vasoconstrictor properties which may account for some of their anti-inflammatory actions (Reid & Brookes, 1968; Kaidbey & Kligman, 1974; Marks & Sawyer, 1986). It has been suggested that these effects may be ascribed to inhibition of the induction of nitric oxide (NO) synthase (Radomski *et al.*, 1990). Thus, the anti-oedema actions of glucocorticosteroids could be due to an inhibition of vasodilatation induced by stimulated NO release. Although the mechanism of action of glucocorticosteroids under certain inflammatory conditions may be partly mediated by an interference with NO production, such a mechanism appears an unlikely explanation for the results obtained in the present study. In our experiments, a short *in vivo* period was used for measurement during which dexamethasone did not alter increased blood flow induced by PGE_2. The induction of NO synthase in endotoxin- and cytokine-activated cultured endothelial cells is a slow and protein-synthesis dependent process with a lag period of 2 h (Radomski *et al.*, 1990).

Since systemically administered, but not locally administered, dexamethasone inhibited neutrophil accumulation, experiments were carried out to investigate the effect of dexamethasone on the neutrophil itself. For these studies we examined the *in vivo* accumulation of neutrophils isolated from steroid-treated donor rabbits. The results demonstrated that pretreatment of the neutrophil donor with dex-

amethasone resulted in an inhibition of ^{111}In-neutrophil accumulation in untreated recipient animals. These findings suggest that dexamethasone, or dexamethasone-induced products, can suppress neutrophil accumulation *in vivo* by an effect on the neutrophil itself, in addition to a possible effect on the endothelium. Adherence of neutrophils to vascular endothelium is the initial response during the extravascular accumulation of neutrophils at sites of inflammation (for review see: Nourshargh & Williams, 1990) and may be a target response for dexamethasone. In man, glucocorticosteroids induce an increase in circulating neutrophils that may be accounted for by reduced neutrophil adherence (Athens *et al.*, 1961), and there are numerous reports on the inhibitory effects of these drugs on other neutrophil responses *in vitro* (Ward, 1966; Majeski & Alexander, 1976; Weissmann *et al.*, 1976) although many of these effects may be related to the high doses employed. Other studies have, however, failed to detect such inhibitory effects. Neutrophils cultured with dexamethasone for 24 h responded normally with respect to chemotaxis and adherence to cultured endothelial cells (Schleimer *et al.*, 1989). More recently dexamethasone has been shown to act on neutrophils to inhibit their adhesion to thrombin and histamine activated endothelial cells (Watanabe *et al.*, 1991) whilst having no effect on neutrophil adhesion to endotoxin-stimulated endothelial cells in the presence or absence of FMLP (Forsyth & Talbot, 1992). In the latter study, dexamethasone was also without an effect on increased expression of the adhesion molecules CD11b/CD18 on the neutrophil and E-selectin and ICAM-1 on endothelial cells. Since thrombin and histamine have been shown to upregulate the expression of the adhesion molecule P-selectin on cultured endothelial cells (McEver *et al.*, 1989; Lorant *et al.*,1991), the study of Watanabe and colleagues (1991) suggests dexamethasone may modulate the expression of the ligand for endothelial P-selectin on the neutrophil. Such an effect has yet to be investigated. Although adhesion of neutrophils to venular endothelial cells is a prerequisite for neutrophil emigration, the effect of glucocorticosteroids on neutrophil adhesion, which *in vivo* may be mediated by a steroid-induced plasma factor (MacGregor, 1976), is contentious. It is possible that dexamethasone suppresses neutrophil accumulation by inhibiting the neutrophil response at another step such as the passage of the neutrophil across the perivascular basement membrane as has been reported (Katori *et al.*, 1990). Although the precise mechanism by which glucocorticosteroids inhibit neutrophil accumulation is still unknown our results clearly demonstrate that dexamethasone or a dexamethasone-induced factor exert an inhibitory effect on circulating neutrophils.

Glucocorticosteroids can influence the development of an inflammatory reaction by inhibiting the generation of inflammatory mediators and activation of pro-inflammatory cells. Since these are amongst the most widely used pharmacological agents in the treatment of both acute and chronic inflammatory conditions, a better understanding of their mechanism of action could lead to the development of novel anti-inflammatory agents lacking the deleterious side effects of the parent compounds. In this study, we have demonstrated that dexamethasone can suppress neutrophil accumulation *in vivo* by an effect on the neutrophil itself, in addition to a possible effect on the endothelium which also modulates oedema formation.

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References

ATHENS, J.W., HAAB, O.P., RAAB, S.O., MAUER, A.M., ASHENBRUCKER, H., CARTWRIGHT, G.E. & WINTROBE, M.M. (1961). Leukokinetic studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects. *J. Clin. Invest.*, **40**, 989–995.

BJORK, J., GOLDSCHMIDT, T., SMEDEGARD, G. & ARFORS, K.-E. (1985). Methylprednisolone acts at the endothelial cell level reducing inflammatory responses. *Acta Physiol. Scand.*, **123**, 221–224.

BLACKWELL, G.J., CARNUCCIO, R., DI ROSA, M., FLOWER, R.J., PARANTE, L. & PERSICO, P. (1980). Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature*, **287**, 147–149.

CARNUCCIO, R., ROSA, M.D., GUERRASIO, B., IUVONE, T. & SAUTEBIN, L. (1987). Vasocortin: a novel glucocorticoid-induced anti-inflammatory protein. *Br. J. Pharmacol.*, **90**, 443–445.

CHURCH, M.K. & MILLER, P. (1978). Time courses of the anti-anaphylactic and anti-inflammatory effects of dexamethasone in the rat and mouse. *Br. J. Pharmacol.*, **62**, 481–486.

CIRINO, G. & FLOWER, R.J. (1987a). Human recombinant lipocortin 1 inhibits prostacyclin production by human umbilical artery in vitro. *Prostaglandins*, **34**, 59–62.

CIRINO, G. & FLOWER, R.J. (1987b). The inhibitory effect of lipocortin on eicosanoid synthesis is dependent upon divalent cation. *Br. J. Pharmacol.*, **92**, 521P.

CIRINO, G., FLOWER, R.J., BROWNING, J.L., SINCLAIR, L.K. & PEPINSKY, R.B. (1987). Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung. *Nature*, **328**, 270–272.

CIRINO, G., PEERS, S.H., FLOWER, R.J., BROWNING, J.L. & PEPINSKY, R.B. (1989). Human recombinant lipocortin 1 has acute local anti-inflammatory properties in the rat paw edema test. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3428–3432.

CLOIX, J.F., COLARD, O., ROTHHUT, B. & RUSSO-MARIE, F. (1983). Characterization and partial purification of 'renocortins': two polypeptides formed in renal cells causing the anti-phospholipase-like action of glucocorticoids. *Br. J. Pharmacol.*, **79**, 313–321.

CRUMPTON, M.J. & DEDMAN, J.R. (1992). Protein terminology tangle. *Nature*, **345**, 212.

DI ROSA, M., FLOWER, R.J., HIRATA, F., PARENTE, L. & RUSSO-MARIE, F. (1984). Nomenclature announcement: antiphospholipase proteins. *Prostaglandins*, **28**, 441–442.

DI ROSA, M., CALIGNANO, A., CARNUCCIO, R., IALENTI, A. & SAUTEBIN, L. (1985). Multiple control of inflammation by glucocorticoids. *Agents Actions*, **17**, 284–289.

ERRASFA, M. & RUSSO-MARIE, F. (1989). A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids *in vivo* in mice. *Br. J. Pharmacol.*, **97**, 1051–1058.

FLOWER, R.J. (1988). Lipocortin and the mechanism of action of the glucocorticoids. *Br. J. Pharmacol.*, **94**, 987–1015.

FORSYTH, K.D. & TALBOT, V. (1992). Role of glucocorticoids in neutrophil and endothelial adhesion molecule expression and function. *Mediat. Inflamm.*, **1**, 101–106.

FOSTER, S.J. & MCCORMICK, M.E. (1985). The mechanism of the anti-inflammatory activity of glucocorticosteroids. *Agents Actions*, **16**, 58–59.

GRIFFITHS, R.J. & BLACKHAM, A. (1988). The effects of dexamethasone on inflammatory response in rabbit skin. *Br. J. Pharmacol.*, **95**, 535P.

HIRATA, F., SCHIFFMANN, E., VENKATASUBRAMANIAN, K., SALMON, D. & AXELROD, J. (1980). A phospholipase A2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2533–2536.

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 radioimmunoassay. *J. Exp. Med.*, **158**, 2177–2182.

KAIDBEY, K.H. & KLIGMAN, A.M. (1974). Assay of topical corticosteroids by suppression of experimental inflammation in humans. *J. Invest. Dermatol.*, **63**, 292–297.

KATORI, M., ODA, T. & NAGAI, K. (1990). A site of action of dexamethasone on leukocyte extravasation in microcirculation. *Agents Actions*, **29**, 24–26.

LORANT, D.E., PATEL, K.D. MCINTYRE, T.M., MCEVER, R.P., PRESCOTT, S.M. & ZIMMERMAN, G.A. (1991). Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J. Cell Biol.*, **115**, 223–234.

MACGREGOR, R.R. (1976). The effect of anti-inflammatory agents and inflammation on granulocyte adherence. Evidence for regulation by plasma factors. *Am. J. Med.*, **61,** 597–607.

MAJESKI, J.A. & ALEXANDER, J.W. (1976). The steroid effect on the in vitro human neutrophil chemotactic response. *J. Surg. Res.*, **21,** 265–268.

MAJNO, G., SHEA, S.M. & LEVENTHAL, M. (1969). Endothelial contraction induced by histamine-type mediators. An electron microscopic study. *J. Cell Biol.*, **42,** 647–672.

MARKS, R. & SAWYER, N. (1986). Glucocorticoid-induced vasoconstriction in human skin. An inhibitory role of phospholipase A2 activity. *Arch. Dermatol.*, **122,** 881–883.

MCEVER, R.P., BECKSTEAD, J.H., MOORE, K.L., MARSHALL-CARLSON, L. & BAINTON, D.F. (1989). GMP-140, a platelet α-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest.*, **84,** 92–99.

MOVAT, H.Z., RETTL, C., BURROWES, C.E. & JOHNSTON, M.G. (1984). The in vivo effect of leukotriene B4 on polymorphonuclear leukocytes and the microcirculation. Comparison with activated complement (C5a des Arg) and enhancement by prostaglandin E2. *Am. J. Pathol.*, **115,** 233–245.

NOURSHARGH, S., RAMPART, M., HELLEWELL, P.G., JOSE, P.J., HARLAN, J.M., EDWARDS, A.J. & WILLIAMS, T.J. (1989). Accumulation of ^{111}In-neutrophils in rabbit skin in allergic and non-allergic inflammatory reactions in vivo: inhibition by neutrophil pretreatment in vitro with a monoclonal antibody recognising the CD18 antigen. *J. Immunol.*, **142,** 3193–3198.

NOURSHARGH, S. & WILLIAMS, T.J. (1990). Neutrophil – endothelial cell interactions in vivo. In *The Endothelium: An Introduction to Current Research*, ed. Warren, J. pp. 171–186. New York: Wiley-Liss.

PEERS, S.H. & FLOWER, R.J. (1991). Site of anti-inflammatory action of dexamethasone in rabbit skin. *Eur. J. Pharmacol.*, **196,** 37–41.

PERRETTI, M. & FLOWER, R.J. (1992). Lipocortin-1 inhibits the neutrophil infiltration elicited by interleukin-1 in the mouse air-pouch. *Br. J. Pharmacol.*, **105,** 44P.

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.

RAMPART, M. & WILLIAMS, T.J. (1988). Evidence that neutrophil accumulation induced by interleukin-1 requires both local protein biosynthesis and neutrophil CD18 antigen expression *in vivo*. *Br. J. Pharmacol.*, **94,** 1143–1148.

REID, J. & BROOKES, D.B. (1968). Topical corticosteroids: an experimental evaluation of the vasoconstrictor test as an index of anti-inflammatory activity. *Br. J. Dermatol.*, **80,** 328–336.

SCHLEIMER, R.P., FREELAND, H.S., PETERS, S.P., BROWN, K.E. & DERSE, C.P. (1989). An assessment of the effects of glucocorticoids on degranulation, chemotaxis, binding to vascular endothelium and formation of leukotriene B_4 by purified human neutrophils. *J. Pharmacol. Exp. Ther.*, **250,** 598–605.

SVENSJO, E. & ROEMPKE, K. (1985). Time-dependent inhibition of bradykinin- and histamine-induced microvascular permeability increase by local glucocorticoid treatment. *Prog. Resp. Res.*, **19,** 173–180.

TSURUFUJI, S., SUGIO, K. & TAKEMASA, F. (1979). The role of glucocorticoid receptor and gene expression in the anti-inflammatory action of dexamethasone. *Nature*, **280,** 408–410.

TSURUFUJI, S., KURIHARA, A. & OJIMA, E. (1984). Mechanisms of anti-inflammatory action of dexamethasone: blockade by hydrocortisone mesylate and actinomycin D of the inhibitory effect of dexamethasone on leukocyte infiltration in inflammatory sites. *J. Pharmacol. Exp. Ther.*, **229,** 237–243.

WARD, P.A. (1966). The chemosuppression of chemotaxis. *J. Exp. Med.*, **124,** 209–226.

WATANABE, M., YAGI, M., OMATA, M., HIRASAWA, N., MUE, S., TSURUFUJI, S. & OHUCHI, K. (1991). Stimulation of neutrophil adherence to vascular endothelial cells by histamine and thrombin and its inhibition by PAF antagonists and dexamethasone. *Br. J. Pharmacol.*, **102,** 239–245.

WEDMORE, C.V. & WILLIAMS, T.J. (1981). Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature*, **289,** 646–650.

WEISSMANN, G., GOLDSTEIN, I. & HOFFSTEIN, S. (1976). Prostaglandins and the modulation of cyclic nucleotides of lysosomal enzyme release. *Adv. Prostaglandin Thromboxane Leukot. Res.*, **2,** 803–814.

WILLIAMS, T.J. (1979). Prostaglandin E_2, prostaglandin I_2 and the vascular changes of inflammation. *Br. J. Pharmacol.*, **65,** 517–524.

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 prostaglandin-mediated vasodilatation in inflammation. *Nature*, **270,** 530–532.

WILLIAMS, T.J. & YARWOOD, H. (1990). Effect of glucocorticosteroids on microvascular permeability. *Am. Rev. Respir. Dis.*, **141,** S39–S43.

YARWOOD, H., NOURSHARGH, S., BRAIN, S.D. & WILLIAMS, T.J. (1988). Suppression of neutrophil accumulation and neutrophil-dependent oedema by dexamethasone in rabbit skin. *Br. J. Pharmacol.*, **95,** 726P.

YARWOOD, H., NOURSHARGH, S., BRAIN, S.D. & WILLIAMS, T.J. (1989). Inhibition of neutrophil accumulation by dexamethasone in vivo: evidence for site of action. *Br. J. Pharmacol.*, **96,** 284P.

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Cholecystokinin as a factor in the enhanced potency of spinal morphine following carrageenin inflammation

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1 Cholecystokinin (CCK) has been shown to diminish opioid analgesia. Here we investigate whether changes in the physiological levels of spinal CCK are responsible for the enhanced potency of spinal morphine in animals following carrageenin inflammation, as compared with normal animals.

2 Single dorsal horn nociceptive neurones were recorded in intact halothane-anaesthetized rats in the presence and absence of carrageenin-induced inflammation and comparisons were made between the two groups of animals. Inflammation was induced by the injection of 100 μl of 2% λ-carrageenin into the hind paw.

3 The inhibitory effect of intrathecal morphine on the C-fibre-evoked responses of the neurones was enhanced in the carrageenin-treated animals such that the effects of 0.25 μg and 10 μg of morphine in normal animals were comparable to those of 0.01 μg and 2.5 μg in the carrageenin animals. The effect of 0.2 mg kg^{-1} of the CCK_B antagonist, L-365,260, on the antinociceptive potency of intrathecal morphine was examined in both groups of animals. In normal animals, L-365,260 produced a significant enhancement in the effect of morphine indicating a tonic CCK modulation in these animals, but it had no effect on the inhibitions produced by either dose of morphine in the carrageenin animals.

4 The inhibition of the C-fibre-evoked response produced by intrathecal morphine in the presence of 1 μg of CCK was examined in both groups of animals. CCK attenuated the effects of morphine only in animals with carrageenin inflammation, having no effect on the action of morphine in normal animals.

5 The effects of both CCK and L-365,260 were therefore dependent on the inflammatory state of the animal, with each drug being active in opposite situations.

6 We propose that in normal animals, morphine may produce a maximal stimulation of the release of CCK such that exogenous CCK is unable to reduce further the analgesic effects under these conditions. However, the differential effects of the agonist and antagonist in the normal and inflamed rats points to a role of CCK in the enhanced opiate actions. This enhancement of the potency of spinal morphine in inflammation is best explained by a reduction in spinal CCK release by morphine in this state.

Keywords: Morphine; cholecystokinin; L-365,260; inflammation; analgesia; spinal cord

Introduction

Over the years it has been demonstrated that there is great capacity for change in not only the transmission but also the modulation of nociceptive information (see recent reviews by Dickenson, 1991; Dubner & Ruda, 1992). Little is known about the transmitter systems responsible for alterations in the ability to produce analgesia. Whereas opiate sensitivity can be diminished in neuropathic pains (Arner & Meyerson, 1988; Portenoy *et al.*, 1990) the opposite can occur in inflammatory conditions. It is well established that there is an enhancement in the potency of systemically administered opiate agonists in a variety of chronic inflammatory states, gauged by behavioural approaches (Kayser & Guilbaud, 1983; Neil *et al.*, 1986; Joris *et al.*, 1990). Recently it has been shown that one site contributing to this increased potency of opiates following carrageenin-induced inflammation is the spinal cord (Hylden *et al.*, 1991; Stanfa *et al.*, 1992). We have demonstrated, in electrophysiological studies, that this enhancement in spinal potency is especially marked for the μ-opiate agonist, morphine compared with that seen with the δ-agonist DSTBULET and the κ-agonist, U69593. In addition, the facilitation of the C-fibre evoked response normally seen with low doses of intrathecal μ-agonists in this model was absent in animals with carrageenin-induced inflammation (Stanfa *et al.*, 1992).

Here we investigate one of the pharmacological systems within the spinal cord which may be responsible for the relatively selective enhancement in the spinal potency of μ-opioids following peripheral inflammation. One candidate for this role is the peptide cholecystokinin (CCK) which acts predominantly at CCK_B receptors in the rat spinal cord (Hill & Woodruff, 1990). Although there are some anomalies in the results from studies using CCK and CCK receptor antagonists, there is good evidence that the peptide CCK selectively interferes with the action of μ- but not δ- or κ-agonists at the level of the spinal cord (Faris *et al.*, 1983; Barbaz *et al.*, 1989; Magnuson *et al.*, 1990). As yet, there has been no attempt to assign a physiological role to CCK in inflammatory states.

In this study we have used sulphated CCK-8 and the CCK_B receptor antagonist, L-365,260, to investigate whether physiological levels of CCK exert a tonic inhibition of the antinociceptive effect of intrathecal morphine in normal (control) animals and whether the same is true in animals with carrageenin-induced inflammation.

In addition, we used the CCK_B receptor antagonist to investigate whether the release of CCK is responsible for the facilitations seen with low doses of morphine in normal animals but absent in animals with carrageenin-induced inflammation (Stanfa *et al.*, 1992).

Methods

The method is essentially that previously described (Stanfa *et al.*, 1992). Briefly, male Sprague Dawley rats (200–250 g) were anaesthetized with 2% halothane in a mixture of N_2O

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and O_2 and after cannulation of the trachea a laminectomy was performed to expose the L1–L3 region of the spinal cord. A tungsten electrode was lowered into the dorsal horn and single unit extracellular recordings were made from convergent neurones responding to both innocuous and noxious stimuli with receptive fields on the ipsilateral hind paw. During the recording the level of halothane was reduced to 1–1.5% which was sufficient to maintain complete areflexia. Electrical stimulation (2 ms wide pulse, 0.5 Hz, 16 stimuli per trial) was used as the test stimulus for the experiment, applied at 3 times the threshold current for C-fibre activation. This type of stimulation provides a constant reproducible test stimulus for the experiment and avoids the problem of altered baseline responses which could result from hyperalgesia to natural stimuli as a consequence of the inflammation. Trials were performed every 10 min and we have previously shown that no change occurs in the thresholds for C-fibre activation during the period of the experiment (Stanfa *et al.*, 1992). C-fibre-evoked responses, which could be separated from the Aβ-fibre evoked responses on the basis of threshold and latency, were quantified from the post-stimulus histogram. The degree of wind-up of the cells (Mendell, 1966; Davies & Lodge, 1987; Dickenson & Sullivan, 1987), W, was also quantified and calculated as the total number of spikes to the 16 stimuli divided by I, where I is the cell's response to the initial C-fibre stimulus × 16. Drugs were administered following 3 stable control trials.

In the animals with peripheral inflammation, 100 μl of 2% λ-carrageenin was injected into the plantar region of the paw 3 h before drug administration. The evoked responses of the cells and the degree of wind-up of the cells was monitored during this 3 h period, with the 3 readings prior to the drug administration used as controls for the subsequent drug effect.

Morphine and CCK were applied directly to the surface of the cord (equivalent to an intrathecal injection) in a volume of 50 μl. CCK was administered 20 min before the administration of morphine. L-365,260 0.2 mg kg^{-1} (in 10% Tween/alcohol) in a volume of 0.25 ml was given subcutaneously 10 min before the application of morphine to the surface of the cord. The evoked response of the cell was then followed for a further 40 min. Intrathecal naloxone at a dose of 1 or 5 μg was used to reverse the effects of morphine.

The effects of L-365,260 alone and the effects of the vehicle alone were examined in separate animals.

Materials

Morphine sulphate (Thornton and Ross), naloxone hydrochloride (Sigma), sulphated cholecystokinin-8 (CCK) (Sigma), λ-carrageenin (Sigma) and L-365, 260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)- N^1-(3-methyl-phenyl)urea) (a gift from Merck Sharp and Dohme) were used in this study.

Statistical analysis

The results are expressed as a percentage of control or as percentage inhibition. The data were analysed either by Student's *t* test or two factor analysis of variance (ANOVA) followed by Newman-Keuls Student's test. *P* values less than 0.05 were regarded as significant.

Results

The convergent neurones used in this study were located in the deeper laminae of the dorsal horn (500–1000 μm, mean depth = 738 ± 31 μm ($n = 40$)), with these neurones forming a similar population to those used in the previous study (Stanfa *et al.*, 1992).

Responses of the dorsal horn neurones following carrageenin injection

The C-fibre evoked response of 14 of the neurones was followed for 3 h after the injection of carrageenin. It was found that the C-fibre-evoked response of 6 (43%) of the neurones increased to a mean 132.5 ± 10% of control, while that of the other 8 (57%) decreased to 88.5 ± 4% of control. The degree and magnitude of this change was significantly correlated with the initial degree of wind-up of the neurones ($r = -0.51, P = 0.032$). Wind-up is the frequency-dependent increase in evoked neuronal responses of the cells to repeated constant intensity stimulation (Mendell, 1966; Davies & Lodge, 1987; Dickenson & Sullivan, 1987). Figure 1 shows that neurones that had little wind-up during the control period (low value of W) tended to have an increased response after carrageenin, whilst those with a large degree of wind-up initially (high W) had a decreased response. This pattern of changes is identical to that previously found in a more comprehensive study in this model (Stanfa *et al.*, 1992). In this earlier study we showed that the direction and magnitude of the change in the C-fibre evoked response following carrageenin was not correlated in any way with the subsequent responses to intrathecal opiates, thus allowing us to discard this as a factor in the altered potency of morphine in these animals. In addition neither the injection of saline into the paw nor the repeated stimulation had any effect on the magnitude of the C-fibre-evoked response (Stanfa *et al.*, 1992).

None of the neurones developed any spontaneous activity during the period of inflammation.

Effect of the CCK_B antagonist L-365, 260 on the potency of morphine in normal animals.

Two doses of intrathecal morphine (0.25 and 10 μg) were tested on the C-fibre-evoked responses of convergent dorsal horn neurones in normal (untreated) rats and caused dose-dependent inhibitions of 7.9 ± 9% ($n = 7$) and 81.3 ± 6% ($n = 6$) respectively (see Figure 2).

In these normal animals, the subcutaneous administration of 0.2 mg kg^{-1} of L-365,260 (the maximum effective dose

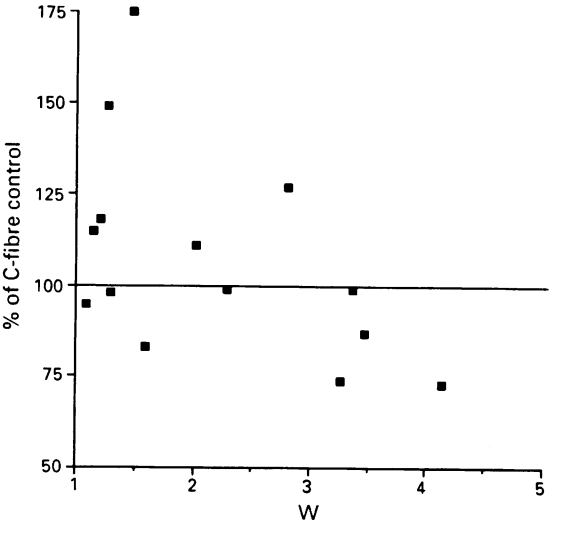


Figure 1 The relationship between the magnitude of the C-fibre evoked response in dorsal horn cells (3 h after the injection of carrageenin into the paw) and the initial degree of wind-up of the cell, W. Cells with little wind-up (low value of W) in the control period tended to have an enhanced C-fibre-evoked response after carrageenin, whilst those cells that exhibited a large degree of wind-up (high W) tended to have a decreased C-fibre evoked response after carregeenin. Correlation coefficient, $r = -0.51$ ($P = 0.032$).

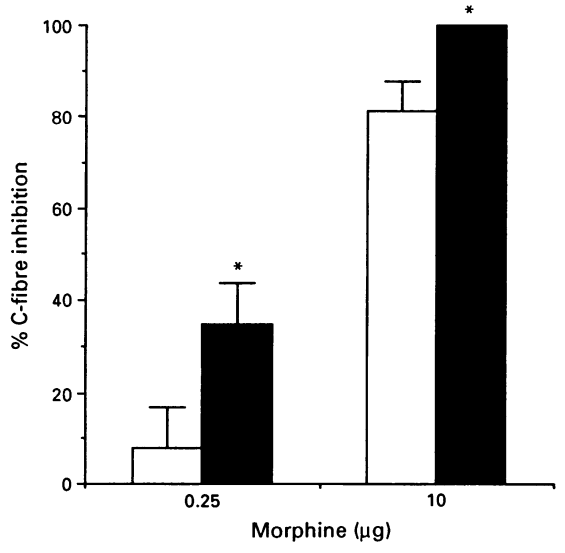


Figure 2 The effect of L-365,260, 0.2 mg kg^{-1}, on the morphine-induced inhibitions of the C-fibre-evoked response in dorsal horn cells in normal animals. Note the significant enhancement of the potency of intrathecal morphine in the presence of L-365, 260 (solid columns) compared with the controls (open columns). $n = 5-7$ for each point. $^*P < 0.05$.

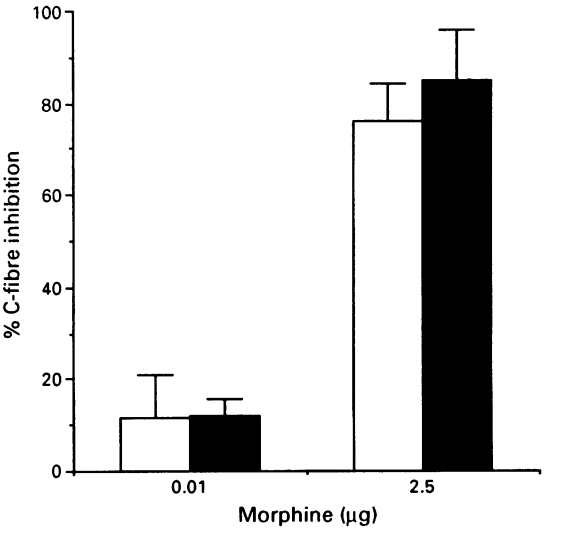


Figure 3 The effect of L-365,260, 0.2 mg kg^{-1}, on the morphine-induced inhibitions of the C-fibre evoked response in dorsal horn cells in animals with carrageenin-induced inflammation. In the animals with peripheral inflammation, L-365,260 no longer potentiates the antinociceptive effects of morphine (solid columns) compared to morphine alone (open columns). $n = 5-9$ for each point.

(Dourish *et al.*, 1990)) 10 min before 0.25 μg and 10 μg of intrathecal morphine led to a significant enhancement in the potency of both doses. This produced a leftward shift in the dose-response curve with the degree of inhibition produced by 0.25 μg being enhanced to 34.7 ± 9% ($n = 6$, $P = 0.033$) and that produced by 10 μg to 99.4 ± 1% ($n = 5$, P = 0.014) (see Figure 2). Intrathecal naloxone at a dose of 5 μg reversed the morphine-induced inhibitions of the C-fibre evoked response to 84 ± 12% of control values within 20 min. These results indicate that in normal (control) animals the endogenous levels of CCK released in the dorsal horn are sufficient to attenuate partly the inhibitory effects of intrathecal morphine.

In some animals the effects of the vehicle alone ($n = 4$) or L-365,260 alone ($n = 4$) were examined. Neither substance had any effect on the C-fibre evoked response when followed over a 90 min time course.

Effect of the CCK_B antagonist L-365,260 on the potency of morphine in animals with carrageenin inflammation

In order to investigate whether endogenous levels of CCK attenuate the analgesic effects of intrathecal morphine in animals with carrageenin inflammation, two doses of morphine were chosen which produced a similar degree of inhibition of the C-fibre evoked response to those used in normal animals. We have previously shown that the dose-response curve for intrathecal morphine is shifted to the left in animals with carrageenin-induced inflammation (Stanfa *et al.*, 1992), hence these doses of morphine are lower than those used in the normal animals.

Intrathecal administration of 0.01 and 2.5 μg of morphine 3 h after the injection of carrageenin into the paw produced inhibitions of the C-fibre-evoked response of 11.5 ± 9% ($n = 5$) and 75.4 ± 8% ($n = 9$) respectively (Figure 3).

When the same two doses were applied 10 min after the subcutaneous administration of 0.2 mg kg^{-1} of L-365,260 no significant enhancement was found in the antinociceptive potency of morphine (inhibitions of 12.0 ± 4 ($n = 6$) and 85.2 ± 11 ($n = 5$) respectively) which is in contrast to the situation in normal animals (see Figure 2). The intrathecal administration of 1 μg of naloxone reversed the inhibitions produced by 2.5 μg of morphine to 78 ± 10% of control values within 20 min. These findings suggest that there is insufficient CCK released in the spinal cord of animals with carrageenin induced inflammation to attenuate significantly the antinociceptive actions of morphine under these conditions.

Effect of CCK on morphine analgesia in normal animals

CCK (1 μg) was given intrathecally 20 min before the application of 10 μg of morphine to the surface of the cord and the inhibition of the C-fibre evoked response of the dorsal horn neurones was followed for 40 min. There was found to be no difference between the inhibition produced by morphine alone and that produced in the presence of 1 μg of CCK (see Figure 4) with morphine alone causing an inhibi-

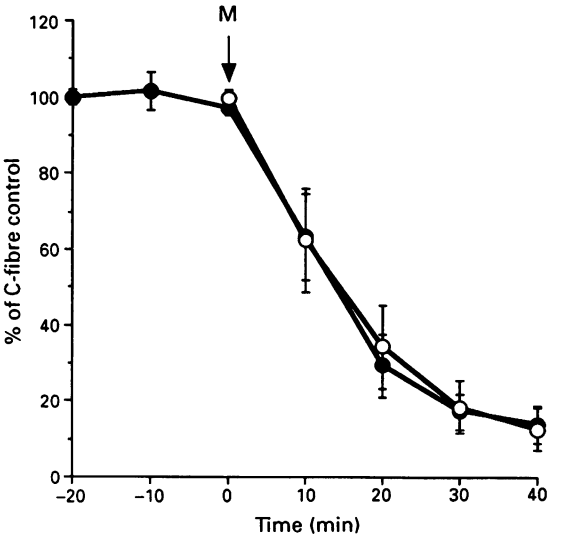


Figure 4 The time course for the inhibition of the C-fibre-evoked response by 10 μg of intrathecal morphine alone (○) and 20 min after the intrathecal administration of 1 μg of cholecystokinin (CCK, ●) in normal animals. In normal animals the presence of CCK had no effect on the potency of morphine. $n = 7-8$.

tion of 87.3 ± 6% ($n = 8$) after 40 min and 86.3 ± 5% ($n = 7$) when 1 μg of CCK was present. The intrathecal administration of 5 μg of naloxone reversed the morphine-induced inhibition of the C-fibre evoked response to 90 ± 12% of control within 20 min.

Effect of CCK on morphine analgesia in animals with carrageenin inflammation

The effect of 1 μg of intrathecal CCK (given 20 min before the intrathecal administration of morphine) was studied on the morphine-induced inhibition of the C-fibre evoked response of 7 dorsal horn neurones in carrageenin animals. Five of these cells showed substantially smaller inhibitions with 2.5 μg of morphine than the control neurones whilst two appeared to be unaffected by the presence of CCK. No other differences were found between the CCK-sensitive and insensitive neurones so all cells were pooled. Thus in the presence of 1 μg of CCK, 2.5 μg of morphine produced a significantly smaller inhibition of the C-fibre evoked response of the neurones ($F_{1,52} = 18.5$; $P < 0.0001$; $n = 7$ cells per group) (see Figure 5). The intrathecal administration of 1 μg of naloxone reversed the morphine-induced inhibition of the C-fibre evoked response to 93 ± 3% of control within 20 min. This demonstrates that exogenous CCK is still capable of reducing the antinociceptive actions of morphine in animals with carrageenin inflammation. Hence it is most likely that a reduced release of the peptide is responsible for the lack of effect of the CCK_B antagonist in these animals.

No significant facilitation of the C-fibre evoked response was seen with CCK as has been reported previously in this model and in other studies (Jeftinija *et al.*, 1981; Willetts *et al.*, 1985; Magnuson *et al.*, 1990), although we previously observed this effect to be maximal 5 min post CCK administration and this time point was not tested in this study.

Effect of L-365,260 on facilitations due to low dose morphine in normal animals

It has been reported that (1) morphine is able to cause the release of CCK in the dorsal horn of the spinal cord (Tang *et al.*, 1984; Benoliel *et al.*, 1991) (and see discussion), (2) CCK itself is capable of causing a facilitation of the C-fibre evoked response of neurones (Jeftinija *et al.*, 1981; Willetts *et al.*, 1985; Magnuson *et al.*, 1990), although this was not seen in the present study and (3) the facilitation of the C-fibre evoked response produced by low doses of intrathecal morphine in normal animals no longer occurs in animals with carrageenin inflammation (Stanfa *et al.*, 1992). The results of the present study suggest that morphine is no longer causing the release of CCK in animals with carrageenin-induced inflammation. This led us to investigate whether a morphine-induced release of CCK may be responsible for the facilitations seen with the low doses of morphine in normal animals, with μ-receptor inhibitions becoming predominant as the dose is increased.

The two doses of morphine chosen for this study are at the lower end of the morphine dose-response curve. The first dose (0.01 μg) is a subthreshold dose and had no significant effect on the C-fibre-evoked response (93.5 ± 9% of control, $n = 6$) in normal animals. The second dose (0.1 μg) produced a facilitation of the C-fibre-evoked response to 126.2 ± 8% of control ($n = 5$) when given intrathecally. As the dose of morphine was increased to 0.25 μg and above, the facilitation turned to dose-dependent inhibitions (see Figure 6). When these low doses of morphine (0.01 and 0.1 μg) were repeated 10 min after the subcutaneous administration of 0.2 mg kg^{-1} L-365,260, 0.01 μg of morphine, previously a subthreshold dose, facilitated the C-fibre evoked response to 118.8 ± 8% of control ($n = 5$) whilst 0.1 μg of morphine failed to have a significant effect on the C-fibre evoked response (102.8 ± 9% of control, $n = 5$) (see Figure 6).

Thus is appears that the CCK_B receptor antagonist, L-365,260, does not block the facilitations produced by low doses of morphine suggesting that this phenomenon is not due to the release of CCK. There has however been a shift in the dose-response curve in the presence of the antagonist in the same way as occurs with the higher doses. As a result of this, the threshold dose for a significant effect (with these low doses, a facilitation) on the C-fibre evoked response has been lowered.

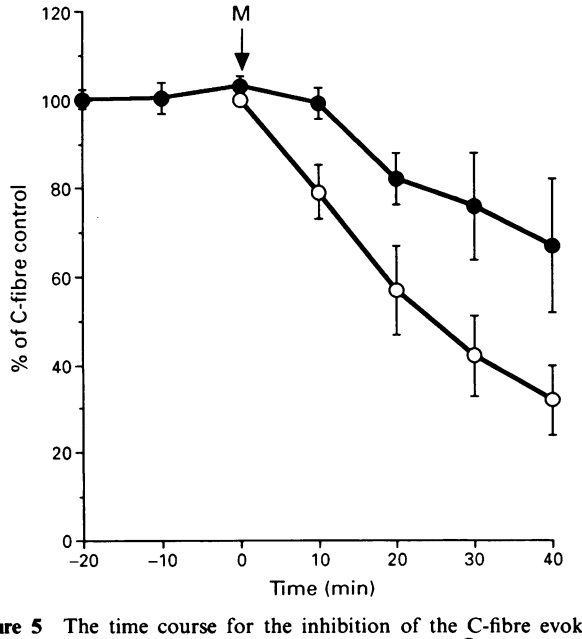


Figure 5 The time course for the inhibition of the C-fibre evoked response by 2.5 μg of intrathecal morphine alone (○) and 20 min after the intrathecal administration of 1 μg of cholecystokinin (CCK, ●) in animals with carrageenin-induced inflammation.
In animals with carrageenin inflammation the presence of CCK leads to a significant decrease in the potency of intrathecal morphine ($P < 0.0001$, ANOVA). $n = 7$ cells per group.

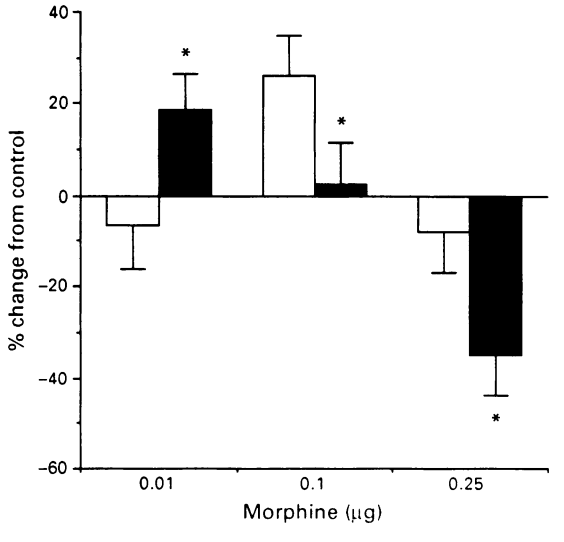


Figure 6 The effects of low doses of intrathecal morphine alone (open columns) and in combination with CCK_B receptor antagonist, L-365,260 (solid columns) on the C-fibre-evoked responses. The vertical axis represents the % change from the controls so that a negative change signifies inhibitory effects and a positive change facilitation. Note that in the presence of the antagonist the dose-response curve is shifted to the left such that the facilitation occurs with a lower dose of morphine but the magnitude remains the same. *$P < 0.05$ compared with morphine alone.

Discussion

The peptide CCK has been widely heralded as an endogenous modulator of the μ-opiate receptor whilst appearing to have little effect on effects mediated by the other subtypes of opiate receptor (Faris *et al.*, 1983; Barbaz *et al.*, 1989; Magnuson *et al.*, 1990). In this study we have used the CCK_B receptor antagonist, L-365,260, to demonstrate that in normal animals, under these experimental conditions, endogenous levels of CCK in the cord are sufficient to attenuate the antinociceptive actions of intrathecal morphine. This finding is in agreement with behavioural studies in normal animals (Baber *et al.*, 1989; Dourish *et al.*, 1990; Wiesenfeld-Hallin *et al.*, 1990) but this interaction has never been studied in models of pain states more relevant to the clinical situation. Here, in animals with carrageenin-induced inflammation, the CCK_B receptor antagonist produced no enhancement of the potency of morphine in direct contrast to the situation in normal animals.

The CCK receptors are mainly of the CCK_B type (Hill & Woodruff, 1990) in the rat spinal cord and although their exact location is not known, they may be located presynaptically since it has been reported that CCK_B receptors undergo axonal transport to the periphery in the vagus nerve (Mercer & Lawrence, 1992). Following early immunocytochemical studies, CCK was thought to be located within C-fibres although recent studies disagree with these findings (Hökfelt *et al.*, 1988; Zouaoui *et al.*, 1990). In addition cDNA probes have failed to localize CCK precursor mRNA within dorsal root ganglia in the rat (Seroogy *et al.*, 1990) suggesting that it is very unlikely that genuine CCK is found in primary afferents. It now seems most likely that the sources of endogenous CCK within the dorsal horn are the rich network of interneurones found in superficial laminae and possibly also some of descending fibres terminating there (Hökfelt *et al.*, 1988).

CCK does not appear to play a major role in nociceptive transmission since the peptide itself produces only mild increases in C-fibre responses (Jeftinija *et al.*, 1981; Willetts *et al.*, 1985; Magnuson *et al.*, 1990). Behavioural nociceptive thresholds are not altered by antagonists in all studies (Baber *et al.*, 1989; Dourish *et al.*, 1990; O'Neill *et al.*, 1990; Wiesenfeld-Hallin *et al.*, 1990) and where analgesic effects have occurred, they have been attributed to indirect opioid mechanisms (Wiesenfeld-Hallin *et al.*, 1990). In the present study both CCK and L-365,260 had no effect on the neuronal responses when given alone. The primary role of CCK in nociception would therefore appear to be as a modulator of opioid analgesia.

The exact sequence of events which triggers the release of CCK is not known although there is evidence that morphine stimulates the release of CCK within the dorsal horn (Tang *et al.*, 1984; Benoliel *et al.*, 1991). One possible physiological role of the morphine induced release of CCK is a mechanism whereby an endogenous 'brake' is applied to the antinociceptive actions of morphine or the endogenous ligand of the μ-receptor in times of acute pain. However, in animals with carrageenin-induced inflammation the antagonist no longer produced an enhancement of the antinociceptive effect of morphine, suggesting that CCK is no longer available. This lack of CCK is probably due to a reduced release of CCK and not an uncoupling of the interaction between morphine and CCK, (the exact nature of which is not known), since exogenous CCK applied to the cord caused a substantial reduction in the antinociceptive potency of morphine in these animals. It is possible that under these conditions of subchronic inflammation, morphine no longer evokes the release of CCK in the spinal cord and it is the absence of this autolimiting control that leads to the leftward shift in the dose-response curve seen in these animals compared with the normal state.

One explanation for the lack of effect of the CCK_B receptor antagonist in the carrageenin animals could be a reduced opiate release of CCK as a result of the use of lower doses of morphine (0.25 and 2.5 μg). This is unlikely since the CCK_B antagonist still produced a leftward shift in the responses to the even lower doses (0.01 and 0.1 μg) of morphine used in normal animals. Different doses of morphine cannot therefore explain these findings leaving the presence of inflammation as the causal factor.

A feature of the morphine dose-response curve in normal animals is the facilitation of the C-fibre evoked response by low doses of morphine (Stanfa *et al.*, 1992). We speculated that this might be due to morphine releasing CCK which in turn causes facilitations of the neurones. These facilitations are absent in animals with carrageenin-induced inflammation where we now believe CCK release to be reduced. However since these facilitations still occur in the presence of L-365,260 it is unlikely that CCK is involved. This facilitation may then be due to an opiate-induced release of other excitatory neuropeptides such as substance P (Wiesenfeld-Hallin *et al.*, 1991). In addition, there are reports of direct opioid receptor-mediated increases in the duration of action potentials in dorsal root ganglion cells (Crain & Shen, 1990). Both mechanisms could be altered in inflammation.

The ability of CCK to modulate the actions of morphine is variable and depends on particular circumstances. CCK clearly attenuated the effects of morphine in the animals with carrageenin inflammation. The negative results obtained with the combination of CCK and morphine in normal animals in our model at first appears to conflict with reports by others showing that exogenous CCK blocks the antinociceptive actions of morphine (Faris *et al.*, 1983; Wiesenfeld-Hellin & Duranti, 1987; Baber *et al.*, 1989; Kellstein *et al.*, 1991). However Barbaz *et al.* (1989) found CCK to be effective in blocking morphine antinociception in the tail flick test but not in the hot plate or abdominal stretch test in the mouse.

We previously reported that 1 μg of exogenous CCK caused a marked inhibition of the antinociceptive effect produced by the potent μ-agonist [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO) (Magnuson *et al.*, 1990). The difference between DAMGO and morphine may be due to morphine stimulating the release of CCK such that maximal CCK_B receptor activation results and so exogenous CCK can have no additional effect. Unlike morphine, DAMGO does not stimulate the release of CCK but in contrast has been shown to reduce the release of CCK at the level of the spinal cord (Rodriguez & Sacristan, 1989; Benoliel *et al.*, 1991). Thus when exogenous CCK is applied with DAMGO, attenuation of the analgesic effect of this opioid can occur. It is not clear how morphine produces the release of CCK, although it has been suggested that it may be through an action on the δ-receptor (Benoliel *et al.*, 1991). We suggest alternative explanations since in our model, in cross-tolerance studies, morphine does not appear to have any actions at the δ-receptor (unpublished observations).

One is that morphine and DAMGO may have different efficacies at the putative subtypes of the μ-receptor such as the μ_1 and μ_2 receptors (Pasternak & Wood, 1986). Indeed CCK blocks the antinociceptive action of meptazinol (a μ_1-agonist) more successfully than that of morphine which binds to both μ_1 and μ_2-receptors (Barbaz *et al.*, 1989). However the idea of μ_1 and μ_2-receptors is a matter of controversy and there is evidence suggesting another receptor subclassification involving receptor complexes (Rothman *et al.*, 1989). Finally, there is a suggestion that DAMGO and morphine can be distinguished on the basis that different agonist-receptor complexes may couple with different G proteins (Sanchez-Blazquez & Garzon, 1991). These possibilities need further study before conclusions can be drawn.

Behavioural studies have suggested that the release of endogenous CCK is governed by the environment to which the rat is exposed. At present the results are conflicting with one group suggesting that CCK is released in 'safe' situations to prevent the analgesic actions of morphine (Wiertelak *et al.*, 1992) whilst another reports that CCK receptor

antagonists do not enhance morphine antinociception in rats in 'familiar' situations but do so in animals exposed to a novel environment (Lavigne *et al.*, 1992) suggesting that CCK release is associated with 'stress'. Our animals, being under general anaesthesia, could arguably be considered as being in a 'safe' situation. Equally the presence of inflammation could activate 'stress' systems although the anaesthetic would prevent any manifestation of these signs. Whatever the case, these results serve to indicate that the release of CCK is not fixed but is modified according to circumstances.

To summarize, we present evidence to show that morphine-mediated CCK release may be abolished in inflammatory states so providing a mechanism for the enhancement in the potency of spinal morphine seen following inflammation. In support of this hypothesis, not only does CCK have no effect on δ-opioid antinociception (Magnuson *et al.*, 1990) but the effectiveness of δ-agonists in carrageenin inflammation is only moderately enhanced (Stanfa *et al.*, 1992). In more general terms, on the basis of studies with CCK antagonists, CCK has been implicated in morphine tolerance (see Baber *et al.*, 1989) where there is a reduced effect of morphine. These findings, together with the present study indicate that the enhanced and reduced effects of μ-opiates may be mediated through opposite extremes of the same mechanisms, in this case involving CCK. Hence further studies on the control of CCK may provide interesting insights into the role of CCK and possibly other neuropeptides in the modulation of nociceptive processing in the dorsal horn under different conditions.

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References

ARNER, S. & MEYERSON, B.A. (1988). Lack of analgesic effect of opioids on neuropathic forms of pain. *Pain*, **33**, 11–23.

BABER, N.S., DOURISH, C.T. & HILL, D.R. (1989). The role of CCK, caerulein, and CCK antagonists in nociception. *Pain*, **39**, 307–328.

BARBAZ, B.S., HALL, N.R. & LIEBMAN, J.M. (1989). Antagonism of morphine analgesia by CCK-8-S does not extend to all assays nor all opiate analgesics. *Peptides*, **9**, 1295–1300.

BENOLIEL, J.J., BOURGOIN, S., MAUBORGNE, A., LEGRAND, J.C., HAMON, M. & CESSELIN, F. (1991). Differential inhibitory/stimulatory modulation of spinal CCK release by μ and δ opioid agonists, and selective blockade of μ-dependent inhibition by κ receptor stimulation. *Neurosci. Lett.*, **124**, 204–207.

CRAIN, S.M. & SHEN, K.-F. (1990). Opioids can evoke direct receptor-mediated excitatory effects on sensory neurones. *Trends Pharmacol. Sci.*, **11**, 77–81.

DAVIES, S.N. & LODGE, D. (1987). Evidence for involvement of N-methyl-D-aspartate receptors in 'wind-up' of class 2 neurones in the dorsal horn of the rat. *Brian Res.*, **424**, 402–406.

DICKENSON, A.H. (1991). Recent advances in the physiology and pharmacology of pain: plasticity and its implications for clinical analgesia. *J. Psychopharmacol.*, **5**, 342–351.

DICKENSON, A.H. & SULLIVAN, A.F. (1987). Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep dorsal horn neurones following C-fibre stimulation. *Neuropharmacology*, **26**, 1235–1238.

DOURISH, C.T., O'NEILL, M.F., COUGHLAN, J., KITCHENER, S.J., HAWLEY, D. & IVERSEN, S.D. (1990). The selective CCK-B receptor antagonist L-365,260 enhances morphine analgesia and prevents morphine tolerance in the rat. *Eur. J. Pharmacol.*, **17**, 35–44.

DUBNER, R. & RUDA, M.A. (1992). Activity-dependent neuronal plasticity following tissue injury and inflammation. *Trends Pharmacol. Sci.*, **15**, 96–103.

FARIS, P.L., KOMISARUK, B.R., WATKINS, L.R. & MAYER, D.J. (1983). Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. *Science*, **219**, 310–312.

HILL, D.R. & WOODRUFF, G.N. (1990). Differentiation of central cholecystokinin receptor binding sites using the non-peptide antagonists MK-329 and L-365,260. *Brain Res.*, **526**, 276–283.

HÖKFELT, T., HERRERA-MARSCHITZ, M., SEROOGY, K., JU, G., STAINES, W.A., HOLETS, V., SCHALLING, M., UNGERSTEDT, U., POST, C., REHFELD, J.F., FREY, P., FISCHER, J., DOCKRAY, G., HAMAOKA, T., WALSH, J.H. & GOLDSTEIN, M. (1988). Immunohistochemical studies on cholecystokinin (CCK)-immunoreactive neurons in the rat using sequence specific antisera and with special reference to the caudate nucleus and primary sensory neurons. *J. Chem. Neuroanat.*, **1**, 11–52.

HYLDEN, J.L.K., THOMAS, D.A., IADAROLA, M.J., NAHIN, R.L. & DUBNER, R. (1991). Spinal opioid analgesic effects are enhanced in a model of unilateral inflammation/hyperalgesia: possible involvement of noradrenergic mechanisms. *Eur. J. Pharmacol.*, **194**, 135–143.

JEFTINIJA, S., MILETIC, V. & RANDIC, M. (1981). Cholecystokinin octapeptide excites dorsal horn neurones both in vivo and in vitro. *Brain Res.*, **213**, 231–236.

JORIS, J., COSTELLO, A., DUBNER, R. & HARGREAVES, K.M. (1990). Opiates suppress carrageenin-induced oedema and hyperthermia at doses that inhibit hyperalgesia. *Pain*, **43**, 95–103.

KAYSER, V. & GUILBAUD, G. (1983). The analgesic effects of morphine, but not those of the enkephalinase inhibitor thiorphan, are enhanced in arthritic rats. *Brain Res.*, **267**, 131–138.

KELLSTEIN, D.E., PRICE, D.D. & MAYER, D.J. (1991). Cholecystokinin and its antagonist lorgumide respectively attenuate and facilitate morphine-induced inhibition of C-fiber evoked discharges of dorsal horn nociceptive neurons. *Brain Res.*, **540**, 302–306.

LAVIGNE, G.J., MILLINGTON, W.R. & MUELLER, G.P. (1992). The CCK-A and CCK-B receptor antagonists, devazepide and L-365,260, enhance morphine antinociception only in non-acclimated rats exposed to a novel environment. *Neuropeptides*, **21**, 119–129.

MAGNUSON, D.S.K., SULLIVAN, A.F., SIMMONET, G., ROQUES, B.P. & DICKENSON, A.H. (1990). Differential interactions of cholecystokinin and FLFQPQRFamide with μ and δ opioid antinociception in rat spinal cord. *Neuropeptides*, **16**, 213–218.

MENDELL, L.M. (1966). Physiological properties of unmyelinated fiber projection to the spinal cord. *Exp. Neurol.*, **16**, 316–332.

MERCER, J.G. & LAWRENCE, C.B. (1992). Selectivity of cholecystokinin (CCK) receptor antagonists, MK-329 and L-365,260, for axonally-transported CCK binding sites on the rat vagus nerve. *Neurosci. Lett.*, **137**, 229–231.

NEIL, A., KAYSER, V., GACEL, G., BESSON, J.-M. & GUILBAUD, G. (1986). Opioid receptor types and antinociceptive activity in chronic inflammation: both κ- and μ-opiate agonists are enhanced in arthritic rats. *Eur. J. Pharmacol.*, **130**, 203–208.

O'NEILL, M.F., DOURISH, C.T., TYE, S.J. & IVERSEN, S.D. (1990). Blockade of CCK-B receptors by L-365,260 induces analgesia in the squirrel monkey. *Brain Res.*, **534**, 287–290.

PASTERNAK, G.W. & WOOD, P.J. (1986). Multiple opiate receptors. *Life Sci.*, **38**, 1889–1898.

PORTENOY, R.K., FOLEY, K.M. & INTURISSI, C.E. (1990). The nature of opioid responsiveness and its implications for neuropathic pain: new hypotheses derived from studies of opioid infusions. *Pain*, **43**, 273–286.

RODRIGUEZ, R.E. & SACRISTAN, M.P. (1989). In vivo release of CCK-8 from the dorsal horn of the rat: inhibition of DAGOL. *FEBS Lett.*, **250**, 215–217.

ROTHMAN, R.B., BYKOV, V., LONG, J.B., BRADY, L.S., JACOBSON, A.E., RICE, K.C. & HOLADAY, J.W. (1989). Chronic administration of morphine and naltrexone up-regulate μ-opioid binding sites labelled by [^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin: further evidence for two μ-binding sites. *Eur. J. Pharmacol.*, **160**, 71–82.

SANCHEZ-BLAZQUEZ, P. & GARZON, J. (1991). Cholera toxin and pertussis toxin on opioid-and α_2-mediated supraspinal analgesia in mice. *Life Sci.*, **48**, 1721–1727.

SEROOGY, K.B., MOHAPATRA, N.K., LUND, P.K., RETHELYI, M., MCGHEE, D.S. & PERL, E.R. (1990). Species-specific expression of cholecystokinin messenger RNA in rodent dorsal root ganglia. *Mol. Brain Research*, **7**, 171–176.

STANFA, L.C., SULLIVAN, A.F. & DICKENSON, A.H. (1992). Alterations in neuronal excitability and the potency of spinal mu, delta and kappa opioids after carrageenin induced inflammation. *Pain*, **50**, 345–354.

TANG, J., CHOU, J., IADAROLA, M., YANG, H.-Y. & COSTA, E. (1984). Proglumide prevents and curtails acute tolerance to morphine in rats. *Neuropharmacol.*, **23**, 715–718.

WIERTELAK, E.P., MAIER, S.F. & WATKINS, L.R. (1992). Cholecystokinin antianalgesia: safety cues abolish morphine analgesia. *Science*, **256**, 830–833.

WIESENFELD-HALLIN, Z. & DURANTI, R. (1987). Intrathecal cholecystokinin interacts with morphine but not substance P in modulating the nociceptive flexion reflex in the rat. *Peptides*, **8**, 153–158.

WIESENFELD-HALLIN, Z., XU, X.-J., HÅKANSON, R., FENG, D.-M. & FOLKERS, K. (1991). Low-dose morphine facilitates the spinal flexor reflex by releasing different neuropeptides in rats with intact and sectioned peripheral nerves. *Brain Res.*, **551**, 157–162.

WIESENFELD-HALLIN, Z., XU, X.-J., HUGHES, J., HORWELL, D.C. & HÖKFELT, T. (1990). PD134308, a selective antagonist of cholecystokinin type B receptor, enhances the analgesic effect of morphine and synergistically interacts with galanin to depress spinal nociceptive reflexes. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 7105–7109.

WILLETTS, J., URBAN, L., MURASE, K. & RANDIC, M. (1985). Actions of cholecystokinin octapeptide on rat spinal dorsal horn neurones. *Ann. N.Y. Acad. Sci.*, **448**, 385–402.

ZOUAOUI, D., BENOLIEL, J.J., CONRATH, M. & CESSELIN, F. (1990). Cholecystokinin-like immunoreactivity in the dorsal horn of the rat spinal cord: an attempt to analyse contradictory results between immunocytochemistry and radioimmunoassay. *Neuropeptides*, **17**, 177–185.

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Nitric oxide affects mammalian distal colonic smooth muscle by tonic neural inhibition

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1 The role of the L-arginine-nitric oxide (NO) pathway in tonic neural inhibition of spontaneous mechanical activity of distal colonic circular smooth muscle (DCCSM) was investigated in male Wistar rats.

2 Muscle strips were mounted in organ baths and spontaneous contractions recorded with isometric force transducers. They were characterized as low frequency (LFCs) 0.41 ± 0.03 N cm^{-2} or high frequency contractions (HFCs) 0.22 ± 0.04 N cm^{-2}. The latter occurred intermittently to produce summation contractions (SCs) range 0.5–12 N cm^{-2}.

3 Tetrodotoxin (100 nM) increased the forces of LFCs and SCs. Increase in force to tetrodotoxin did not occur after incubation of the muscle with N^G-monomethyl-L-arginine (L-NMMA) 500 μM, an inhibitor of NO biosynthesis.

4 L-NMMA but not its enantiomer D-NMMA increased the force of LFCs (EC_{50}:200 μM) and SCs (EC_{50}:175 μM) in a concentration-dependent manner which was reversed by L-arginine but not by D-arginine.

5 Muscle, precontracted by acetylcholine, relaxed to sodium nitroprusside (EC_{50}:1.8 μM) NO gas (EV_{50}:70 μl) and NO solutions (EC_{50}:4 μM) in a concentration-dependent manner. Guanosine 3′:5′-cyclic monophosphate tissue concentrations (pmol mg^{-1} protein) were elevated in muscle after relaxation by sodium nitroprusside (500 μM) from 0.32 ± 0.06 to 1.2 ± 0.37 and by 1 ml of NO gas from 0.49 ± 0.05 to 1.54 ± 0.14.

6 These data suggest that DCCSM is under tonic neural inhibition mediated by NO biosynthesis.

Keywords: Nitric oxide; neural inhibition; smooth muscle; colonic; guanosine 3′:5′-cyclic monophosphate (cyclic GMP)

Introduction

Spontaneous mechanical activity of colonic circular smooth muscle is under non-adrenergic non-cholinergic (NANC) tonic neural inhibition (TNI) (Crema *et al.*, 1968; Koch *et al.*, 1988), but the mediator(s) responsible for this is unknown (Wood, 1981). Following identification of nitric oxide (NO) as an endothelial derived relaxing factor (Palmer *et al.*, 1987), it has been proposed as a neurotransmitter in the gastrointestinal tract (Bult *et al.*, 1990) and brain (Knowles *et al.*, 1990). This is substantiated by the identification of NO synthase in the myenteric nerve plexus (Bredt *et al.*, 1990). Synthesis of NO from L-arginine (Palmer *et al.*, 1988) is inhibited by certain L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) (Rees *et al.*, 1989) and N^G-nitro-L-arginine (L-NOARG) (Moore *et al.*, 1990), but not their enantiomers, for example, D-NMMA. Inhibitory L-arginine analogues block certain NANC-mediated motor events such as muscle relaxation produced by electrical field stimulation of the canine ileocolonic region (Boeckxstaens *et al.*, 1990), inhibitory junction potentials in the canine proximal colon (Dalziel *et al.*, 1991), descending inhibition in the canine proximal colon (Hata *et al.*, 1990), adaptive relaxation of the stomach (Desai *et al.*, 1991), and NANC relaxation of the internal anal sphincter (Tottrup *et al.*, 1992; Burleigh, 1992). NO elevates intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels by increasing the activity of soluble guanylate cyclase (Knowles *et al.*, 1990). Cyclic GMP inhibits the release of calcium from intracellular stores and causes muscle relaxation (Nakatsu & Diamond, 1987). Here tonic neural inhibition of distal colonic circular muscle is investigated to examine if it is mediated by NO. Initial characterization of spontaneous mechanical activity allowed the effects of NO and inhibitors of its biosynthesis to be studied.

Methods

Full thickness strips of distal colonic tissue from adult non-fasted male Wistar rats (wt, 300–450 g) were mounted in organ baths (10 ml) (Soham Scientific, England) so that circular muscle bundles ran in the longitudinal axis and, perfused by oxygenated Krebs Henseleit (KH) solution (composition mM: NaCl 118, KCl 4.69, $MgSO_4$ 1.13, $CaCl_2$ 2.56, $NaHCO_3$ 25, KH_2PO_4 1.15, glucose 5.5). This solution had a pH of 7.4 when gassed with 95% oxygen and 5% carbon dioxide at 37°C, which was monitored with a PW 9418 Pye Unicam pH meter and Russell electrode and replaced if the pH varied beyond 7.2 or 7.6. Each muscle strip was attached to an isometric force transducer (Harvard apparatus, Kent, England) and a fixed point in the bath. The transverse orientation of longitudinal muscle prevented it from contributing to measured mechanical force. Tissues were allowed to stabilize for 2 h before they were stretched by 2 mm increments to the optimal length for contraction (Lo) (Merlo & Cohen, 1988). The force of muscle contraction was normalized for cross sectional area obtained by the following equation: XS = M/Lo × d (Cohen *et al.*, 1986). Where XS (cm^2) = cross sectional area of muscle, M(g) = mass of circular muscle layer (after removal of the mucosa and submucosa), Lo (cm) = optimal length for muscle contraction, d (g ml^{-1}) = density of colonic muscle in bath numerically equal to specific gravity of colonic muscle in KH solution (1.056) (Gordan & Siegman, 1971). Drugs were added in

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volumes no greater than 0.1 ml (1% of the organ bath volume). Control (distilled water) in volumes of this size were found to have no effect on the mechanical activity of these preparations.

Cyclic GMP measurement

Muscle strips were placed in 10 ml organ baths beside but free from strips that were attached to isometric force transducers. Sodium nitroprusside (SNP), NO gas or control (KH) were added to the organ bath medium. Unattached muscle strips were removed and immediately frozen in liquid nitrogen when the force of spontaneous contraction of the transducer attached strips began to fall. Cyclic GMP concentrations in these muscle strips were measured by radioimmunoassay as described.

Tissue preparation for cyclic GMP radioimmunoassay

Mucosa was removed from colonic strips which were freeze dried, weighed and homogenized before removal of protein with 6% trichloracetic acid to give a 10% homogenate. The supernatant obtained by centrifugation at 6000 *g* for 15 min at 2–8°C was washed with 5 vol of water-saturated ether, until the pH of the aqueous layer was approximately 6.7. The lower aqueous layers were taken to dryness under oxygen-free nitrogen at 60°C. The residue was redissolved in radioimmunoassay buffer and acetylated with concentrated triethanolamine:acetic acid anhydride, (2:1) and analysed for cyclic GMP content. The protein content of the tissue plug obtained from the initial centrifugation was determined by the method of Lowry *et al.* (1951).

Cyclic GMP radioimmunoassay

Radioimmunoassay was performed according to the protocol of the Amersham radioimmunoassay kit code RPA 525. Briefly, 100 µl of cyclic GMP antiserum was added to acetylated standards and unknowns and allowed to stand for 1 h at room temperature; 100 µl of [I^{125}]-cyclic GMP was added and allowed to stand for 20 h at 4°C, before the addition of 0.5 ml of a second antibody to bind excess [^{125}I]-cyclic GMP. This was centrifuged at 6000 *g* for 10 min and the radioactivity in the antibody-bound fraction measured with a gamma counter. Results are expressed as pmol cyclic GMP mg^{-1} protein.

Preparation of NO gas and solutions

Authentic NO gas (99.9% ARGO International Ltd., Essex, England) was passed through a glass column containing sodium hydroxide pellets as 'scrubbing' agents to remove higher oxides of nitrogen. NO solutions were prepared as described by Shikano *et al.* (1987). Briefly, 10 ml of distilled water was degassed by perfusion with oxygen-free nitrogen gas (BOC) for 3 h at 20°C then perfused with 500 ml of 'scrubbed' NO gas at 0.1% and vortexed for 20 min at 0–1°C to obtain NO-saturated stock solution. Dilutions of stock were prepared with degassed distilled water and added to organ baths in 0.1 ml volumes. A saturated solution of NO gas at 0°C contains between 1–1.5 mmol l^{-1} NO (Ignarro *et al.*, 1987). In the current experiments organ bath concentrations of NO were estimated assuming a stock solution concentration of 1 mM.

Samples of scrubbed NO gas were added to volumes of oxygen-free nitrogen (BOC) in gas-tight containers through self-sealing rubber caps via Hamilton gas-tight syringes; 1 ml samples of NO gas or dilutions were added to organ baths over 5 s. Samples (1 ml) of nitrogen gas (controls) had no effect on muscle.

Materials

The following drugs were obtained from Sigma Chemicals Ltd., Poole, England: atropine sulphate, propranolol hydrochloride, salbutamol, methysergide, phentolamine, hexamethonium bromide, phenylephrine hydrochloride, histamine diphosphate salt, chlorpheniramine maleate, tetrodotoxin, N^G-monomethyl-L-arginine, N^G-monomethyl-D-arginine, L-arginine and D-arginine, indomethacin, sodium nitroprusside, tetrodotoxin and methylene blue. Indomethacin was dissolved in 0.01 M Na_2CO_3 and further diluted with KH. Sodium nitroprusside was dissolved in 5% dextrose and diluted with KH immediately before use. Liquid nitrogen was obtained from B.O.C., England. All drugs were prepared daily and dissolved in distilled water where appropriate.

Statistics

Student's *t* test for paired data was used for analysis of data where appropriate. $P<0.05$ was taken as statistically significant. Data are given as means ± standard errors of means. Two way analysis of variance was used to test the difference between concentration-response curves where indicated.

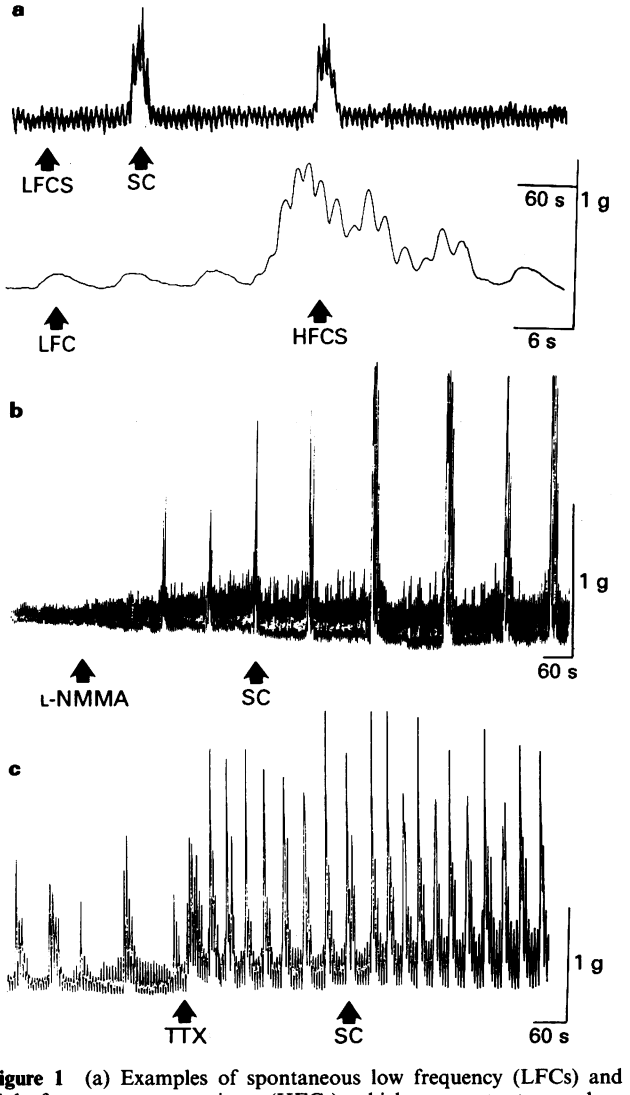


Figure 1 (a) Examples of spontaneous low frequency (LFCs) and high frequency contractions (HFCs) which summate to produce summation contractions (SCs). (b) Typical response of circular muscle to N^G-monomethyl-L-arginine (L-NMMA) 100 µM. (c) Typical response to tetrodotoxin (TTX) 100 nM.

Results

Spontaneous contractions of muscle were characterized by three types of mechanical activity. Regular phasic contractions termed low frequency contractions (LFCs) 0.41 ± 0.03 N cm^{-2} ($n = 52$), higher frequency contractions of lower amplitude termed high frequency contractions (HFCs) 0.22 ± 0.04 N cm^{-2} ($n = 30$) and contractions of larger amplitude and duration formed by summation of these, termed summation contractions (SCs) range 0.5–12 N cm^{-2} ($n = 45$). SCs were variable in force and frequency and often absent. LFCs were always present and showed little variation (Figure 1a).

Blockade of α-(phentolamine, 10 μM) and β-(propranolol, 10 μM) adrenoceptors, muscarinic (atropine 10 μM), nicotinic (pentolinium, 10 μM). 5-hydroxytryptamine (5-HT) (methysergide, 10 μM) and histamine, H_1 (chlopheniramine, 10 μM)-receptors did not significantly alter spontaneous muscle activity. The forces of LFCs and SCs were increased by tetrodotoxin, (TTX) 100 nM, and further elevated by L-NMMA (500 μM, $P<0.05$). However, TTX had no significant effects after L-NMMA (500 μM) (Figure 2). Muscle contractions induced by 5-HT 10 μM (approximate EC_{50}) (1.0 ± 0.07 N cm^{-2}) were attenuated by tetrodotoxin (TTX) 100 nM (0.1 ± 0.1, $P<0.01$) ($n = 5$) but not significantly altered by L-NMMA 500 μM (1.5 ± 0.15 to 1.2 ± 0.2) ($n = 7$).

Effect of L-NMMA

L-NMMA produced concentration-dependent increases in the forces of LFCs and SCs (EC_{50} 200 μM and 175 μM respectively) and frequency of SCs (EC_{50} 20 μM) (Figure 3), whereas D-NMMA (500 μM) had no significant effect (Figures 4 and 5). L-Arginine inhibited the effect of L-NMMA in a concentration-dependent manner in contrast to D-arginine which at high concentrations increased the forces of LFCs (Figures 4 and 5). L-Arginine (6 mM) had no significant effects on the force of SCs ($8.14 \pm 4\%$ relaxation, $n = 7$) or LFCs ($0.6 \pm 2\%$ relaxation, $n = 6$), L-arginine (10 mM) relaxed SCs ($25 \pm 6\%$ relaxation, $n = 7$, $P<0.05$) compared to control (equal volume of distilled water) but not LFCs ($7 \pm 8\%$ relaxation, $n = 7$). D-Arginine (1 mM) did not affect the forces of LFCs ($4.8 \pm 5\%$ relaxation, $n = 6$) or SCs ($7.3 \pm 4\%$ relaxation, $n = 6$). L-Canavanine 500 μM, an inhibitor of the inducible NO synthase of macrophages (Iyengar *et al.*, 1987) had no effect on spontaneous contractions ($n = 4$, not

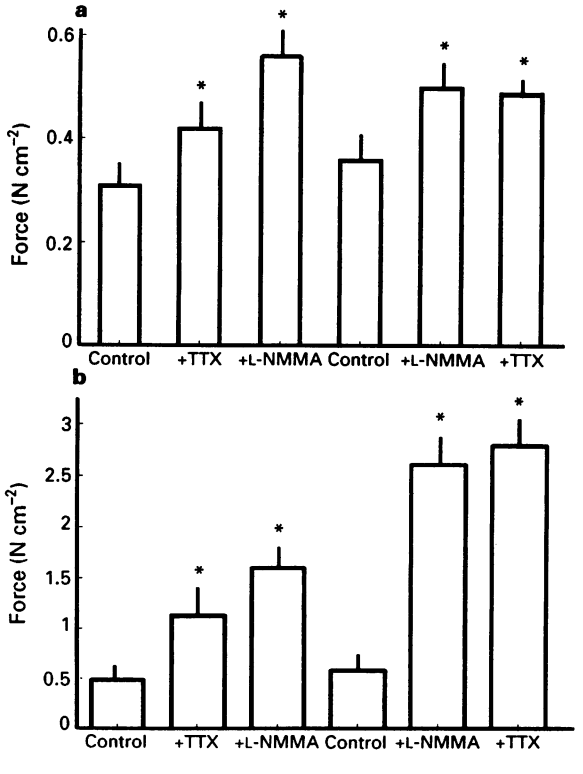


Figure 2 Effect of tetrodotoxin (TTX) 100 nM on spontaneous (a) low frequency (LFC) and (b) summation contractions (SCs) before and after N^G-monomethyl-L-arginine (L-NMMA) 500 μM. Means and standard errors for 9–10 values are given.
Significant differences from control (distilled water) values were tested with Student's *t* test for paired data and are denoted by asterisks. $*P<0.05$.

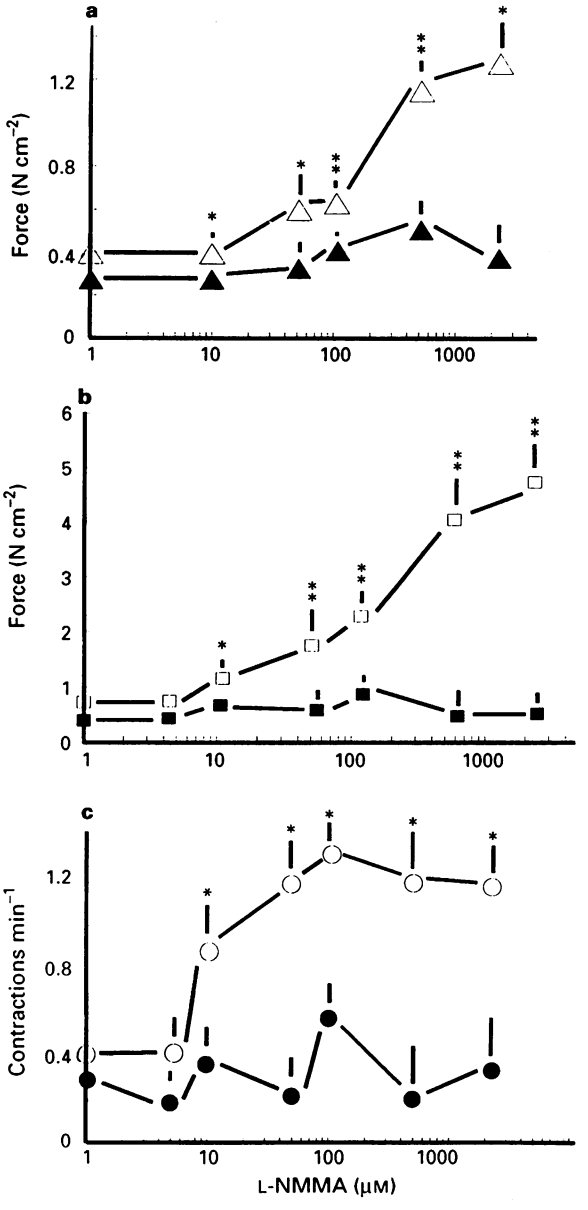


Figure 3 Concentration-response curves to N^G-monomethyl-L-arginine (L-NMMA). The effects of L-NMMA (open symbols) on muscle strip contraction force and frequency was compared to those of control (closed symbols). Because of the variability between tissues from different animals, control values are also given. (a) Forces of low frequency contractions. (b) Forces of summation contractions (SCs) and (c) frequencies of SCs. Means for 10–25 values are shown with standard errors.
Significant differences from the control values (equal volumes of distilled water) at each L-NMMA concentration were tested by Student's *t* test for paired data and are denoted by $*P<0.05$; $**P<0.01$.

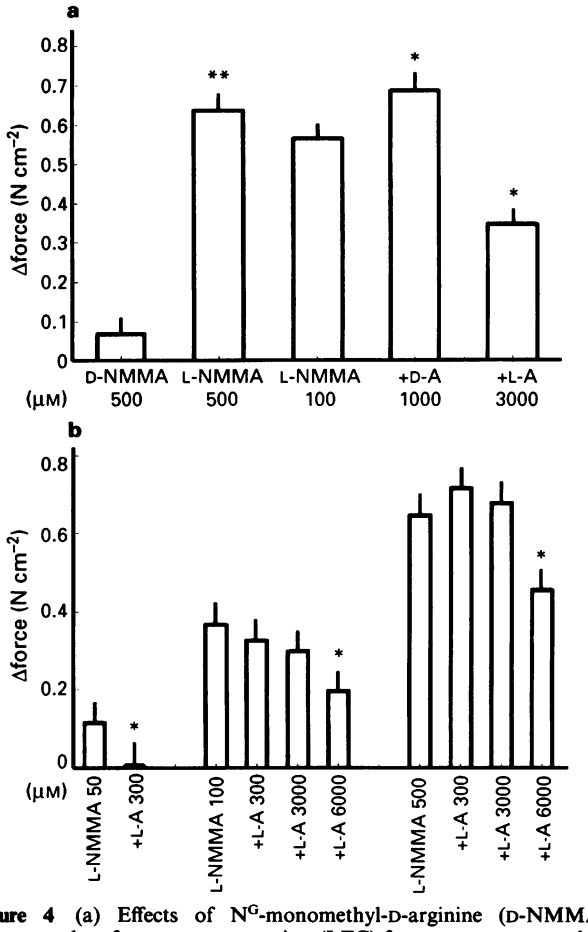


Figure 4 (a) Effects of N^G-monomethyl-D-arginine (D-NMMA) 500 μM on low frequency contraction (LFC) forces were compared to those of N^G-monomethyl-L-arginine (L-NMMA) 500 μM. LFCs increased to L-NMMA 100 μM upon which the effects of D-arginine (D-A, 1000 μM) were compared to L-arginine (L-A, 3000 μM). L-NMMA (500 μM) and (100 μM) increased LFC forces compared to control (equal volumes of distilled water) ($P<0.01$). (b) Concentration-dependent inhibitory effects of L-arginine (L-A) on LFCs after stimulation by L-NMMA. LFC forces increased to each concentration of L-NMMA compared to control (equal volumes of distilled water) ($P<0.01$). All data are means and standard errors of 10–15 experiments. For both graphs significant differences from LFC forces after L-NMMA alone were tested by Student's t test for paired data and are denoted by asterisks: $*P<0.05$; $**P<0.01$.

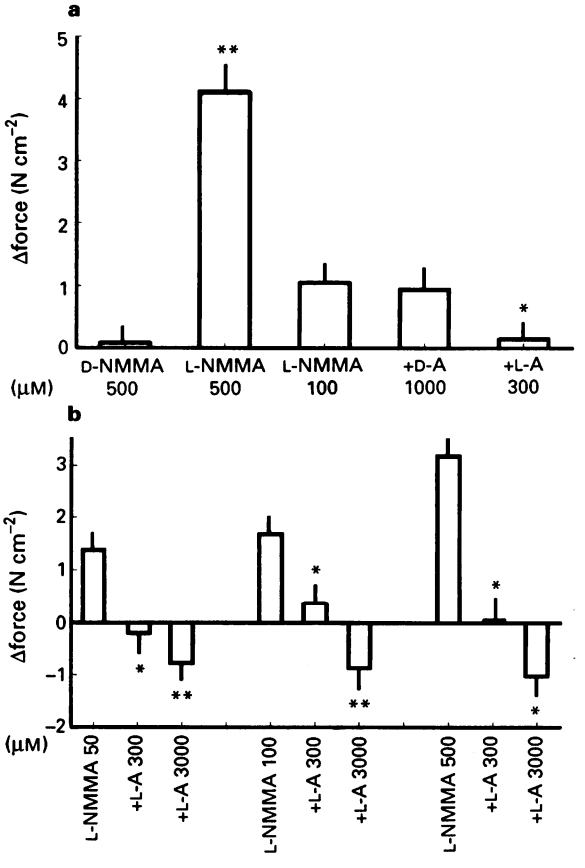


Figure 5 (a) Effects of N^G-monomethyl-D-arginine (D-NMMA) 500 μM on summation contraction (SC) forces were compared to those of N^G-monomethyl-L-arginine (L-NMMA) 500 μM. Muscle strips were treated with L-NMMA 100 μM and the effects of D-arginine (D-A) 1000 μM on SC forces compared to L-arginine (L-A) (300 μM). L-NMMA (500 μM) and (100 μM) increased SC forces compared to control (equal volumes of distilled water) ($P<0.01$). (b) Inhibitory effect of L-A on SC force after simulation by L-NMMA. SC forces increased to each concentration of L-NMMA compared to control (equal volumes of distilled water) ($P<0.01$). Means and standard errors for 10–15 values are given. Significant differences from L-NMMA alone are denoted by asterisks: $*P<0.05$ and $**P<0.01$.

shown). The frequency of LFCs (10 ± 0.1 contractions min^{-1}) was not significantly altered by L-NMMA or L-arginine ($n = 25$) (not shown).

Effect of nitric oxide on mechanical activity and tissue cyclic GMP concentration

Muscle strips contracted to acetylcholine (ACh 100 μM, approximately EC_{50}) and relaxed to NO solutions (EC_{50}:4 μM), NO gas (EV_{50}:70 μl) and SNP (EC_{50}:1.8 μM) in a dose-dependent manner (Figures 6 and 7). The effect of the latter was inhibited by methylene blue (10 μM) (Figure 6). TTX 100 nM did not affect relaxations by SNP 2.0 μM ($n = 10$) or 1 ml NO gas ($n = 5$) (not shown).

Tissue cyclic GMP concentrations (pmol mg^{-1} protein) increased to SNP (500 μM) from 0.32 ± 0.6 to 1.2 ± 0.37 ($n = 11$, $P<0.05$) and to 1 ml pure NO gas from 0.49 ± 0.5 to 1.54 ± 0.14 ($n = 6$, $P<0.01$).

Discussion

We have identified low and high frequency spontaneous mechanical contractions (LFCs and HFCs respectively) of distal colonic circular smooth muscle (DCCSM) of the rat. HFCs are intermittent and produce summation contractions (SCs). Similar low frequency and summation contractions of circular muscle from the human sigmoid colon under NANC regulation have been reported by others, but the inhibitory mediator could not be identified (Koch *et al.*, 1988). Smith *et al.* (1987) observed low frequency electrical pacemaker activity arising from the circular smooth muscle layer and high frequency activity from the longitudinal layer. Simultaneous recording of electrical and mechanical events demonstrated that mechanical low frequency contractions coincide with electrical slow wave activity (Koch *et al.*, 1988) which may arise from the interstitial cells of Cajal (Huizinga *et al.*, 1990). Such electrical pacemaker systems may initiate the mechanical activity observed in our experiments.

Spontaneous mechanical activity was not affected by propranolol, atropine, phentolamine or pentolinium, but the removal of neural activity by TTX increased the force of LFCs and SCs. When SCs were absent, they invariably appeared in the presence of TTX, and when present their force and frequency were increased. This suggests mechanical activity is under tonic neural inhibition (TNI) and agrees with the findings of others (Crema *et al.*, 1968; Koch *et al.*, 1988). Investigations of neurotransmitters possibly respon-

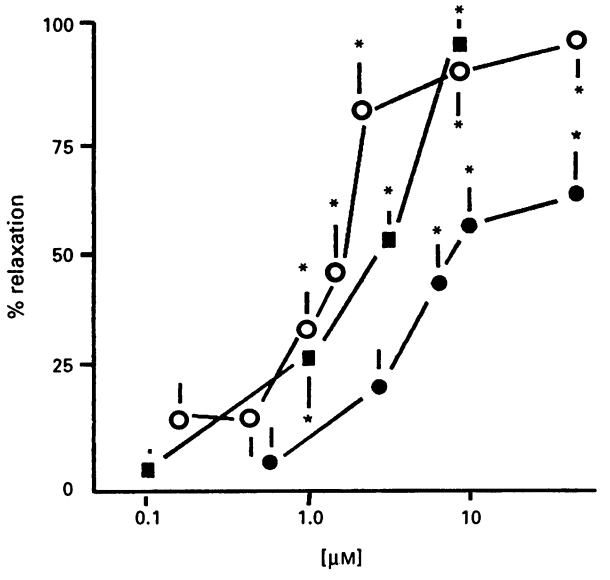


Figure 6 Colonic circular muscle contracted by acetylcholine (ACh) 100 μM relaxed to sodium nitroprusside (SNP) (○) in a concentration-dependent manner. Preincubation of muscle with methylene blue 10 μM (●) reduced relaxation of muscle to SNP and shifted the concentration-response curve to the right in the manner of a non-competitive inhibitor. Data are mean percentage relaxations and standard errors of 8–10 experiments.

Statistical differences from control values (equal volumes of distilled water) were tested by Student's *t* test for paired data and are indicated by asterisks: $*P<0.05$. Curves are statistically different by two way analysis of variance ($F=7.9$, $P<0.05$). Precontracted muscle strips relaxed to NO solutions in a concentration dependent manner (■). Data are mean percentage relaxations and standard errors of 17–25 experiments. Statistical differences from controls (equal volumes of distilled water) were tested by Student's *t* test for paired data and are indicated by asterisks. $*P<0.01$.

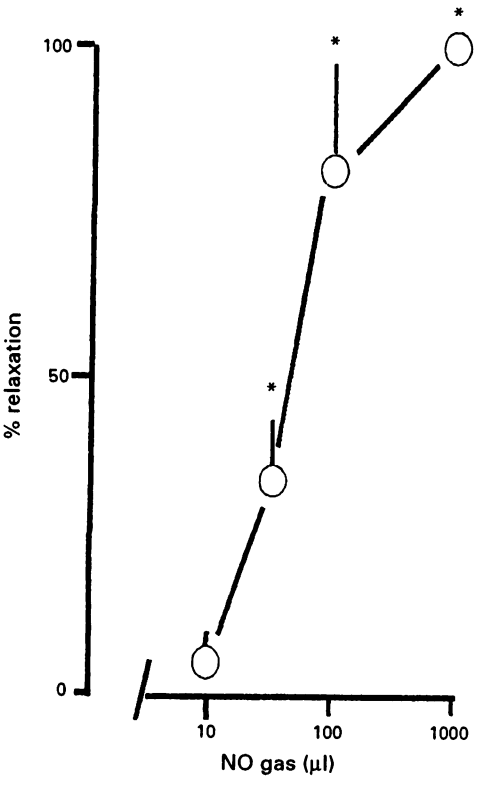


Figure 7 Colonic circular muscle contracted by acetylcholine (ACh) 100 μM relaxed to NO gas in a volume-dependent manner. Volumes of NO gas were made up to 1 ml with oxygen-free nitrogen gas before addition to organ baths. Data are mean percentage relaxations and standard errors of 7–15 experiments. Comparison with the effect of controls (equal volumes of nitrogen gas) were made by Student's *t* test for paired data.
Significant differences: * $P<0.01$.

sible for NANC inhibition in the gut, such as vasoactive intestinal peptide (Goyal & Rattan, 1980; Daniel *et al.*, 1983) and adenosine-5'-triphosphate (Burnstock *et al.*, 1970; Westfall *et al.*, 1982) have been inconclusive.

L-NMMA increased the forces of LFCs and SCs after which TTX had no effect, suggesting that TNI is mediated by NO biosynthesis. An alternative explanation could be that L-NMMA itself has a neurotoxic effect. However, contraction of muscle to 5-HT is mediated by neural elements (Wiley & Owyan, 1987) and was absent in the presence of TTX but not affected by L-NMMA. L-NMMA produced a further small increase in mechanical activity after TTX suggesting additional extraneural NO biosynthesis. SCs were often absent but were consistently stimulated by low concentrations of L-NMMA, whereas LFCs were always present, upon with L-NMMA had a smaller effect. This suggests that NO biosynthesis has greater inhibitory influence over SCs that LFCs. The reason for this is unclear. Perhaps electrical activity responsible for HFCs is transmitted from longitudinal muscle to circular muscle and is more sensitive to blockade by NO biosynthesis near the myenteric plexus which separates these muscle layers, than circular muscle is to NO-mediated tonic neural inhibition from separate inhibitory neural elements, deep in the circular muscle layer.

The stimulatory effect of L-NMMA is likely to be a consequence of stereospecific competitive inhibition of NO biosynthesis as D-NMMA was inactive and L-arginine, but not D-arginine, inhibited its effect on the forces of SCs and LFCs in a concentration-dependent manner. Complete reversal of the effects of L-NMMA on SCs occurred with a 2–6 fold molar excess of L-arginine. Much greater concentrations of L-arginine are required to reverse the effects of L-NMMA on LFCs suggesting that the effect of L-NMMA may not be entirely due to NO synthase inhibition.

L-arginine had no significant effect on LFCs and inhibited the effects of L-NMMA on SCs at much lower concentrations than those required to inhibit SCs of untreated muscle, indicating that the former was an effect on NO biosynthesis. TTX did not inhibit the effects of high concentrations of L-arginine on SCs which were consequently unlikely to be mediated by neural elements. Macrophages were unlikely to be an important source of NO as L-canavanine, a specific inhibitor of macrophage NO synthase, had no effect on spontaneous muscle contractions.

It is possible that NO is an intracellular second messenger for an unidentified inhibitory neurotransmitter, rather than providing intercellular communication. Evidence for this has been provided by Boeckxstaens *et al.* (1991) who demonstrated that L-NMMA and L-NOARG inhibited adenosine-5'-triphosphate and γ-aminobutyric acid relaxation of noradrenaline contracted canine ileocolonic junction and ileal circular smooth muscle. Alternatively, if NO is released by neural elements as a myomodulator, it might diffuse bound to a carrier molecule such as cysteine affording it stability, or thiol containing proteins such as tissue-type plasminogen activator or albumen (Ignarro, 1990; McCall & Vallance, 1992).

The inhibitory nature of NO and SNP help substantiate a role for NO as an inhibitory myomodulator, although its site(s) of biosynthesis remains unclear. Muscle relaxation to NO was associated with an increase in cyclic GMP concentration, and was inhibited by the soluble guanylate cyclase inhibitor, methylene blue. This suggests that cyclic GMP is the second messenger of NO in DCCSM, as is the case in other tissues (Katsuki *et al.*, 1977; Schultz *et al.*, 1977).

In conclusion, we provide evidence that NO biosynthesis

mediates NANC tonic neural inhibition of spontaneous mechanical activity in DCCSM. This finding has implications for investigation of motility disorders of the colon, particularly enteric neuropathies, such as Hirschsprung's disease and may facilitate the development of new therapeutic agents for functional bowel disorders.

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References

BOECKXSTAENS, G.E., PELKMANS, 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 canine ileocolonic junction. *Eur. J. Pharmacol.*, **190**, 239–246.

BOECKXSTAENS, G.E., PELKMANS, P.A., BULT, H., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991). Evidence for nitric oxide as mediator of non-adrenergic, non-cholinergic relaxations induced by ATP and GABA in the canine gut. *Br. J. Pharmacol.*, **102**, 434–438.

BREDT, D., HWANG, P. & SNYDER, S. (1990). Localization of nitric oxide synthase indicating a role for nitric oxide. *Nature*, **347**, 768–770.

BULT, H., BOECKXSTAENS, G.E., PELKMANS, 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.

BURLEIGH, D. (1992). N^G-nitro-L-arginine reduces nonadrenergic, noncholinergic relaxations of human gut. *Gastroenterology*, **102**, 679–683.

BURNSTOCK, G., CAMPBELL, G., SATCHELL, D. & SMYTHE, A. (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br. J. Pharmacol.*, **40**, 668–688.

COHEN, J.D., KAO, H.W., TANI, S.T., LECHAGO, J. & SNAPE, W.J. Jr. (1986). Effect of acute experimental colitis on rabbit colonic smooth muscle. *Am. J. Physiol.*, **251**, G538–G545.

CREMA, A., DEL TACCA, M., FRIGO, G.M. & LECCHINI, S. (1968). Presence of a non-adrenergic inhibitory system in the human colon. *Gut*, **9**, 633–637.

DALZIEL, H., THORNBURY, K., WARD, S. & SANDERS, K. (1991). Involvement of nitric oxide synthetic pathway in inhibitory junction potentials in canine proximal colon. *Am. J. Physiol.*, **260**, G789–G792.

DANIEL, E.E., HELMY-ELKHOLY, A., JAGER, L.P. & KANNAN, M.S. (1983). Neither a purine nor VIP is the mediator of inhibitory nerves of opossum oesophageal smooth muscle. *J. Physiol.*, **336**, 243–260.

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**, 447–479.

GORDAN, A.R. & SIEGMAN, M.S. (1971). Mechanical properties of smooth muscle. Length-tension and force-velocity relations. *Am. J. Physiol.*, **221**, 1243–1249.

GOYAL, R.K. & RATTAN, S. (1980). VIP as a possible neurotransmitter of non-cholinergic, non-adrenergic inhibitory neurones. *Nature*, **288**, 378–380.

HATA, F., ISHII, T., KANADA, A., YAMANOI, N., KATAOKA, T., TAKEUCHI, T. & YAGASAKI, O. (1990). Essential role of nitric oxide in descending inhibition in the rat proximal colon. *Biochem. Biophys. Res. Commun.*, **172**, 1400–1406.

HUIZINGA, J.D., BEREZIN, I., DANIE, E.E. & CHOW, E. (1990). Inhibitory innervation of colonic smooth muscle cells and interstitial cells of Cajal. *Can. J. Pharmacol.*, **69**, 447–454.

IGNARRO, L.J. (1990). Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension*, **16**, 5, 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 pharmacological and chemical properties identical to those of nitric oxide radical. *Circ.*, **61**, 866–879.

IYENGAR, R., STUEHR, D.J. & MARLETTA, M.A. (1987). Macrophage synthesis or nitrite, nitrate and N-nitrosamines: precursors and role of respiratory burst. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 6369–6377.

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. Cycl. Nucleotide Res.*, **3**, 239–247.

KNOWLES, R.G., PALACIOS, M., PALMER, R.M.J. & MONCADA, S. (1990). Kinetic characteristics of nitric oxide synthase from rat brain. *J. Biochem.*, **269**, 207–210.

KOCH, T.R., CARNEY, J.A., GO, V.L.V. & SZURSZEWSKI, J.H. (1988). Spontaneous contractions and some electrophysiological properties of circular muscle from normal sigmoid colon and ulcerative colitis. *Gastroenterology*, **95**, 77–84.

LOWRY, O.H., ROSEBROUGH, J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurements with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MCCALL, T. & VALLANCE, P. (1992). Nitric oxide takes centre-stage with newly defined roles. *Trends Pharmacol. Sci.*, **13**, 1–6.

MERLO, A. & COHEN, S. (1988). Neuropeptide responses and mechanics of the proximal and distal feline colon in vitro. *Am. J. Physiol.*, **255**, G787–G793.

MOORE, P.K., AL-SWAYEH, O.H., CHONG, N.W.S., 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.

NAKATSU, K. & DIAMOND, J. (1987). Role of cGMP in relaxation of vascular and other smooth muscle. *Can. J. Physiol. Pharmacol.*, **67**, 251–262.

PALMER, R.M.J., FERRIDGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524.

PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

REES, D.D., PALMER, R.M.J., HODSON, H.F. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

SCHULTZ, K., SCHULTZ, K.D. & SCHULTZ, G. (1977). Sodium nitroprusside and other smooth muscle relaxants increase cyclic GMP levels in rat ductus deferens. *Nature*, **265**, 750–751.

SHIKANO, K., OHLSTEIN, E.H. & BERKOWITZ, B.A. (1987). Differential selectivity of endothelium-derived relaxing factor and nitric oxide in smooth muscle. *Br. J. Pharmacol.*, **92**, 483–485.

SMITH, T., READ, J. & SANDERS, K. (1987). Interaction of two electrical pacemakers in muscularis of canine proximal colon. *Am. J. Physiol.*, **252**, C290–C299.

TOTTRUP, A., BIRGITTE, G. & SVANE, D. (1992). Involvement of the L-arginine–nitric oxide pathway in internal anal sphincter relaxation. *Gastroenterology*, **102**, 409–415.

WESTFALL, D.P., HOGABOOM, G.K., COLBY, J., O'DONNELL, J.P. & FEDAN, J.S. (1982). Direct evidence against a role of ATP as the non-adrenergic, non-cholinergic inhibitory neurotransmitter in guinea pig taenia coli. *Proc. Nat. Acad. Science.*, **79**, 7041–7045.

WILEY, J. & OWYAN, C. (1987). Participation of serotonin and substance P in the action of cholecystokinin on colonic motility. *Am. J. Physiol.*, **252**, G431–G435.

WOOD, J.W. (1981). Physiology of the enteric nervous system. In *Physiology of the Gastrointestinal Tract*. ed. Johnson, L.R. pp. 1–37. New York: Raven Press.

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Quinapril treatment and arterial smooth muscle responses in spontaneously hypertensive rats

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1 The effects of long-term angiotensin-converting enzyme inhibition with quinapril on arterial function were studied in spontaneously hypertensive rats, Wistar-Kyoto rats serving as normotensive controls.

2 Adult hypertensive animals were treated with quinapril (10 mg kg^{-1} day^{-1}) for 15 weeks, which reduced their blood pressure and the concentrations of atrial natriuretic peptide in plasma and ventricular tissue to a level comparable with that in normotensive rats.

3 Responses of mesenteric arterial rings *in vitro* were examined at the end of the study. Compared with normotensive and untreated hypertensive rats, responses to noradrenaline were attenuated in hypertensive animals on quinapril, both force of contraction and sensitivity being reduced. Quinapril also attenuated maximal contractions but not sensitivity to potassium chloride. Nifedipine less effectively inhibited vascular contractions in normotensive and quinapril-treated than in untreated hypertensive rats.

4 Arterial relaxation responses by endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitrite, isoprenaline) mechanisms were similar in normotensive and quinapril-treated rats and more pronounced than in untreated hypertensive rats.

5 Cell membrane permeability to ions was evaluated by means of potassium-free solution-induced contractions of endothelium-denuded denervated arterial rings. These responses were comparable in normotensive and quinapril-treated rats and less marked than in untreated hypertensive rats.

6 Intracellular free calcium concentrations in platelets and lymphocytes, measured by the fluorescent indicator quin-2, were similar in normotensive and quinapril-treated rats and lower than in untreated hypertensive rats.

7 In conclusion, quinapril treatment improved relaxation responses and attenuated contractions in arterial smooth muscle of hypertensive rats. These changes may be explained by diminished cytosolic free calcium concentration, reduced cell membrane permeability, and alterations in dihydropyridine-sensitive calcium channels following long-term angiotensin-converting enzyme inhibition.

Keywords: Angiotensin-converting enzyme inhibition; atrial natriuretic peptide; arterial smooth muscle; blood pressure; quinapril; spontaneously hypertensive rats

Introduction

The principal mechanism of blood pressure reduction by angiotensin-converting enzyme (ACE) inhibitors is a fall in peripheral arterial resistance resulting from decreased angiotensin II formation (Frohlich, 1989). However, during chronic therapy, in particular, some discrepancy remains between the antihypertensive effect of ACE inhibitors and the suppression of systemic angiotensin II levels (Waeber *et al.*, 1989). Hence other mechanisms of blood pressure reduction may be operative during long-term ACE inhibition, namely decreased degradation of kinins, enhanced formation of vasodilator prostaglandins (Schrör, 1990), sympatho-inhibitory action, and inhibition of angiotensin II formation at tissue level (Frohlich, 1989).

During the past few years the vascular endothelium has been discovered to be an important regulator of both normal and pathological blood vessel tone (for a review, see Vane *et al.*, 1990 and Moncada *et al.*, 1991). ACE is largely located on the luminal surface of the arterial endothelium (Caldwell *et al.*, 1976). Moreover, the vascular wall appears to have a renin-angiotensin system of its own which ACE inhibitors directly inhibit (Levy *et al.*, 1990), even more effectively than ACE circulating in the blood stream (Baudin & Drouet, 1989). A short four day-long ACE inhibitor treatment has been shown to augment endothelium-dependent but not to affect endothelium-independent vascular relaxation in spontaneously hypertensive rats (SHR) (Clozel *et al.*, 1990). In addition, ACE inhibitors have been reported to increase the production of endothelium-derived relaxing factor(s) *in vitro* (Mombouli *et al.*, 1991). Wiemer *et al.* (1991) have recently shown that ramiprilat prevents the degradation of bradykinin liberated from the endothelial cells, which in turn stimulates the release of relaxing factors from the endothelium. This mechanism may partly explain the endothelium-mediated effects of ACE inhibitors.

Chronic ACE inhibition has been found to prevent effectively cardiac hypertrophy and to attenuate vascular responses to noradrenaline (NA) in SHR and Milan hypertensive rats (Lee *et al.*, 1991; Mulvany *et al.*, 1991), and to reduce pressor responses to infused NA also in man (Malini *et al.*, 1990). In addition it has been suggested that long-term ACE inhibition reduces arterial tone in SHR by reducing intracellular free calcium concentration ($[Ca^{2+}]_i$) in vascular smooth muscle (Sada *et al.*, 1990). Even brief 4-week ACE inhibitor therapy during the development of hypertension can induce a long-term blood pressure reduction in SHR, with associated permanent changes in vascular resistance properties. In contrast, when ACE inhibitor treatment is introduced in the established phase of hypertension, the vascular changes

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attained are transient and cardiovascular hypertrophy and enhanced resistance responses redevelop if therapy is withdrawn (Adams *et al.*, 1990; Harrap *et al.*, 1990).

In the present study, we elucidated the effects of long-term treatment with quinapril, an orally active non-sulphydryl compound (Kaplan *et al.*, 1984) inducing prolonged inhibition of ACE especially in vascular, heart and kidney tissues (Fabris *et al.*, 1990), on arterial responses in SHR. Quinapril therapy was started in the established phase of hypertension to examine if blood pressure normalization can reverse the abnormalities of arterial function found in this type of genetic hypertension. We investigated whether the altered responses to NA following ACE inhibition (Lee *et al.*, 1991; Mulvany *et al.*, 1991) are associated with changes in vascular calcium sensitivity or in sensitivity to other contractile agents. Moreover, the effects of long-term quinapril therapy on both endothelium-dependent and endothelium-independent vascular relaxation mechanisms were studied. Since the arterial smooth muscle contractile response induced by a K^+-free solution is known to differentiate clearly normotensive and hypertensive blood vessels (Arvola *et al.*, 1992), and is also an indirect method for the assessment of vascular permeability, this response was included in the study protocol. The efficacy of the quinapril treatment was evaluated by atrial natriuretic peptide (ANP) determinations from plasma and ventricular tissue, the concentrations of which are known to correlate positively with cardiac hypertrophy (Kinnunen *et al.*, 1990). Furthermore, platelets and lymphocytes were used as cell models to study the associated changes in $[Ca^{2+}]_i$.

Methods

Animals and experimental design

Male SHR of the Okamoto-Aoki strain and age-matched Wistar-Kyoto (WKY) rats were obtained from Møllegaard's Breeding Centre, Ejby, Denmark. The rats were housed 4–5 animals to a cage in a standard experimental animal laboratory (illuminated from 06 h 00 min to 18 h 00 min, room temperature + 22°C), and had free access to drinking fluid (tap water) and food pellets (Ewos, Södertälje, Sweden). At 8 weeks of age the SHR were divided into two groups of equal mean systolic blood pressures, which were measured from unanaesthetized rats by the tail cuff method at + 28°C (Model 129 Blood Pressure Meter; IITC Inc., Woodland Hills, Ca., U.S.A.). Beginning at the age of 15 weeks, SHR ($n = 10$) were given quinapril 0.12 mg ml^{-1} of drinking fluid (the average daily dose thus being 10 mg kg^{-1} body weight), while untreated SHR ($n = 12$) and normotensive WKY rats ($n = 10$) were kept on normal drinking fluid. Daily chow consumption was measured by weighing the pellets. Weekly indirect blood pressure measurements and the quinapril therapy continued for 15 more weeks until the animals were 30 weeks old. Thereafter, quinapril administration was withdrawn 1 day before the rats were decapitated, exsanguinated and blood samples drawn into polystyrene tubes containing heparin (100 units ml^{-1}) as anticoagulant for $[Ca^{2+}]_i$ determinations, and chilled tubes on ice containing EDTA (2.7 mM) as anticoagulant for plasma ANP assays. The hearts were removed and weighed and the superior mesenteric arteries carefully excised and cleaned of adherent connective tissue. The experimental design of the study was approved by the Animal Experimentation Committee of the University of Tampere, Finland.

Mesenteric arterial responses in vitro

Four successive 3-mm-long standard sections of the mesenteric artery were cut, beginning 0.5 cm distally from the mesenteric artery-aorta junction. The rings were placed between stainless steel hooks and mounted in an organ bath chamber in physiological salt solution (PSS) (pH 7.4) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, and aerated with 95% O_2 and 5% CO_2. The rings were equilibrated for 1 h at + 37°C with a resting tension of 1.5 g. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph (FT03 transducer, Model 7 E Polygraph; Grass Instrument Co., Quincy, Ma., U.S.A.). The presence of intact endothelium in vascular preparations was confirmed by observing an almost complete relaxation response to 1 μM acetylcholine (ACh) in 1 μM NA-precontracted rings.

Vascular preparation 1: After a 30 min stabilization period, concentration-response curves for NA and 30 min later for potassium chloride (KCl), were cumulatively determined from the most proximal section of each mesenteric artery. In solutions containing high concentrations of potassium (20–125 mM), NaCl was replaced with KCl on an equimolar basis.

Vascular preparation 2: The next vascular ring was used to study the effect of organ bath calcium concentration on NA-induced contractions, i.e. the calcium-sensitivity of the rings during α-adrenoceptor agonist stimulation. A modification of the method described by Mulvany & Nyborg (1980) was applied. Briefly, after 30 min of stabilization the calcium stores of vascular rings were depleted by bathing them in a Ca^{2+}-free PSS for 40 min and by twice contracting them maximally with 10 μM NA during this period. Thereafter 10 μM NA-induced contractions were repeatedly elicited in increasing organ bath calcium concentrations (0.01–2.5 mM). After each contraction had developed fully, NA was rinsed from the baths with PSS containing the next concentration of calcium to be studied, the following contraction to 10 μM NA being induced in 2 min. When this procedure was completed, the same scheme of repeated 10 μM NA-induced contractions in increasing organ bath calcium concentrations (0.01–2.5 mM) was elicited in the presence of 0.5 nM nifedipine.

Vascular preparation 3: Vascular relaxation responses to ACh, isoprenaline and sodium nitrite ($NaNO_2$) were examined in the third section of each mesenteric artery. The rings were precontracted with 1 μM NA, and after the contraction had developed fully increasing concentrations of relaxing agent were added cumulatively to the organ bath. The next concentration of relaxing compound was added only after the previous level of relaxation was stable. The rings were allowed a 20-min recovery period in resting tension between the study of each relaxant.

Vascular preparation 4: Vascular endothelium was denuded from the most distal section of each mesenteric artery by gently rubbing with a jagged injection needle (Arvola *et al.*, 1992). The absence of endothelium was confirmed with a complete lack of relaxation to 1 μM ACh in 1 μM NA-precontracted vascular rings. Thereafter chemical sympathectomy was performed by the method described by Aprigliano & Hermsmeyer (1976): the rings were denervated *in vitro* by exposing them to a buffer-free solution containing 1.2 mM 6-hydroxydopamine, and by vigorous gassing with nitrogen for 15 min, followed by a 2-h recovery period in normal PSS. After this maximal 125 mM KCl-induced contractions (serving as reference responses) were elicited, and after another 30 min the rings were exposed to K^+-free buffer solution (pH 7.4), which was prepared by substituting KH_2PO_4 and KCl of normal PSS with NaH_2PO_4 and NaCl, respectively, on an equimolar basis. The omission of potassium induced gradual contractions in all vascular rings. After the contraction had reached a plateau, 1 mM potassium was re-added and the subsequent relaxation registered.

After registration of responses all preparations were dried

overnight at + 100°C and weighed. Vascular contractile responses were related to tissue dry weight. NA-induced contractions in different organ bath calcium concentrations were normalized by relating them to the maximal contractile response in 2.5 mM calcium in each arterial ring. The effect of nifedipine on NA-induced contractions in different organ bath calcium concentrations was calculated by relating the response in the presence of nifedipine to that without it. The relaxations were presented as percentage of pre-existing contractile force. In each ring EC_{50} was calculated from plots of the concentration of contractile agent versus the percentage of maximal response, or from plots of the concentration of relaxing compound versus the percentage of 1 μM NA-induced precontraction. The mean relaxation rate after maximal responses to 125 mM KCl was calculated from force of contraction and the time required to return to resting tension after rinsing the vascular rings with normal PSS. The contractions induced by K^+-free medium and the relaxation responses following the re-addition of potassium were normalized by relating them to the previously determined maximal KCl-induced (125 mM) contraction or tissue dry weight in each ring. After potassium repletion, the greatest reduction in smooth muscle contractile force during a 1 min period was considered the maximal relaxation rate.

Morphological studies

From six animals in each study group, vascular rings were prepared for light microscopy from the most proximal part of the remaining section of each mesenteric artery. The rings were fixed in 2% glutaraldehyde at + 4°C and postfixed in 2% osmiumtetroxide. After washing, they were stained with 1% uranyl acetate and dehydrated with acetone series. Thereafter the samples were embedded in Epon (LX-112 Resin, Ladd, Burlington, Vt., U.S.A.). Thin (2 μm) transverse sections were stained with 1% toluidine blue, and examined and photographed under light microscopy (Nikon Microphot-FXA, Japan). In each vascular ring the thickness of medial smooth muscle (determined as the mean medial thickness of four arterial wall quadrants) was measured from the photographs.

Atrial natriuretic peptide determinations

Left and right auricles were removed to be used for atrial ANP measurements, and the remaining atrial tissue was carefully cut separate from ventricular tissue. The ventricles were cut into superior (about 15–20% of total weight) and inferior parts, the latter being used for ventricular ANP determinations to avoid contamination of atrial tissue in these samples. All samples were blotted dry, weighed, and stored at − 70°C until assayed.

Blood samples were centrifuged (2,000 *g*, 10 min, at + 4°C), and ANP extracted from plasma as previously described (Ruskoaho *et al.*, 1989). ANP extraction from heart tissue was performed by homogenizing the atrial and ventricular samples with 3 ml of 0.3 M HCl as previously described (Ruskoaho & Leppäluoto, 1988). For the radioimmunoassay (RIA) the atrial and ventricular extracts were diluted 3×10^5 and 1.2×10^3 fold, respectively, with the RIA buffer. Then the tissue extracts and the plasma samples were incubated in duplicates of 100 μl with 100 μl of the specific rabbit ANP antiserum in the final dilution of $1{:}2.5 \times 10^4$. ANP was determined from plasma and tissue samples by RIA as earlier described by Ruskoaho *et al.* (1989). Tissue ANP is expressed as concentration per milligram tissue wet weight.

Measurement of intracellular free calcium in platelets and lymphocytes

The cytosolic free calcium concentration was measured as previously described (Pörsti *et al.*, 1992). Briefly, whole blood was diluted 1:2 with RPMI-1640 medium (Flow Laboratories, Irvine, Scotland, U.K.) and 4 ml of the sample was layered on 3 ml of Ficoll-Paque solution (Pharmacia, LKB Biotechnology Inc., Piscataway, N.J., U.S.A.). After centrifugation (25 min, 120 *g*, + 20°C) the plasma was removed and platelets and lymphocytes were harvested from plasma-Ficoll interface. The cells were resuspended in 4 ml of RPMI-1640 medium, and 0.4 ml of ACD solution (8% citric acid, 2.2% trisodium citrate, 2.45% dextrose, pH 6.5) was added. Platelets and lymphocytes were separated by centrifugation (10 min, 100 *g*, + 20°C), and lymphocytes were washed with RPMI-1640 and resuspended in HEPES medium (NaCl 145, $MgSO_4$ 5, glucose 10 and HEPES 10 mM; pH 7.4). The platelets were spun down from the supernatant (10 min, 500 *g*, + 20°C), washed twice with HEPES, and finally resuspended in this medium. The cell suspensions ($1-2 \times 10^9$ platelets ml^{-1}, $3-4 \times 10^6$ lymphocytes ml^{-1}) were loaded with 50 μM acetoxymethyl ester of quin-2 for 30 min at + 37°C, and washed twice with HEPES to remove extracellular quin-2. Finally the cells were resuspended in + 37°C Hanks' balanced salt solution ($2-2.5 \times 10^8$ platelets ml^{-1}; $1-2 \times 10^6$ lymphocytes ml^{-1}) containing 1.1 mM calcium, and fluorescence was recorded with a Shimadzu RF-5000 spectrofluorometer (Shimadzu Corp., Kyoto, Japan). The excitation and emission wavelengths were set at 339 nm and 492 nm, respectively. Calibration of the signal was performed basically according to the method described by Tsien *et al.* (1982). Maximal fluorescence was measured after 2 μM ionomycin and 5 mM $CaCl_2$ were added to the solution; minimal fluorescence after 25 mM Tris-EGTA (pH 8.6) and 0.1% Triton X100 were added.

Drugs

The following drugs were used: quinapril hydrochloride (Warner-Lambert Co., Ann Arbor, Mi., U.S.A.), acetylcholine chloride, calcium salt of ionomycin, 6-hydroxydopamine hydrobromide, isoprenaline hydrochloride, ammonium salt of heparin, synthetic rat ANP_{1-28} (Sigma Chemical Co., St. Louis, Mo., U.S.A.), acetoxymethyl ester of quin-2, Triton X100 (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.), noradrenaline hydrogentartrate (Fluka Chemie AG, Buchs, Switzerland), nifedipine (Orion Pharmaceutical Co., Espoo, Finland) and sodium nitrite (E. Merck AG, Darmstadt, Germany). Quinapril was dissolved directly in tap water. The stock solutions of the compounds used in the *in vitro* studies were dissolved in distilled water, with the exception of ionomycin (in absolute ethanol), quin-2 (in dimethylsulphoxide), nifedipine (in 50% ethanol) and 6-hydroxydopamine (directly in buffer-free PSS). All solutions were freshly prepared before use and protected from light.

Statistics

Statistical analysis was carried out using one-way analysis of variance (ANOVA), supported by Bonferroni confidence intervals in the case of pairwise comparisons between the study groups. When the data consisted of repeated observations at successive time points analysis of variance for repeated measurements was applied. The results are expressed as mean ± s.e.mean. The data were analyzed with BMDP Statistical Software (Los Angeles, Ca., U.S.A.).

Results

Blood pressure, heart weight, plasma and tissue ANP concentrations, intracellular free calcium, and body weight

The systolic blood pressure of SHR was already higher at the beginning of the study than in WKY rats. During the 7-week

period before quinapril administration, blood pressures in the two SHR groups were elevated equally. Quinapril treatment beginning at the age of 15 weeks reduced blood pressure in SHR to normotensive level during the follow-up period, while in untreated SHR the rise in blood pressure continued (Figure 1 and Table 1). Cardiac hypertrophy was totally prevented in SHR by quinapril, relative heart weights of treated SHR not differing from those of WKY rats. Platelet and lymphocyte $[Ca^{2+}]_i$, measured by the fluorescent indicator quin-2, were comparable in WKY rats and quinapril-treated SHR, and significantly lower than in untreated SHR (Table 1). In addition, quinapril therapy reduced plasma and ventricular ANP, the concentrations in treated SHR being comparable to those in WKY rats and markedly lower than in untreated SHR (Figure 2). The total amounts of ANP in the ventricular tissue of untreated SHR, quinapril-treated SHR, and WKY rats were 157.4 ± 10.0***, 38.3 ± 5.4**,††† and 70.0 ± 5.6 ng, respectively (**$P<0.01$; ***$P<0.001$ compared with WKY rats; †††$P<0.001$ versus SHR). ANP content in the right atrium was similar in all groups, the concentration in the left atrium being higher in quinapril-treated than in untreated SHR (Figure 2).

SHR on oral quinapril gained somewhat less weight than untreated SHR and WKY rats (Table 1). However, no signs of compromised well-being of the animals (e.g. in appearance, activity, behaviour) were observed by our experienced experimental animals laboratory staff. In addition, average daily chow consumption was similar in the SHR groups: 11 ± 3 and 12 ± 3 g kg^{-1} body weight daily in treated and untreated SHR, respectively.

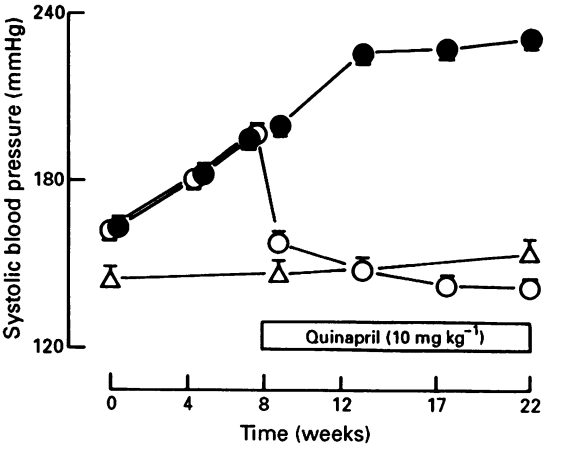


Figure 1 Systolic blood pressures in untreated spontaneously hypertensive rats (SHR, ●), SHR treated with quinapril (○) and untreated normotensive Wistar-Kyoto rats (WKY, △). The 15 week-long quinapril treatment (10 mg kg^{-1} body weight per day; indicated by the horizontal bar) of SHR was initiated at the age of 15 weeks and it effectively lowered blood pressure ($P<0.001$ versus untreated SHR, ANOVA for repeated measurements). Final systolic blood pressures did not significantly differ between quinapril-treated SHR and untreated WKY rats. Symbols indicate means with s.e.mean, $n = 10-12$ in each group.

Mesenteric arterial responses in vitro

When related to tissue dry weight, NA-induced maximal contractile force in isolated arterial rings was clearly lower in untreated SHR than WKY rats. However, 3-mm-long vascular rings from SHR, excised from a standard section of mesenteric artery, weighed more than those from WKY rats, and without relation to tissue weight maximal contractions were similar in these two strains. Untreated SHR had higher sensitivity to NA, i.e. lower EC_{50} values, than WKY rats (Figure 3 and Table 2). Contractile force generation to NA was reduced in SHR by quinapril therapy and was markedly lower than in WKY rats, whether related to tissue dry weight or not. In addition, sensitivity to NA was lowest in SHR on quinapril. Vascular rings of quinapril-treated SHR, too, weighed more than in WKY rats (Figure 3 and Table 2). The tissue weight-related contractile force induced by high concentrations of KCl was also lower in untreated SHR than WKY rats, and without the relation maximal contractions were again similar in these groups. SHR on quinapril showed clearly attenuated KCl-induced contractions when compared with WKY rats, but only differed from untreated SHR at high KCl concentrations (80–125 mM). Sensitivity to KCl was comparable in untreated SHR and WKY rats, but was slightly higher in quinapril-SHR than in the other groups (Figure 3 and Table 2).

The effect of organ bath calcium concentration on 10 μM NA-induced contractions, i.e. the calcium sensitivity of the vascular rings during α-adrenoceptor stimulation, was similar in all three study groups (Figure 4). However, the inhibitory effect of 0.5 nM nifedipine on NA-evoked contractions in different organ bath calcium concentrations was lower in

Table 1 Blood pressure, body weight and heart weight during the study, and intracellular free calcium concentrations at the close of the 22-week study

Variable	*Group* SHR	QSHR	WKY
Systolic blood pressure (mmHg)			
week 0	164 ± 2*	162 ± 3*	145 ± 4
week 7	196 ± 2***	197 ± 2***	149 ± 4
week 22	232 ± 3***	142 ± 3†††	155 ± 5
Body weight (g)			
week 0	151 ± 3	158 ± 5	159 ± 6
week 7	316 ± 4	316 ± 5	324 ± 6
week 22	412 ± 5	377 ± 5***†††	430 ± 7
Heart weight (g kg^{-1} body weight)	3.5 ± 0.1***	2.8 ± 0.1†††	2.9 ± 0.1
Intracellular free calcium concentration (nM)			
Platelets	105 ± 2***	91 ± 3†	83 ± 4
Lymphocytes	115 ± 3***	90 ± 4†††	94 ± 4

Values are mean ± s.e.mean; $n = 10-12$ in each group. SHR and QSHR, untreated and quinapril-treated spontaneously hypertensive rats, respectively; WKY, Wistar-Kyoto rats. The 15-week long quinapril treatment commenced at study week 7 when the animals were 15 weeks old.

*$P<0.05$, ***$P<0.001$ compared with WKY rats, †$P<0.05$, †††$P<0.001$ QSHR versus SHR (Bonferroni test).

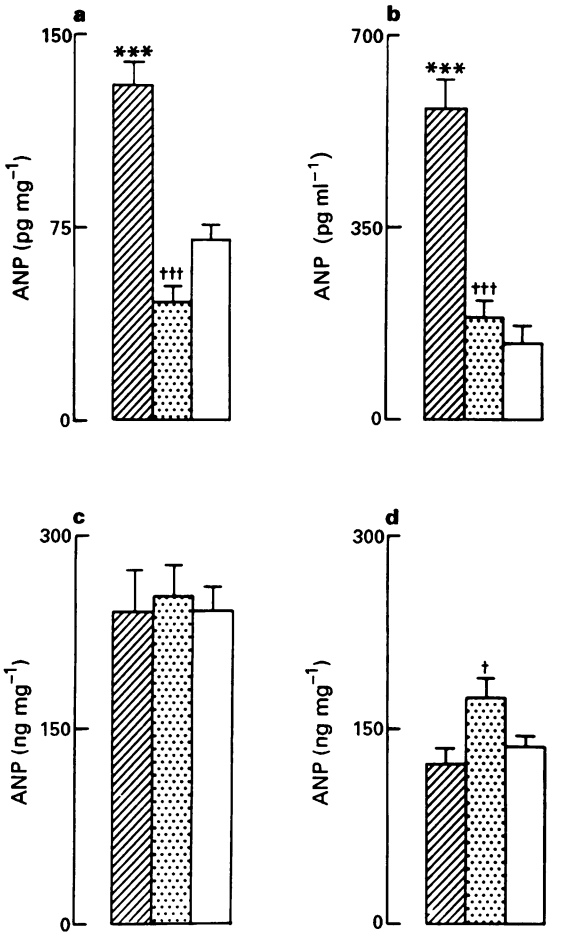


Figure 2 Atrial natriuretic peptide (ANP) concentrations in ventricular tissue (a), plasma (b), and in right (c) and left atrial tissues (d) of untreated spontaneously hypertensive rats (SHR, hatched columns), SHR treated with quinapril (stippled columns) and untreated Wistar-Kyoto rats (WKY, open columns). Columns indicate means with s.e.mean, $n = 6-10$ in each group. Statistical analysis was by one-way ANOVA followed by the Bonferroni test for individual pairs: ***$P<0.001$ compared with WKY rats; †$P<0.05$; †††$P<0.001$ quinapril-treated SHR versus untreated SHR.

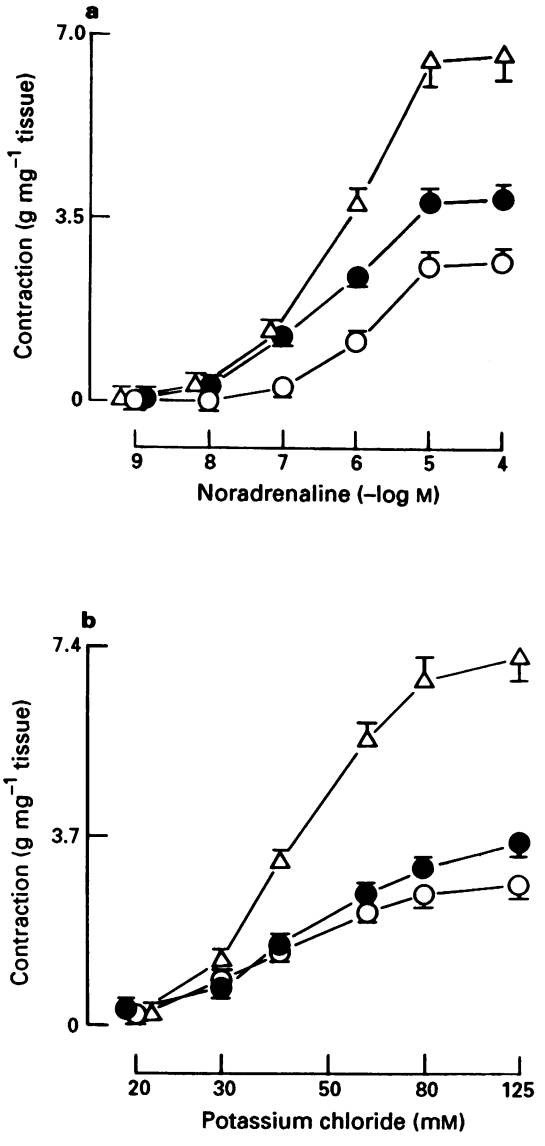


Figure 3 Contractile responses (related to tissue dry weight) of isolated endothelium-intact mesenteric arterial rings from untreated spontaneously hypertensive rats (SHR, ●), SHR treated with quinapril (○) and untreated Wistar-Kyoto rats (WKY, △). (a) Cumulative concentration-response curves to noradrenaline. Contractile force generation was lower in SHR than WKY rats ($P<0.001$). Quinapril-treated SHR showed reduced contractions when compared with untreated SHR ($P<0.01$) and WKY rats ($P<0.001$). (b) Cumulative concentration-response curves to high concentrations of potassium chloride. Contractile force generation was again lower in SHR than WKY rats ($P<0.001$), and quinapril-treated SHR showed reduced contractions when compared with WKY rats ($P<0.001$), but differed from untreated SHR only at the highest concentrations of potassium chloride (80–125 mM, $P<0.01$). Symbols indicate means with s.e.mean, $n = 8-10$ in each group. Statistical analysis was by ANOVA for repeated measurements.

WKY rats and quinapril-treated SHR than in untreated SHR (Figure 4).

The relaxation responses of endothelium-intact mesenteric arterial preparations induced by ACh, $NaNO_2$, and isoprenaline were greatly impaired in untreated SHR when compared with WKY rats. All these relaxations were markedly improved in SHR by quinapril treatment, the responses not differing from those of WKY rats. High concentrations of ACh (10 μM) induced slight recontractions in untreated SHR, such phenomena being absent in WKY rats and quinapril-treated SHR (Figure 5 and Table 3). In addition, the rate of relaxation after washout of maximal (125 mM) KCl-induced contractions was similar in WKY rats and quinapril-SHR and markedly faster than in untreated SHR (Table 3).

In endothelium-denuded mesenteric arterial rings subjected to denervation *in vitro* with 6-hydroxydopamine, the time to onset of K^+-free solution-induced contraction was longer and the plateau phase of the response was attained later in WKY rats and quinapril-SHR than in untreated SHR. When related either to the 125 mM KCl-induced reference contractions or to tissue weight, maximal K^+-free responses were lower in WKY rats and quinapril-SHR than in untreated SHR (Figure 6 and Table 4). The potassium relaxation response of endothelium-denuded denervated arterial rings, induced by re-addition of 1 mM potassium upon maximal K^+-free contractions, was markedly impaired in untreated SHR when compared with WKY rats. Quinapril therapy clearly improved this response, the rate of potassium relaxation not differing between quinapril-SHR and WKY rats (Figure 6 and Table 4).

Morphological studies

Medial smooth muscle thickness, measured from mesenteric rings photographed under light microscopy, was moderately reduced in quinapril-treated SHR when compared with untreated SHR, but still remained clearly more pronounced

Table 2 Parameters of contractile responses and average medial thickness in isolated endothelium-intact mesenteric arterial rings

Variable	*Group* *SHR*	*QSHR*	*WKY*
EC_{50}			
Noradrenaline (nM)	419 ± 39*	1219 ± 254*††	731 ± 88
Potassium chloride (mM)	44 ± 1	37 ± 2*††	42 ± 1
Maximal force (g mg^{-1} tissue)			
Noradrenaline	3.9 ± 0.3***	2.7 ± 0.3***†	6.7 ± 0.5
Potassium chloride	3.7 ± 0.2***	2.8 ± 0.2***†	7.2 ± 0.5
Maximal force (g)			
Noradrenaline	2.7 ± 0.2	1.6 ± 0.1**††	2.5 ± 0.2
Potassium chloride	2.4 ± 0.1	1.7 ± 0.1***††	2.7 ± 0.1
Vascular ring dry weight (mg)	0.69 ± 0.04***	0.63 ± 0.06*	0.38 ± 0.03
Average medial thickness (μm)	117 ± 3***	103 ± 3***†	77 ± 4

Values are mean ± s.e.mean, $n = 8-10$ in each group, $n = 6$ for morphological studies. SHR and QSHR, untreated and quinapril-treated spontaneously hypertensive rats, respectively; WKY, Wistar-Kyoto rats. EC_{50} is the concentration of agonist producing 50% of maximal contractile response.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with WKY rats, †$P<0.05$, ††$P<0.01$ QSHR versus SHR (Bonferroni test).

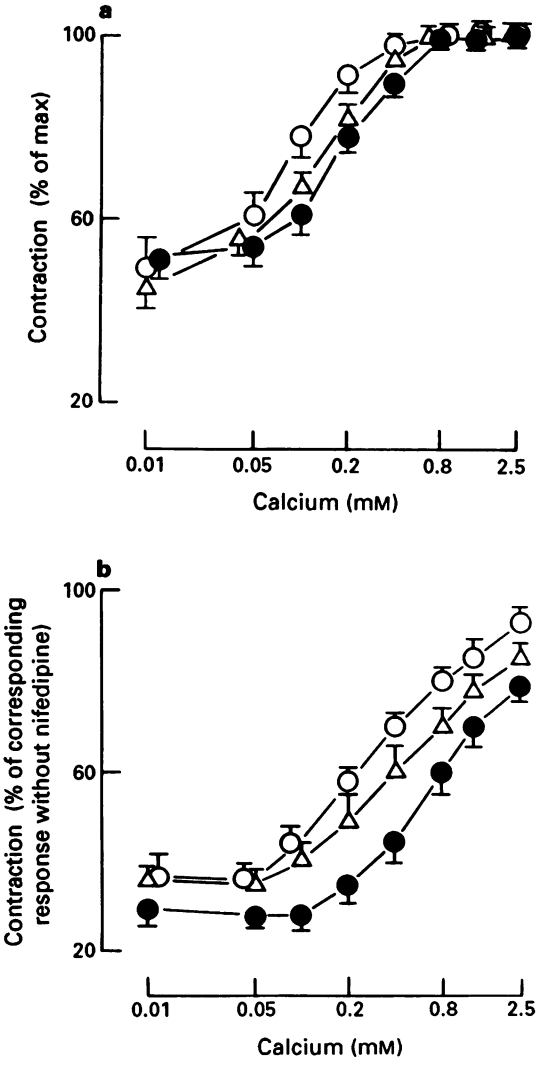


Figure 4 Contractile responses of isolated endothelium-intact mesenteric arterial rings from untreated spontaneously hypertensive rats (SHR, ●), SHR treated with quinapril (○) and untreated Wistar-Kyoto rats (WKY, △). (a) Effect of organ bath calcium concentration on 10 μM noradrenaline-induced contractions. No significant differences were found between the groups. (b) Effect of 0.5 nM nifedipine on 10 μM noradrenaline-induced contractions in different organ bath calcium concentrations. Nifedipine inhibited contractions of WKY rats ($P<0.05$) and quinapril-treated SHR ($P<0.01$) less effectively than those of untreated SHR. Symbols indicate means with s.e.mean, $n = 8-10$ in each group. Statistical analysis was by ANOVA for repeated measurements.

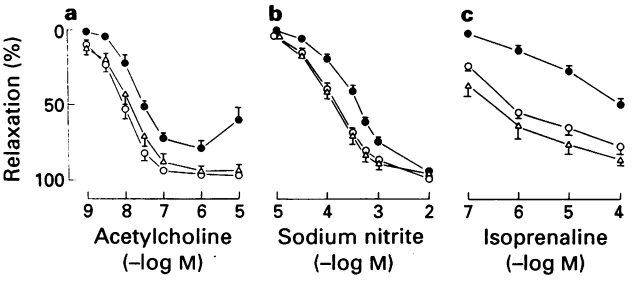


Figure 5 Cumulative relaxation responses of isolated endothelium-intact mesenteric arterial rings from untreated spontaneously hypertensive rats (SHR, ●), SHR treated with quinapril (○) and untreated Wistar-Kyoto rats (WKY, △). Relaxations were induced by acetylcholine (a), sodium nitrite (b) and isoprenaline (c) after full precontraction with 1 μM noradrenaline. Quinapril-treated SHR and untreated WKY rats showed more pronounced relaxations than untreated SHR ($P<0.05$ for acetylcholine, $P<0.001$ for sodium nitrite and isoprenaline). Symbols indicate means with s.e.mean, $n = 8-10$ in each group. Statistical analysis was by ANOVA for repeated measurements.

than in WKY rats (Figure 7 and Table 2). The results of medial thickness in the study groups paralleled well with vascular ring dry weights (Table 2).

Discussion

In the present study, blood pressure was totally normalized and cardiac hypertrophy abolished in SHR by quinapril therapy, the results being consistent with previous investigations using other ACE inhibitors (Lee *et al.*, 1991; Frohlich & Horinaka, 1991). The renin-angiotensin-aldosterone system thus seems to be one of the most important factors supporting elevated blood pressure in SHR. Recent investigations have shown that the pathogenesis of hypertension in SHR may be related to the renin gene (Kurtz *et al.*, 1990; Morris, 1991). Given the suggested central role of the renin gene, it is

Table 3 Parameters of relaxation of isolated endothelium-intact mesenteric arterial rings

Variable	*Group* SHR	QSHR	WKY
EC_{50}			
Acetylcholine (nM)	27 ± 4*	11 ± 3††	12 ± 2
Sodium nitrite (μM)	459 ± 55***	165 ± 26†††	174 ± 44
Isoprenaline (μM)	94 ± 22***	1.2 ± 0.6†††	0.9 ± 0.3
Maximal relaxation (% of 1 μM noradrenaline -induced precontraction)			
Acetylcholine	79 ± 5*	97 ± 2††	94 ± 3
Sodium nitrite	95 ± 2	99 ± 1	96 ± 2
Isoprenaline	53 ± 4**	77 ± 5†	87 ± 4
Mean rate of relaxation after washout of 125 mM KCl (g mg^{-1} tissue min^{-1})	0.27 ± 0.02***	0.90 ± 0.11†††	1.08 ± 0.11

Values are mean ± s.e.mean, $n = 8-10$ in each group. SHR and QSHR, untreated and quinapril-treated spontaneously hypertensive rats, respectively; WKY, Wistar-Kyoto rats. EC_{50} is the concentration producing 50% relaxation of 1 μM noradrenaline-induced precontraction.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with WKY rats, †$P<0.05$, ††$P<0.01$, †††$P<0.001$ QSHR versus SHR (Bonferroni test).

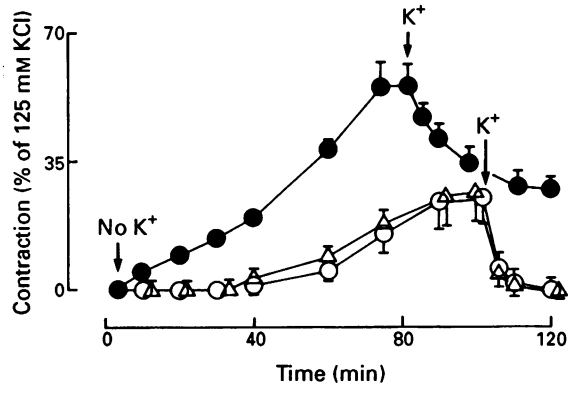


Figure 6 Contractile responses induced by K^+-free buffer solution (100% contraction represents the force attained by 125 mM KCl) and the subsequent relaxation responses to re-addition of 1.0 mM K^+ in isolated endothelium-denuded and sympathectomized mesenteric arterials rings from untreated spontaneously hypertensive rats (SHR, ●), SHR treated with quinapril (○) and untreated Wistar-Kyoto rats (WKY, △). The responses of WKY rats and quinapril-treated SHR did not differ, the rate of contractile force development being slower ($P<0.01$) and the rate of relaxation being faster ($P<0.05$) than in untreated SHR. Symbols indicate means with s.e.mean, $n = 6$ in each group. Statistical analysis was by one-way ANOVA followed by the Bonferroni test for individual pairs.

not surprising that inhibition of angiotensin II formation, the major effector of the renin-angiotensin system, was highly effective in lowering blood pressure and remodelling arterial function in the present study. Achieved normotension and reduced cardiac afterload also clearly reduced ventricular tissue ANP concentrations, which probably reflects diminished synthesis and storage of ANP, since hypertension-induced ventricular hypertrophy in SHR is known to be associated with increased synthesis, storage and release of ANP (Arai *et al.*, 1988; Ruskoaho *et al.*, 1989). The finding that plasma ANP was similar in WKY rats and quinapril-SHR and markedly lower than in untreated SHR supports this conclusion. Elevated ANP content in the left atrium of SHR on quinapril, in turn, probably results from reduced ANP release, since the rate of ANP synthesis in atrial tissue of SHR is known to be constant (Kinnunen *et al.*, 1991). Thus, the results on plasma and tissue ANP concentrations confirm the normalization of blood pressure in quinapril-treated SHR.

Weight gain was somewhat attenuated in SHR on oral quinapril, although chow consumption was comparable to that in untreated SHR. Previously, treatment with various other ACE inhibitors has been reported to result in reduced body weight in hypertensive rats (Adams *et al.*, 1990; Harrap *et al.*, 1990, Mulvany *et al.*, 1991). The explanation of this phenomenon remains unclear. However, in addition to other

Table 4 Parameters of K^+-free buffer solution-induced contractions and subsequent relaxations induced by 1.0 mM potassium in isolated endothelium-denuded and sympathectomized mesenteric arterial rings

Variable	*Group* SHR	QSHR	WKY
Contraction to 125 mM KCl (g mg^{-1} tissue)	4.6 ± 0.4	4.2 ± 0.4	4.9 ± 0.6
Potassium-free contraction			
Time to onset (min)	4.2 ± 1.5**	51.0 ± 5.7††	46.4 ± 9.9
Time to maximum (min)	73.3 ± 3.7**	102.7 ± 6.5††	96.2 ± 6.3
Maximal force			
(g mg^{-1} tissue)	2.6 ± 0.3*	0.9 ± 0.2†	1.1 ± 0.4
(% of 125 mM KCl)	56.5 ± 7.2*	23.7 ± 7.3†	22.4 ± 7.6
Potassium relaxation rate			
(mg mg^{-1} tissue min^{-1})	87 ± 20*	354 ± 82†	504 ± 44
(% of 125 mM KCl min^{-1})	2.0 ± 0.5*	8.3 ± 2.0†	10.3 ± 0.9

Values are mean ± s.e.mean, $n = 6$ in each group. SHR and QSHR, untreated and quinapril-treated spontaneously hypertensive rats, respectively; WKY, Wistar-Kyoto rats.
*$P<0.05$, **$P<0.01$ compared with WKY rats, †$P<0.05$, ††$P<0.01$, QSHR versus SHR (Bonferroni test).

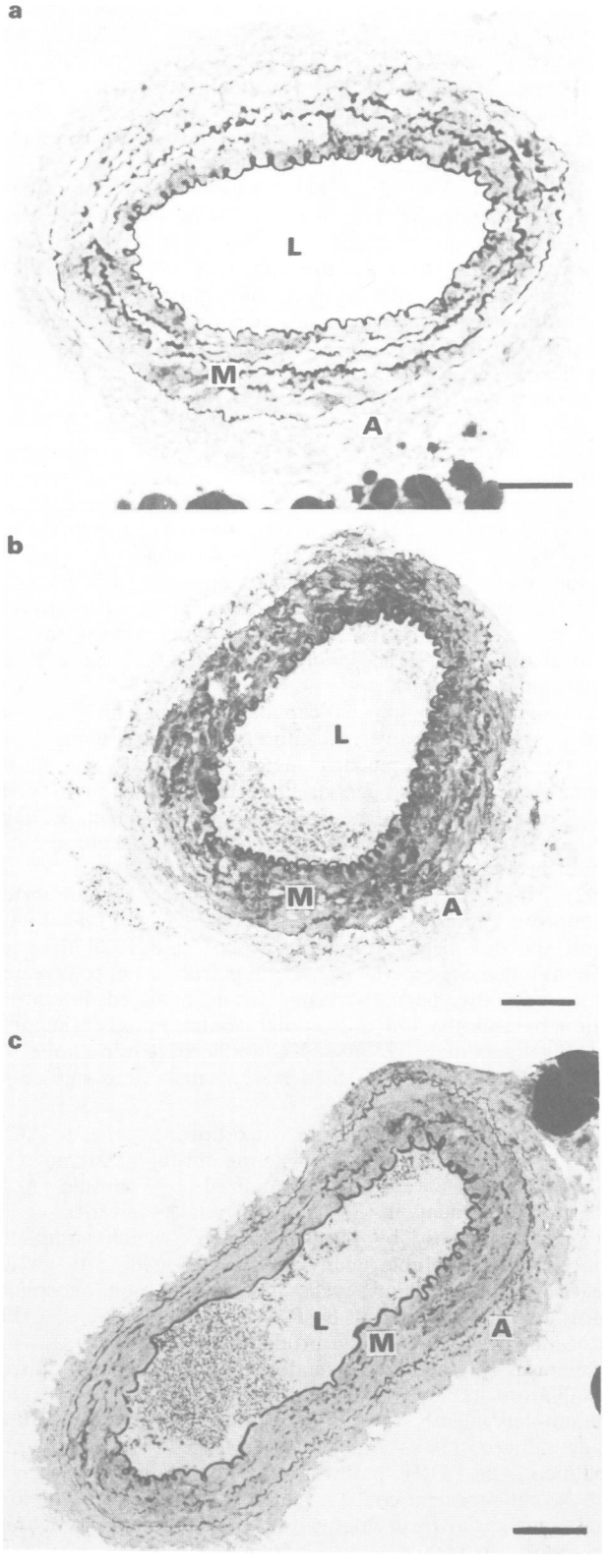


Figure 7 Light microscopy of representative mesenteric arterial rings from untreated spontaneously hypertensive rats (SHR, a), SHR treated with quinapril (b) and untreated Wistar-Kyoto rats (c). A = adventitia, M = media, L = lumen; calibration bar = 100 μm.

functions, angiotensin II acts as a growth factor in the cardiovasular system (Dzau *et al.*, 1991). Moreover, enhanced responses to angiotensin II and other growth factors have been reported in both vascular smooth muscle cells and skin fibroblasts of SHR (Paquet *et al.*, 1989; Guicheney *et al.*, 1991). Thus, reduction of angiotensin II formation by ACE inhibitors may affect growth of vascular and nonvascular cells in experimental animals, especially in SHR. Attenuated weight gain may also result from unspecific effects on other factors affecting growth, or from reduced plasma aldosterone concentration and the subsequent natriuresis and diuresis following long-term ACE inhibition (Säynävälammi *et al.*, 1986).

The concentration of intracellular free calcium is a major factor determining arterial tone (Stull *et al.*, 1991). Vascular smooth muscle cells in SHR are characterized by abnormal calcium handling already at the prehypertensive stage: although basal $[Ca^{2+}]_i$ in 4-week-old animals is similar to that in WKY rats, stimulation with caffeine or angiotensin II results in higher values in SHR. In 8-week-old SHR with manifest hypertension basal $[Ca^{2+}]_i$, too, is higher than in WKY rats (Sugiyama *et al.*, 1990). Elevated $[Ca^{2+}]_i$ has also been found in platelets (Pörsti *et al.*, 1992) and lymphocytes of SHR (Furspan *et al.*, 1989). The possibility that elevated $[Ca^{2+}]_i$ in these circulating blood cells might only be a secondary phenomenon to high blood pressure and shear stress has raised concern (Young *et al.*, 1988). However, Oshima *et al.* (1991) reported that both basal and thrombin-stimulated $[Ca^{2+}]_i$ in platelets and concanavalin A-stimulated $[Ca^{2+}]_i$ in thymic lymphocytes of 4-week-old SHR are higher than in WKY rats. Thus, abnormalities in calcium metabolism appear to be prior events rather then subsequent to the development of hypertension in SHR, and elevated $[Ca^{2+}]_i$ can be found in circulating and noncirculating cells that are shielded from shear stress. In the present study, $[Ca^{2+}]_i$ was clearly higher in platelets and lymphocytes of SHR when compared with WKY rats, and was significantly reduced by quinapril therapy in both of these cell types in SHR. Sada *et al.* (1990) have recently reported that ACE inhibitor treatment both normalizes $[Ca^{2+}]_i$ and decreases Ca^{2+}-dependent tone in vascular smooth muscle of SHR. Thus, the reduction in blood pressure following long-term ACE inhibitors is associated with improved cellular calcium regulation in SHR.

In the present study, quinapril treatment of SHR was started when the animals were 15 weeks old and their systolic blood pressures had risen to nearly 200 mmHg, to examine whether long-term ACE inhibition at this advanced stage can reverse the abnormalities of arterial function associated with genetic hypertension. Morphological studies of the vasculature showed that medial smooth muscle hypertrophy was slightly reduced in treated SHR, but still remained clearly more pronounced than in WKY rats, in spite of the marked blood pressure reduction by quinapril. Thus, the hypertension-induced thickening of the mesenteric arterial wall could be only moderately reversed by the present ACE inhibitor treatment, corresponding to previous reports in which structural vascular changes in SHR could be effectively prevented only by very early introduction of antihypertensive therapy (Lee *et al.*, 1991).

The results presented here were obtained with selected segments of the mesenteric artery *in vitro* to study the effects of long-term ACE inhibition on arterial function. The fact that blood vessels from different parts of the vasculature may differ substantially must be kept in mind when relating the results to the whole vascular system. When smooth muscle contractions were induced by depolarizing the cell membrane with high concentrations of KCl, SHR showed comparable sensitivity but attenuated tissue weight-related force generation when compared with WKY rats. This type of 'forced' contraction depends mainly upon calcium influx through the voltage-dependent channels, a minor portion of the response resulting from the release of stored calcium (Johns *et al.*, 1987; Karaki & Weiss, 1988). We have previously reported that maximal tissue weight-related contractions induced by both high concentrations of KCl and NA are similar in SHR and WKY rats (Pörsti *et al.*, 1992). Those rats, however, were 10 weeks younger than the present 30-week-old animals, and an impairment of vascular contractions at the later and

more advanced stage of hypertension appears to be a likely explanation. Nonetheless, normalization of blood pressure in SHR by quinapril did not augment the KCl-induced contractile force generation of vascular rings to the level of WKY rats, but even further attenuated the response at higher KCl concentrations. Thus, the reduced arterial contractile response of 30-week-old SHR may be attributed rather to properties of the hypertrophied vascular wall than high blood pressure *per se*. Another possible explanation for these deviations in the results between different studies may be inborn differences between SHR and WKY rats, since recent investigations have shown clear genetic dissimilarities between these two strains (Johnson *et al.*, 1992; St. Lezin *et al.*, 1992).

In addition to cell membrane depolarization and the subsequent calcium entry, liberation of endogenous NA from adrenergic nerve-endings and α-adrenoceptor stimulation participate in the contractile response evoked by a high concentration of KCL (Xiao & Rand, 1991). The relative proportion of endogenous NA in KCl-induced contractions seems more pronounced in mesenteric rings from normotensive rats, since after denervation *in vitro* with 6-hydroxydopamine, maximal responses were no longer greater in WKY rats than in SHR. Moreover, ACE inhibition is known to eliminate the angiotensin II-induced facilitation of NA release in SHR (Adams *et al.*, 1990). Thus, the lower KCl-induced contractions in quinapril-treated SHR when compared with untreated SHR and WKY rats may also reflect diminished transmitter release from vascular adrenergic nerve endings.

Smooth muscle contractions were also induced by activating α-adrenoceptors with NA, which causes a rapid calcium release from the sarcoplasmic reticulum and prevents calcium re-uptake, the sustained contraction resulting from calcium influx through the receptor-linked channel (Johns *et al.*, 1987; Karaki & Weiss, 1988). Sensitivity to NA was highest in untreated SHR, corresponding to the elevated $[Ca^{2+}]_i$ in these animals. When compared with untreated SHR and WKY rats, both contractile force generation and sensitivity to NA were clearly reduced in quinapril-treated SHR. Freslon & Giudicelli (1983) and Lee *et al.* (1991) have reported attenuated responses to NA in captopril-treated SHR, the suggested mechanism being reduced vascular wall hypertrophy with lower smooth muscle mass following chronic ACE inhibition. Attenuation of pressor responses to infused NA has also been reported in lisinopril-treated essentially hypertensive patients (Malini *et al.*, 1990). The present results, however, do not support the view that ACE inhibitor therapy attenuates arterial contractions by diminishing smooth muscle mass, since medial thickness of the mesenteric artery was only slightly reduced. Moreover, reduced smooth muscle mass is not equivalent to smaller contractions, since normotensive WKY rats with clearly lower medial thickness (and vascular ring weight) showed more pronounced responses than SHR. Interestingly, vascular sensitivity to depolarization with KCl was slightly higher in SHR on quinapril than in untreated SHR and WKY rats. Thus the lower sensitivity to NA in quinapril-treated SHR suggests an effect of chronic ACE inhibition on postsynaptic α-adrenoceptor-mediated responses, the mechanism of which remains to be studied, but may be attributed to alterations in dihydropyridine-sensitive calcium channels (see below). In addition, differences in neuronal catecholamine uptake after ACE inhibition may have affected the results on mesenteric arterial NA sensitivity, since the present responses were not performed in the presence of a neuronal uptake inhibitor.

The fact that lower blood pressure alone does not explain reduced sensitivity to vasoconstrictors is supported by the study of Bund *et al.* (1991), who found increased sensitivity to NA in the parts of the arterial tree in SHR which were protected from hypertension by partial ligature. Recently Dohi *et al.* (1992) reported that perfusion with 0.1 μM angiotensin II stimulated the production of the potent vasoconstrictor peptide endothelin in SHR mesenteric arteries but not in those from WKY rats. Endothelin production, in turn, augmented pressor responses to NA, and was suggested to have an important role in the amplification of the pressor effects of the renin-angiotensin system in SHR. The possibility that quinapril-induced reduction of angiotensin II formation attenuates sensitivity to NA via reduced endothelin production remains to be studied.

The calcium sensitivity of NA-induced smooth muscle contractions was not altered by quinapril treatment in SHR and, moreover, no significant differences were found between SHR and WKY rats. However, the inhibitory effect of the dihydropyridine Ca^{2+}-entry blocker, nifedipine, on these contractions was lower in normotensive WKY rats and quinapril-treated SHR than in untreated SHR, suggesting significant differences in voltage-dependent calcium entry. We have previously reported that the arteries of SHR are more sensitive to effects of dihydropyridines than those of WKY rats (Arvola *et al.*, 1992; Pörsti, 1992). Rusch & Hermsmeyer (1988) have shown that the relative proportions of voltage-dependent calcium currents are different in SHR and WKY rats. The long-lasting current, sensitive to dihyropyridines (Bean *et al.*, 1986), is greater in SHR, while the transient dihydropyridine-resistant current predominates in WKY rats. Therefore, ACE inhibitor treatment seems to be associated with normalization of the function of voltage-dependent calcium channels in SHR, as suggested earlier by Sada *et al.* (1990).

Vascular endothelium is capable of controlling arterial tone by releasing many vasoactive substances, among them endothelium-derived relaxing factor (EDRF) (Vane *et al.*, 1990; Moncada *et al.*, 1991). Endothelial function in SHR has been reported to be impaired with smaller EDRF-mediated attenuation of vascular contractile reponses and diminished endothelium-dependent relaxation (Arvola *et al.*, 1992). Moreover, ACE inhibitor therapy has been reported to improve endothelial function in SHR (Clozel *et al.*, 1990). Thus, the diminished responses to NA and KCl in endothelium-intact mesenteric arterial rings from quinapril-treated SHR may also partially result from increased inhibitory action by endothelium or vascular contractions. In support of this, maximal 125 mM KCl-induced contractions of endothelium-denuded and denervated rings were similar in both SHR groups.

In the present study, vascular relaxation responses to ACh, $NaNO_2$ and isoprenaline, each acting through different cellular pathways, were all normalized by chronic ACE inhibitor treatment in SHR. ACh relaxes arteries endothelium-dependently by releasing EDRF, which stimulates smooth muscle soluble guanylate cyclase, leading to an increase in intracellular cyclic guanosine monophosphate (Moncada *et al.*, 1991). EDRF is probably nitric oxide (Moncada *et al.*, 1991), the production of which is also the mechanism of action of endothelium-independent nitrovasodilators like $NaNO_2$ (Feelish, 1991). Since both endothelium-dependently and -independently produced nitric oxide-induced relaxations were enhanced after quinapril treatment, the EDRF pathway may not be improved *per se* but the enhancement could result from changes in a common final route distal from guanylate cyclase. In support of this, the relaxation mediated by β-adrenoceptor stimulation with isoprenaline, leading to increased intracellular cyclic adenosine monophosphate, was also markedly improved by quinapril. In addition, the relaxation rate after washout of maximal (125 mM) KCl-induced contraction was comparable in WKY rats and quinapril-treated SHR and clearly faster than in untreated SHR. Since the concentration of intracellular free calcium, a major factor determining arterial tone (Stull *et al.*, 1991), was significantly reduced in SHR platelets and lymphocytes by quinapril, improved cellular calcium regulation remains a prime candidate to explain the augmented vascular relaxation responses. The discrepancy between the present relaxation results and the report of Clozel *et al.* (1990) who found that 4-days of cilazapril

therapy augmented relaxation responses to ACh but not to sodium nitroprusside in SHR, could result from the fact that endothelial function is first to recover following the reduction of blood pressure while prolonged therapy is required to induce alterations in vascular smooth muscle function.

Vascular contractions induced by Na^+, K^+-ATPase inhibition with a K^+-free medium in endothelium-denuded denervated arterial rings result from sodium leak to the cell, which causes depolarization and increases calcium influx through voltage-dependent channels (Mulvany, 1985). We have previously shown that the K^+-free contractions in these conditions are more pronounced in SHR when compared with WKY rats because of increased cell membrane permeability to sodium and enhanced voltage-dependent calcium entry (Arvola *et al.*, 1992). In the present study, the K^+-free contractions of SHR after chronic ACE inhibition appeared identical to those in WKY rats. Thus, long-term quinapril administration seems to be associated with normalization of arterial cell membrane permeability in SHR. Furthermore, vascular Na^+, K^+-ATPase activity was evaluated indirectly by re-addition of potassium upon full K^+-free medium-induced contractions. The return of potassium activates Na^+, K^+-ATPase, leading to cell membrane repolarization and thereby smooth muscle relaxation (Bonaccorsi *et al.*, 1977). In addition, the rate of potassium relaxation also reflects general relaxation mechanisms (e.g. contractile protein dephosphorylation, calcium sequestration and extrusion) (Johns *et al.*, 1987), but our previous results suggest that potassium relaxation rate is indicative of Na^+, K^+-ATPase activity in rat mesenteric arterial smooth muscle (Arvola *et al.*, 1992). The present results showed a normalized potassium relaxation rate in SHR after quinapril therapy, and thus suggest improved vascular Na^+K^+-ATPase activity following long-term ACE inhibition.

In conclusion, long-term ACE inhibitor therapy with quinapril effectively lowered blood pressure and abolished cardiac hypertrophy in SHR. The treatment was associated with normalization of (1) ANP concentrations in plasma and ventricular tissue, (2) arterial relaxation responses by endothelium-dependent and -independent mechanisms, (3) function of arterial dihydropyridine-sensitive calcium channels, (4) cell membrane permeability of vascular smooth muscle, and (5) concentration of intracellular free calcium in circulating platelets and lymphocytes. However, arterial contractile responses were not normalized but were attenuated in quinapril-treated SHR when compared with untreated SHR and WKY rats. The attenuated vascular contractions may be attributed to the prevailing vascular wall hypertrophy in spite of the considerable blood pressure reduction, while at the same time cellular calcium regulation and the functions of vascular endothelium, cell membrane and voltage-dependent calcium channels were normalized by quinapril in SHR. Thus, enhanced arterial relaxation and attenuated contractile responses may take part in the long-term antihypertensive effect of quinapril in this type of genetic hypertension.

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References

ADAMS, M.A., BOBIK, A. & KORNER, P.I. (1990). Enalapril can prevent vascular amplifier development in spontaneously hypertensive rats. *Hypertension*, **16**, 252–260.

APRIGLIANO, O. & HERMSMEYER, K. (1976). *In vitro* denervation of the portal vein and caudal artery of the rat. *J. Pharmacol. Exp. Ther.*, **198**, 568–577.

ARAI, H., NAKAO, K., SAITO, Y., SUGAWARA, A., YAMADA, T., ITOH, H., SHIONO, S., MUKOYAMA, M., OHKUBO, H., NAKANISHI, S. & IMURA, H. (1988). Augmented expression of atrial natriuretic peptide gene in ventricles of spontaneously hypertensive rats (SHR) and SHR-stroke prone. *Circ. Res.*, **62**, 926–930.

ARVOLA, P., PÖRSTI, I., VUORINEN, P., PEKKI, A. & VAPAATALO, H. (1992). Contractions induced by potassium-free solution and potassium relaxation in vascular smooth muscle of hypertensive and normotensive rats. *Br. J. Pharmacol.*, **106**, 157–165.

BAUDIN, B. & DROUET, L. (1989). In vitro interactions between ramiprilat and angiotensin I-converting enzyme in endothelial cells. *J. Cardiovasc. Pharmacol.*, **14**, (suppl. 4), S37–S42.

BEAN, B.P., STUREK, M., PUGA, A. & HERMSMEYER, K. (1986). Calcium channels in muscle cells isolated from rat mesenteric arteries: modulation by dihydropyridine drugs. *Circ. Res.*, **59**, 229–235.

BONACCORSI, A., HERMSMEYER, K., APRIGLIANO, O., SMITH, C.B. & BOHR, D.F. (1977). Mechanisms of potassium relaxation of arterial muscle. *Blood Vessels*, **14**, 261–276.

BUND, S.J., WEST, K.P. & HEAGERTY, A.M. (1991). Effects of protection from pressure on resistance artery morphology and reactivity in spontaneously hypertensive and Wistar-Kyoto rats. *Circ. Res.*, **68**, 1230–1240.

CALDWELL, P.R.B., SEEGAL, B.C. & HSU, K.C. (1976). Angiotensin converting enzyme: vascular endothelial localization. *Science*, **191**, 1050–1051.

CLOZEL, M., KUHN, H. & HEFTI, F. (1990). Effects of angiotensin converting enzyme inhibitors and of hydralazine on endothelial function in hypertensive rats. *Hypertension*, **16**, 532–540.

DOHI, Y., HAHN, A.W.A., BOULANGER, C.M., BÜHLER, F.R. & LÜSCHER, T.F. (1992). Endothelin stimulated by angiotensin II augments contractility of spontaneously hypertensive rat resistance arteries. *Hypertension*, **19**, 131–137.

DZAU, V.J., GIBBONS, G.H. & PRATT, R.E. (1991). Molecular mechanisms of vascular renin-angiotensin system in myointimal hyperlasia. *Hypertension*, **18**, (suppl. II), II-100–II-105.

FABRIS, B., CHEN, B., PUPIC, V., PERICH, R. & JOHNSTON, C.I. (1990). Inhibition of angiotensin-converting enzyme (ACE) in plasma and tissue. *J. Cardiovasc. Pharmacol.*, **15**, (suppl. 2), S6–S13.

FEELISCH, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.*, **17**, (suppl. 3), 25–33.

FRESLON, J.L. & GIUCIDELLI, J.F. (1983). Compared myocardial and vascular effects of captopril and dihydralazine during hypertension development in spontaneously hypertensive rats. *Br. J. Pharmacol.*, **80**, 533–543.

FROHLICH, E.D. (1989). Angiotensin converting enzyme inhibitors. Present and future. *Hypertension*, **13**, (suppl. I), I-125–I-130.

FROHLICH, E.D. & HORINAKA, S. (1991). Cardiac and aortic effects of angiotensin converting enzyme inhibitors. *Hypertension*, **18**, (suppl. II), II-2–II-7.

FURSPAN, P.B., RINALDI, G.J., HOFFMAN, K. & BOHR, D.F. (1989). Dietary calcium and cell membrane abnormality in genetic hypertension. *Hypertension*, **13**, 727–730.

GUICHENEY, P., WAUQUIER, I., PAQUET, J.L. & MEYER, P. (1991). Enhanced response to growth factors and to angiotensin II of spontaneously hypertensive rat skin fibroblasts in culture. *J. Hypertens.*, **9**, 23–27.

HARRAP, S.B., VAN DER MERWE, W.M., GRIFFIN, S.A., MACPHERSON, F. & LEVER, A.F. (1990). Brief angiotensin converting enzyme inhibitor treatment in young spontaneously hypertensive rats reduces blood pressure long-term. *Hypertension*, **16**, 603–614.

JOHNS, A., LEIJTEN, P., YAMAMOTO, H., HWANG, K. & VAN BREEMEN, C. (1987). Calcium regulation in vascular smooth muscle contractility. *Am. J. Cardiol.*, **59**, 18A–23A.

JOHNSON, M.L., ELY, D.L. & TURNER, M.E. (1992). Genetic divergence between the Wistar-Kyoto rat and the spontaneously hypertensive rat. *Hypertension*, **19**, 425–427.

KAPLAN, H.R., COHEN, D.M., ESSENBURG, A.D., MAJOR, T.C., MERTZ, T.E. & RYAN, M.J. (1984). CI-906 and CI-907: New orally active nonsulfhydryl angiotensin-converting enzyme inhibitors. *Fed. Proc.*, **43**, 1326–1329.

KARAKI, H. & WEISS, G.B. (1988). Calcium release in smooth muscle. *Life Sci.*, **42**, 111–122.

KINNUNEN, P., TASKINEN, T., JÄRVINEN, M. & RUSKOAHO, H. (1991). Effect of phorbol ester on the release of atrial natriuretic peptide from the hypertrophied rat myocardium. *Br. J. Pharmacol.*, **102**, 453–461.

KINNUNEN, P., TASKINEN, T., LEPPÄLUOTO, J. & RUSKOAHO, H. (1990). Release of atrial natriuretic peptide from rat myocardium *in vitro*: effect of minoxidil-induced hypertrophy. *Br. J. Pharmacol.*, **99**, 701–708.

KURTZ, T.W., SIMONET, L., KARBA, P.M., WOLFE, S., CHAN, L. & HJELLE, B.L. (1990). Cosegregation of the renin allele of the spontaneously hypertensive rat with an increase in blood pressure. *J. Clin. Invest.*, **85**, 1328–1332.

LEE, R.M.K.W., BERECEK, K.H., TSOPORIS, J., MCKENZIE, R. & TRIGGLE, C.R. (1991). Prevention of hypertension and vascular changes by captopril treatment. *Hypertension*, **17**, 141–150.

LEVY, B.I., BENESSIANO, J., POITEVIN, P. & SAFAR, M.E. (1990). Endothelium-dependent mechanical properties of the carotid artery in WKY and SHR. Role of angiotensin converting enzyme inhibition. *Circ. Res.*, **66**, 321–328.

MALINI, P.L., STROCCHI, E., VALTANCOLI, G. & AMBROSIONI, E. (1990). ACE inhibition and pressor responsiveness to noradrenaline in hypertensive patients. *J. Clin. Pharmacol.*, **30**, 422–424.

MOMBOULI, J.-V., NEPHTALI, M. & VANHOUTTE, P.M. (1991). Effects of the converting enzyme inhibitor cilazaprilat on endothelium-dependent responses. *Hypertension*, **18**, (suppl. II), II-22–II-29.

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

MORRIS, B.J. (1991). Molecular genetics links renin to hypertension. *Mol. Cell. Endocrinol.*, **75**, C13–C18.

MULVANY, M.J. (1985). Changes in sodium pump activity and vascular contraction. *J. Hypertens.*, **3**, 429–436.

MULVANY, M.J. & NYBORG, N. (1980). An increased calcium sensitivity of mesenteric resistance vessels in young and adult spontaneously hypertensive rats. *Br. J. Pharmacol.*, **71**, 585–596.

MULVANY, M.J., PERSSON, A.E.G. & ANDRESEN, J. (1991). No persistent effect of angiotensin converting enzyme inhibitor treatment in Milan hypertensive rats despite regression of vascular structure. *J. Hypertens.*, **9**, 589–593.

OSHIMA, T., YOUNG, E.W. & MCCARRON, D.A. 1991. Abnormal platelet and lymphocyte calcium handling in prehypertensive rats. *Hypertension*, **18**, 111–115.

PAQUET, J.L., BAUDOUIN-LEGROS, M., MARCHE, P. & MEYER, P. (1989). Enhanced proliferating activity of cultured smooth muscle cells from SHR. *Am. J. Hypertens.*, **2**, 108–110.

PÖRSTI, I. (1992). Arterial smooth muscle contractions in spontaneously hypertensive rats on a high calcium diet. *J. Hypertens*, **10**, 255–263.

PÖRSTI, I., ARVOLA, P., WUORELA, H. & VAPAATALO, H. (1992). High calcium diet augments vascular potassium relaxation in hypertensive rats. *Hypertension*, **19**, 85–92.

RUSCH, N.J. & HERMSMEYER, K. (1988). Calcium currents are altered in the vascular smooth muscle cell membrane of spontaneously hypertensive rats. *Circ. Res.*, **63**, 997–1002.

RUSKOAHO, H. & LEPPÄLUOTO, J. (1988). Immunoreactive atrial natriuretic peptide in ventricles, atria, hypothalamus, and plasma of genetically hypertensive rats. *Cir. Res.*, **62**, 384–394.

RUSKOAHO, H., KINNUNEN, P., TASKINEN, T., VUOLTEENAHO, O., LEPPÄLUOTO, J. & TAKALA, T.E.S. (1989). Regulation of ventricular atrial natriuretic peptide release in hypertrophied rat myocardium. Effect of exercise. *Circulation*, **80**, 390–400.

SADA, T., KOIKE, H., IKEDA, M., SATO, K., OZAKI, H. & KARAKI, H. (1990). Cytosolic free calcium of aorta in hypertensive rats. Chronic inhibition of angiotensin converting enzyme. *Hypertension*, **16**, 245–251.

SÄYNÄVÄLAMMI, P., PÖRSTI, I., NURMI, A.-K., SEPPÄLÄ, E., METSÄ-KETELÄ, T., TUOMISTO, L., MANNINEN, V. & VAPAATALO, H. (1986). Inhibition of angiotensin converting enzyme with quinapril (CI-906): investigation of antihypertensive mechanisms in spontaneously hypertensive rats. *J. Pharmacol. Exp. Ther.*, **237**, 246–251.

SCHRÖR, K. (1990). Converting enzyme inhibitors and the interaction between kinins and eicosanoids. *J. Cardiovasc. Pharmacol.*, **15**, (suppl. 6), S60–S68.

ST. LEZIN, E., SIMONET, L., PRAVENCE, M. & KURTZ, T.W. (1992). Hypertensive strains and normotensive 'control' strains. How closely are they related? *Hypertension*, **19**, 419–424.

STULL, J.T., GALLAGHER, P.J., HERRING, B.P. & KAMM, K.E. (1991). Vascular smooth muscle contractile elements. Cellular regulation. *Hypertension*, **17**, 723–732.

SUGIYAMA, T., YOSHIZUMI, M., TAKAKU, F. & YAZAKI, Y. (1990). Abnormal calcium handling in vascular smooth muscle cells of spontaneously hypertensive rats. *J. Hypertens.*, **8**, 369–375.

TSIEN, R.Y., POZZAN, T. & RINK, T.J. (1982). Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell. Biol.*, **94**, 325–334.

VANE, J.R., ÄNGGÅRD, E.E. & BOTTING, R.M. (1990). Regulatory functions of the vascular endothelium. *N. Engl. J. Med.*, **323**, 27–36.

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.

WIEMER, G., SCHÖLKENS, B.A., BECKER, R.H.A. & BUSSE, R. (1991). Ramiprilat enhances endothelial autocoid formation by inhibiting breakdown of endothelium-derived bradykinin. *Hypertension*, **18**, 558–563.

XIAO, X.H. & RAND, M.J. (1991). Mechanisms of vasoconstrictor responses to KCl in rat isolated perfused tail arteries: interaction with the α2-adrenoceptor agonist UK14304. *Eur. J. Pharmacol.*, **196**, 133–136.

YOUNG, E.W., BUKOSKI, R.D. & MCCARRON, D.A. (1988). Calcium metabolism in experimental hypertension. *Proc. Soc. Exp. Biol. Med.*, **187**, 123–141.

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Effects of P1060 and aprikalim on whole-cell currents in rat portal vein; inhibition by glibenclamide and phentolamine

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1 Smooth muscle cells of the rat portal vein were dispersed by enzymatic treatment and recordings of whole-cell currents were made by the voltage-clamp technique. The effects of the potassium (K) channel openers, P1060 (0.3–10 μM) and aprikalim (3–30 μM) on these currents were investigated. Antagonism of these agents by glibenclamide and phentolamine was also studied.

2 When cells were clamped at − 10 mV, P1060 (1 μM) and aprikalim (3 μM) each induced a slowly-developing K-current (I_{KCO}), the noise of which gradually increased. The rate of onset of I_{KCO} was greater for P1060 than for aprikalim. Current-voltage plots showed that P1060 and aprikalim each caused an approximately 25 mV negative shift of the reversal potential at zero current.

3 P1060 (1 μM) and aprikalim (3 μM) each inhibited the slowly activating, slowly inactivating delayed rectifier current, I_{TO}.

4 Addition of MgATP (5 mM) to the recording pipette inhibited the generation of I_{KCO} by P1060 (1 μM) and reduced the accompanying inhibition of I_{TO}.

5 Stationary fluctuation analysis of the current noise associated with I_{KCO} induced by P1060 (1 μM) or aprikalim (3 μM) at a holding potential of − 10 mV indicated that the unitary conductance of the underlying K-channels was 10.5 pS at 0 mV under the quasi-physiological conditions of the experiment.

6 In the absence of K-channel openers, neither phentolamine (30–100 μM) nor glibenclamide (1 μM) affected the magnitude of control non-inactivating currents. However, phentolamine (30–100 μM), but not glibenclamide (1 μM) inhibited the control delayed rectifier current, I_{TO}.

7 After induction of I_{KCO} by P1060 (1 μM) or aprikalim (3 μM), subsequent exposure to glibenclamide (1 μM) or phentolamine (30 μM) inhibited this current. After aprikalim-induced reduction of I_{TO} had developed, subsequent exposure to glibenclamide was able partially to reverse the inhibition of I_{TO} whereas phentolamine was without effect. Pre-exposure to glibenclamide (1 μM) prevented both the generation of I_{KCO} by aprikalim (3 μM) and the inhibitory effect of this agent on I_{TO}.

8 It is concluded that P1060 and aprikalim each induce the current I_{KCO} by opening the same small conductance, ATP-sensitive K-channel (K_{ATP}), an effect which can be inhibited by glibenclamide or phentolamine. The opening of K_{ATP} by both P1060 and aprikalim probably involves competition between these agents and ATP for the ATP-control site associated with the channel. Inhibition of the delayed rectifier current, I_{TO}, by P1060 and aprikalim was glibenclamide-sensitive and may be caused by the induction of a state of run-down in the channel which underlies this current.

Keywords: P1060; aprikalim; phentolamine; glibenclamide; rat portal vein; voltage clamp; ATP-sensitive potassium channels

Introduction

The potassium (K) channel openers such as levcromakalim, pinacidil and aprikalim comprise a structurally-diverse group of compounds which cause membrane hyperpolarization and relaxation of smooth muscle (Edwards & Weston, 1990). The target channel of these agents in smooth muscle is the subject of some debate and several authors have suggested that a calcium-activated K-channel is involved (Hu *et al.*, 1990; Silberberg & van Breemen, 1990). However, indirect evidence such as the ability of the sulphonylureas like glibenclamide to inhibit the actions of these agents has always raised the possibility that they exert their effects in smooth muscle via the opening of an ATP-sensitive K-channel (K_{ATP}).

Until recently, direct evidence of this was lacking. However, Noack *et al.* (1992a,c) recently showed that exposure to levcromakalim or depletion of intracellular ATP induced K-currents in the rat portal vein. These currents (I_{KCO} and I_{met}, respectively) were inhibited by glibenclamide and were carried by a K-channel with a unitary conductance in the range 10–20 pS (stationary fluctuation analysis; quasi-physiological K-gradient). Noack *et al.* (1992a,c) concluded that I_{KCO} and I_{met} were carried by the same K-channel and that this channel possessed characteristics similar to those of a Type 1 K_{ATP} channel (Ashcroft & Ashcroft, 1990).

The ability of the sulphonylureas to inhibit the K-channel openers extends to a growing number of chemically-diverse agents (see review by Edwards & Weston, 1993). Perhaps the best known of these other substances is the α-adrenoceptor antagonist, phentolamine, which was demonstrated by McPherson & Angus (1989) to inhibit the effects of the K-channel openers in dog coronary artery. Since this report, it has been shown that inhibition of the openers by phentolamine extends not only to other smooth muscle preparations (Murray *et al.*, 1989) but also to the pancreatic β-cell (Plant & Henquin, 1990). In addition, the ability of other imidazolines such as efaroxan and antazoline to inhibit K_{ATP} has been reported in insulin-secreting cells (Dunne, 1991; Chan *et al.*, 1991; Jonas *et al.*, 1992).

The present study in the rat portal vein was designed to investigate whether the structurally-dissimilar K-channel openers P1060 (a potent, non-chiral pinacidil analogue) and aprikalim (a thioformamide) exert effects on K-currents similar to those previously reported for levcromakalim (Noack *et al.*, 1992a,c). In addition, the inhibitory actions of the sulphonylurea, glibenclamide, were compared with those of the 2-substituted imidazoline, phentolamine.

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Methods

All experiments were performed on single smooth muscle cells acutely isolated from male Sprague-Dawley rats (150–200 g), previously killed by stunning and cervical dislocation.

Isolation of cells

Portal veins were dissected into a nominally calcium-free physiological salt solution (Klöckner & Isenberg, 1985) and cleaned of extraneous connective tissue under a dissecting microscope. The vein was opened along its longitudinal axis and placed in a collagenase solution originally described for the separation of guinea-pig bladder smooth muscle cells (Klöckner & Isenberg, 1985). The tissue was agitated for 35 min in the enzyme solution at 37°C, washed in the same solution free of enzyme and cut into 4 segments. These segments were then triturated with a wide bore, smooth tipped pipette in Kraftbrühe (KB-medium; Klöckner & Isenberg, 1985). Cells were stored at 4°C in KB-medium and used within 9 h of separation. All experiments were performed at room temperature (22–26°C). Patch pipettes were made from Pyrex glass (H15/10, Jencons, UK) and had a resistance of 2–4 MOhm when filled with internal solution.

Single-cell electrophysiology

The whole-cell configuration of the patch clamp technique (Hamill *et al.*, 1981) was employed using an Axopatch-1C amplifier (Axon Instruments). The settling time of the system was less than 500 μs.

Voltage commands and data acquisition were performed on-line with an AT-compatible computer equipped with an Axon TL-1 interface. For cell stimulation and for recording and analysing data, the pClamp 5.5 programme was used (Axon Instruments). Data were stored on a digital audio tape recorder (Sony) and the evoked membrane currents were monitored continuously on a Gould Windograf recorder. The data described were not corrected by linear leak subtraction.

The bath containing the isolated cells was perfused with a Ca^{2+}-free physiological salt solution at approximately 0.7 ml min^{-1} using a peristaltic pump (LKB Microperpex); a second identical pump was used to remove the solution from the recording chamber. The effects of drugs were examined by adding appropriate quantities to the reservoir perfusing the bath so that responses could be examined under equilibrium conditions. Results are expressed as the mean ± s.e.mean with the number of observations in parentheses.

Drugs and solutions

The composition of the solutions used was as follows (mM): pipette (internal) 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, pH 7.3. In certain experiments 5 mM MgATP was also included in the pipette solution from which $MgCl_2$ was then omitted; bath (external) NaCl 125, KCl 4.8, $MgCl_2$ 3.7, KH_2PO_4 1.2, glucose 11, HEPES 10, EGTA 1, pH 7.3 aerated with O_2.

The enzyme solution for cell separation comprised (mM): KOH 130, $CaCl_2$ 0.05, taurine 20, pyruvate 5, creatine 5, HEPES 10, collagenase (1 mg ml^{-1}; Sigma Type VIII), pronase (0.2 mg ml^{-1}; Calbiochem), fatty acid-free albumin (1 mg ml^{-1}; Sigma). The pH was adjusted to 7.4 with methanesulphonic acid.

The following drugs were used: aprikalim (formerly RP52891, Rhône Poulenc Rorer), P1060 (N'-cyano-N-(3-pyridyl)-N''-(*t*-butyl)guanidine; Leo), glibenclamide (Sigma), phentolamine hydrochloride (Sigma). These agents were made up as 20 mM stock solutions in dimethyl sulphoxide (except phentolamine hydrochloride which was dissolved in twice distilled water).

Results

In the voltage clamp mode, the whole-cell currents recorded from freshly isolated rat portal vein cells were identical to those reported by Noack *et al.* (1992a,c). When cells were held at − 90 mV and stepped for 500 ms to test potentials ranging between − 80 mV and + 30 mV (10 mV increments), a current with fast activation and fast inactivation kinetics was observed. This current exhibited activation and inactivation time-courses very similar to those of an A-current. In addition, when cells were depolarized to test potentials positive to − 30 mV, a slowly activating, slowing inactivating current developed (Figure 1). This delayed rectifier-type current was designated I_{TO} (transient outward current) by Noack and co-workers (1992a). Following the complete inactivation of I_{TO}, a steady current level that exhibited no inactivation was observed. This current alone was available when cells were clamped at − 10 mV to inactivate the transient currents described above (Figure 1) and was designated I_{NI} (Noack *et al.*, 1992a). The magnitude of I_{TO} was determined by digital subtraction of I_{NI} (induced when stepping to test potentials from a holding potential of − 10 mV) from those currents induced when stepping to identical test potentials from − 90 mV. Measurement of I_{TO} at 200 ms after stepping from the command potential ensured that the A-like current had completely inactivated and therefore did not contaminate the values obtained for I_{TO}.

Generation of I_{KCO} by P1060 and aprikalim

P1060 and aprikalim each caused a concentration-dependent increase in the outward current when the cells were clamped at − 10 mV (Figure 2). This current was designated I_{KCO} and its magnitude was concentration-dependent over the range 300 nM–10 μM for P1060 and 3 μM–30 μM for aprikalim. When the effects of approximately EC_{80} concentrations of P1060 (1 μM) and aprikalim (3 μM) on I_{KCO} were compared, the maximum increase in I_{KCO} following exposure to P1060 was achieved after 7.0 ± 1.2 min compared with 11.4 ± 2.6 min in the presence of aprikalim (see Figure 2; $P<0.05$, Student's t test, $n = 5–6$).

Single-channel conductance underlying the effects of P1060 and aprikalim

The effects of P1060 and aprikalim were compared with those previously obtained for levcromakalim and for ATP-depletion (Noack *et al.*, 1992a,c). From the current noise signals (see Figure 2) generated by these K-channel openers at a constant holding potential (− 10 mV), recording segments of 2 s duration were sampled for further analysis. From these, amplitude histograms were constructed and Gaussian distributions fitted to them by computer. For each cell, the maximum number of possible observations was obtained and ranged from 5 to 54 (mean = 14, $n = 11$). This number was dependent on the rate of onset of the increase in holding current for each individual cell following exposure to P1060 or aprikalim. The mean current (μ) and current variance (σ^2) obtained at different stages of the experiment were analysed as previously described (Noack *et al.*, 1992a).

Using data derived from a total of 8 cells of comparable size, I_{KCO} induced by P1060 (1 μM) and by aprikalim (3 μM) was calculated to consist of single channel currents of 0.41 pA ± 0.08 pA ($n = 4$) and 0.41 ± 0.05 pA ($n = 4$), respectively. There was thus no difference between the target channels of these two agents. When the single channel conductance was calculated according to the Goldman-Hodgkin-Katz equation for both P1060 and aprikalim, a value of 10.5 pS (at 0 mV and under the quasi-physiological potassium gradient of the experiments) was obtained. For both agents, the best fitted curves gave values for the number of channels (N) which contributed to the generation of I_{KCO} in the range 200 to 2000 per cell.

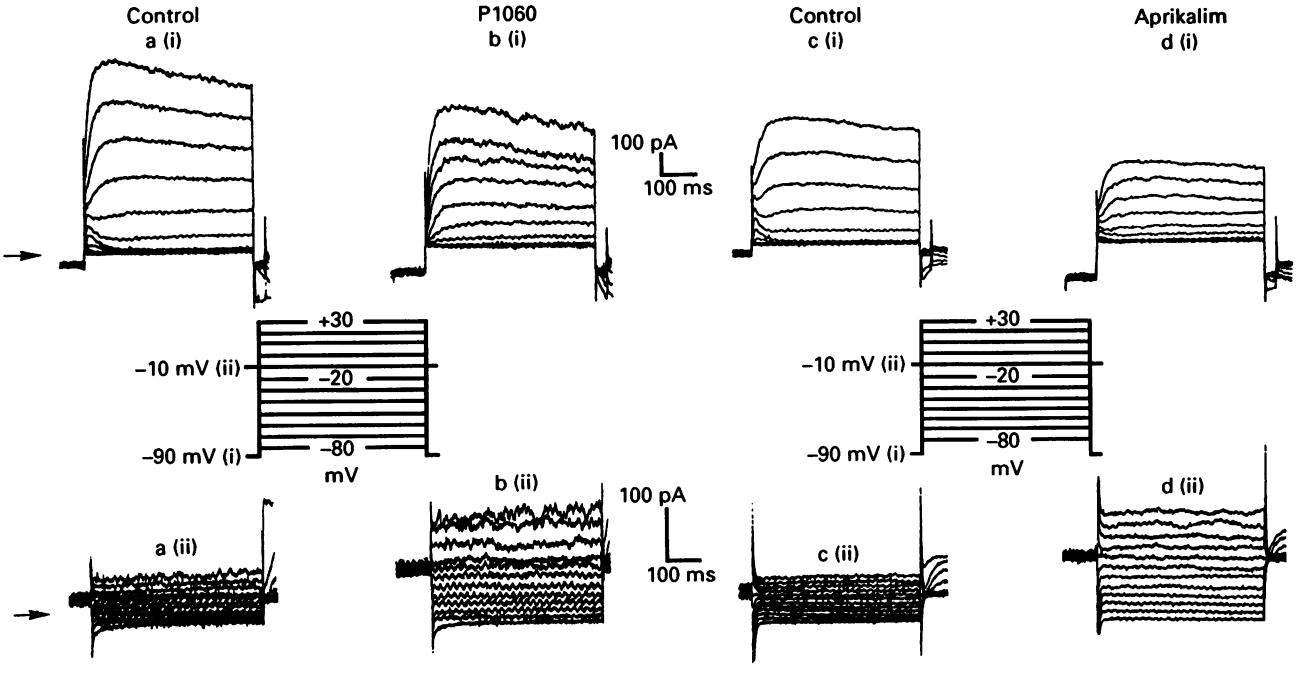


Figure 1 Typical traces demonstrating the inhibition of the delayed rectifier current (I_{TO}) and the induction of the non-inactivating current (I_{KCO}) by (a,b) P1060 (1 μM) and (c,d) aprikalim (3 μM). Control currents (a,c) and currents in the presence of P1060 (b) or aprikalim (d) are displayed for comparison. The upper traces show the delayed rectifier current (I_{TO}) derived by digital subtraction of the non-inactivating currents available when stepping to a series of test potentials from a holding potential of − 10 mV (ii), from the total currents available when stepping to the same series of test potentials from a holding potential of − 90 mV (i). The lower traces are the currents obtained when stepping to the test potentials from a holding potential of − 10 mV. Note the inhibition of I_{TO} in b(i) and d(i) and the generation of I_{KCO} in b(ii) and d(ii). Traces a,b and c,d were derived from two different cells. The arrows indicate the zero current level.

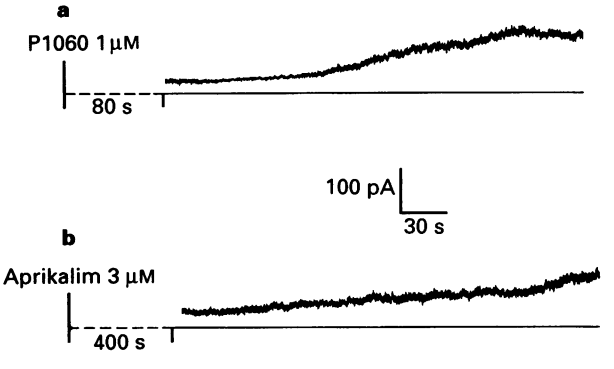


Figure 2 Typical development of I_{KCO} in two different cells from rat portal vein following exposure to (a) P1060 (1 μM) and (b) aprikalim (3 μM). Holding potential − 10 mV. Note the characteristic increase in current noise during the development of I_{KCO} and that the rate of onset of I_{KCO} was faster for P1060 than for aprikalim.

Noise analysis theory predicts a decrease in current noise when the mean value for the open probability is larger than 0.5. Values obtained for μ and σ^2 indicated that both aprikalim and P1060 could each increase the open probability to a value in the range 0.5–0.6 (6 out of 11 cells).

Effects of P1060 and aprikalim on I-*V relationships*

To determine the effects of P1060 and aprikalim in more detail, current-voltage (*I*-V) experiments were carried out in the presence of P1060 (1 μM) or aprikalim (3 μM), concentrations which represented the respective EC_{80} values for the generation of I_{KCO}. Stepping to a range of test potentials from alternate holding potentials of − 90 mV and − 10 mV allowed the generation of total currents (inactivating plus non-inactivating currents) or non-inactivating currents alone, respectively. Subsequent digital subtraction of the non-activating current from the total current at each test potential permitted the evaluation of the effects of P1060 and aprikalim on the delayed rectifier current component, I_{TO}.

On stepping to a series of test potentials from a holding potential of − 10 mV, the generation of I_{KCO} by P1060 (1 μM) and aprikalim (3 μM) was characterized by an increase in the magnitude of the evoked non-inactivating currents over the voltage range employed (− 80 to + 30 mV; Figure 3). At 0 mV, for example, the magnitude of currents in the presence of P1060 and aprikalim was 142.4 ± 26.9 pA and 88.9 ± 14.42 pA, respectively ($n = 6$) compared with control values of 22.9 ± 3.5 pA and 15.3 ± 4.3 pA, respectively ($n = 6$). It can be seen from the *I*-V plots (Figure 3) that the reversal potential of the non-inactivating current complex was moved in a hyperpolarizing direction in the presence of P1060 and aprikalim. Prior to exposure to either agent, control currents reversed near − 30 mV but in the presence of these openers the reversal potential was shifted to approximately − 55 mV (Figure 3). For both P1060 and aprikalim, respective control and test curves intersected at approximately − 78 mV, a value close to the theoretical value for E_K (− 83 mV) in these experiments and indicating that I_{KCO} was a K-current.

P1060 (1 μM) and aprikalim (3 μM) each inhibited the delayed rectifier current, I_{TO}. In the case of aprikalim, this inhibition was evident at all test potentials over the activation range of I_{TO}, whereas P1060 only inhibited I_{TO} at potentials positive to 0 mV (control 280.4 ± 28.0 pA, P1060 240.5 ± 21.9 pA, $n = 7$; control 252.7 ± 61.0 pA, aprikalim 121.5 ± 25.3 pA, $n = 7$ at 0 mV, Figure 3). Treatment of cells with vehicle alone (dimethyl sulphoxide, 0.06%) had no effect on the rat portal vein K-currents ($n = 3$, data not shown).

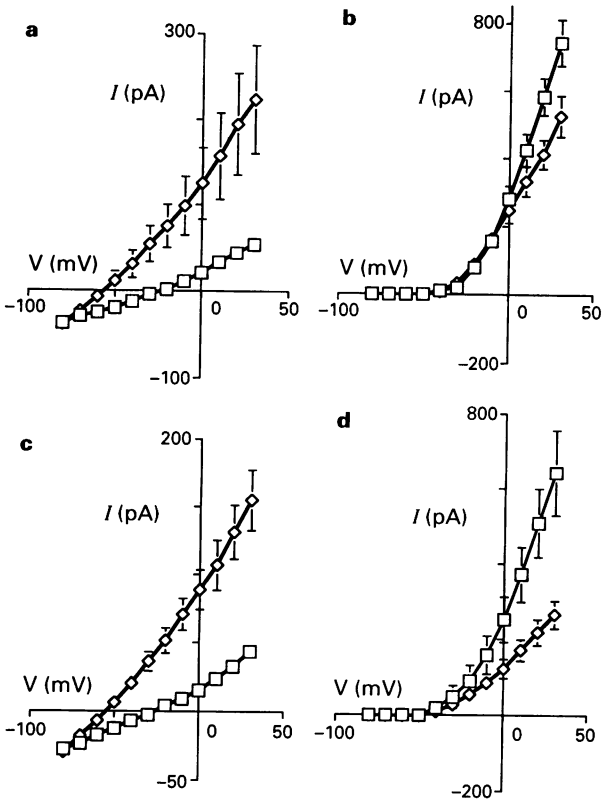


Figure 3 The effects of P1060 (1 μM, ◇; a,b) and aprikalim (3 μM, ◇; c,d) on the whole-cell currents in isolated single cells from rat portal vein. (a) and (c) illustrate the generation by these K-channel openers of I_{KCO} on stepping to test potentials from a holding potential of − 10 mV. (b) and (d) show the effects of the K-channel openers on the transient outward current (I_{TO}) calculated by digitally subtracting the current available when stepping from a holding potential of − 10 mV from that available when stepping from a holding potential of − 90 mV. (□) Control currents. Each point represents the mean value derived from 6 observations ± s.e.mean.

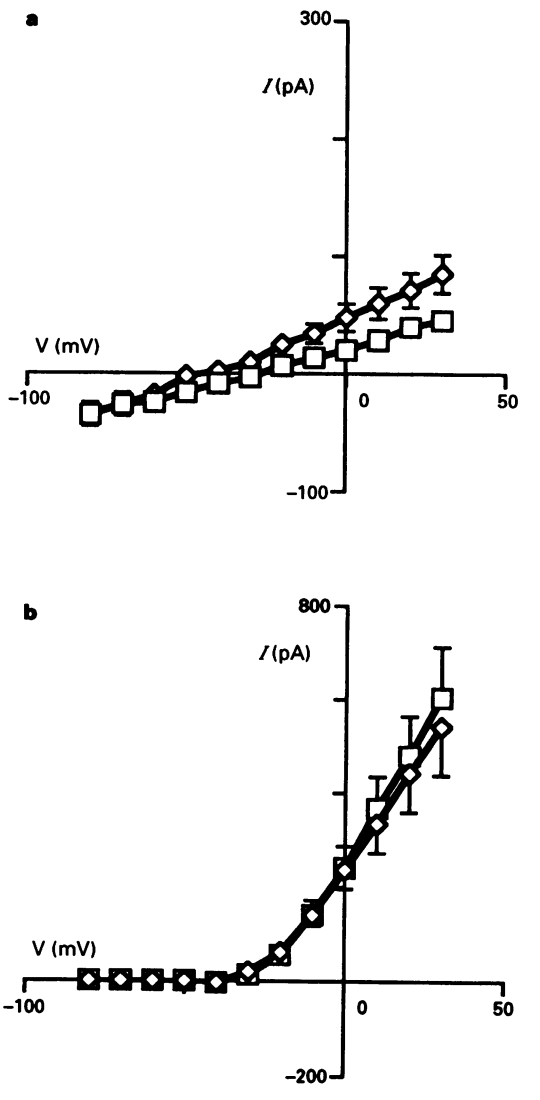


Figure 4 The effect of P1060 (1 μM, ◇) on currents induced in single cells from rat portal vein when the pipette (intracellular) solution contained 5 mM MgATP. (a) Generation of I_{KCO} by P1060 on stepping to test potentials from a holding potential of − 10 mV. (b) Effects of P1060 on the transient outward current (I_{TO}) calculated by digitally subtracting the current available when stepping from a holding potential of − 10 mV from that available when stepping from a holding potential of − 90 mV. (□) Control currents obtained in the presence of 5 mM intracellular ATP. Each point represents the mean value derived from 5 observations ± s.e.mean.

Effect of addition of MgATP to the recording pipette

Since I_{KCO} generated by levcromakalim is carried by ATP-sensitive K-channels (Noack *et al.*, 1992c), we investigated whether the addition of a relatively high concentration (5 mM) of MgATP to the pipette solution could modify the effects of P1060 on K-currents in the rat portal vein. With this concentration of MgATP in the pipette, neither the time course of development (data not shown) nor the magnitude of I_{NI} or I_{TO} was affected (compare control values in Figures 3 and 4). In comparison, inclusion of 5 mM MgATP in the pipette inhibited the stimulation of I_{KCO} by 1 μM P1060 and reduced the inhibitory effect of P1060 on I_{TO} (compare Figures 3 and 4).

Effects of glibenclamide and phentolamine

Addition of glibenclamide (1 μM) to the recording chamber had no significant effect on either I_{NI} or I_{TO} (Figure 5). Phentolamine (30 μM–100 μM) had no effect on I_{NI} but caused concentration-dependent inhibition of I_{TO} (Figure 5). The degree of inhibition of the effects of the K-channel openers by glibenclamide or phentolamine was dependent on the timing of exposure to these agents. Thus, if tissues were first exposed to P1060 (1 μM) or aprikalim (3 μM), the I_{KCO} which developed was fully reversed by subsequent exposure to glibenclamide (1 μM; Figure 6) but only partially reversed by phentolamine (30 μM; Figure 6). Aprikalim (3 μM)-induced inhibition of I_{TO} was partially reversed by the subsequent addition of glibenclamide (1 μM; Figure 7) to the recording chamber whereas phentolamine was without effect. However, prior exposure to glibenclamide (1 μM) completely prevented both the development of I_{KCO} by aprikalim (3 μM) and the associated inhibition of I_{TO} (Figure 7). Pretreatment with phentolamine also prevented the development of I_{KCO} but failed to prevent the inhibition of I_{TO} by aprikalim (Figure 8).

Discussion

Similarities between structurally-different K-channel openers

The present study in rat portal vein has demonstrated that the K-channel openers P1060 (a cyanoguanidine, pinacidil

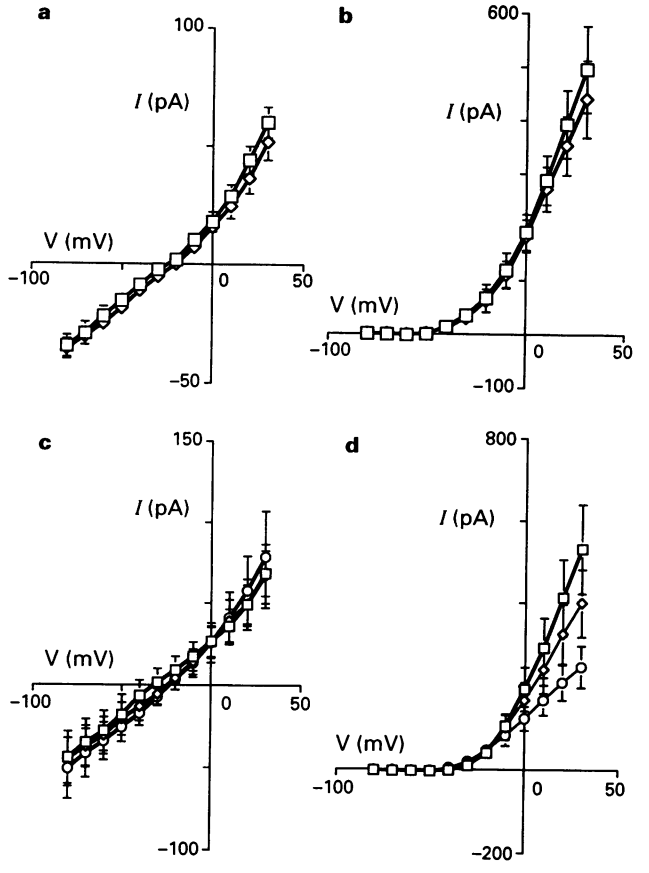


Figure 5 Effects of glibenclamide (1 μM, ◇; a,b) and phentolamine (30 μM, ◇; 100 μM, ○; c,d) on currents in rat portal vein cells. (a) and (c) show effects on non-inactivating currents obtained on stepping to test potentials from − 10 mV. (b) and (d) illustrate the effects on the transient outward current (I_{TO}) calculated by digitally subtracting the current available when stepping from − 10 mV from that available when stepping from − 90 mV. (□) Indicates control currents. Each point represents the mean value derived from 3 to 7 observations ± s.e.mean.

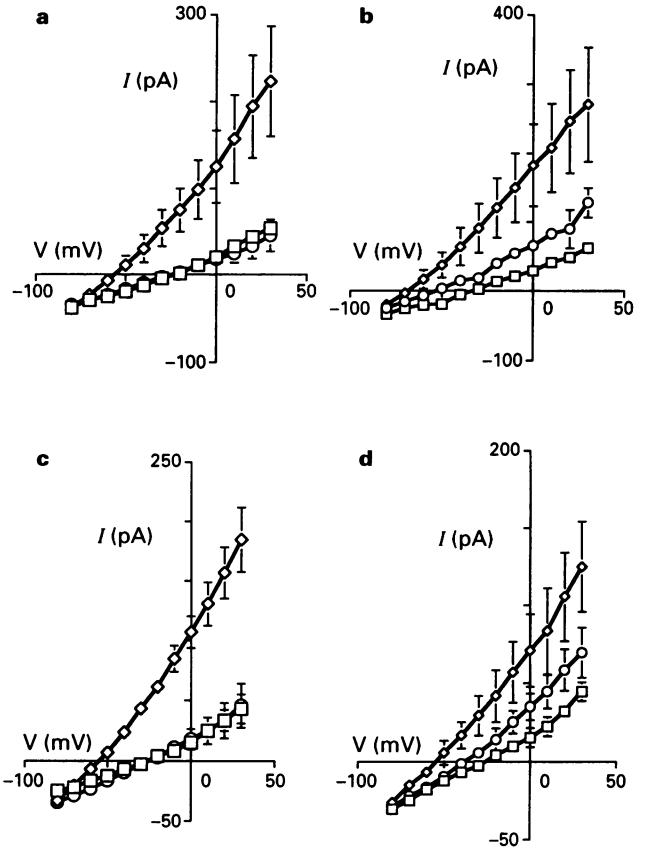


Figure 6 Effect of glibenclamide (1 μM; a,c) and phentolamine (30 μM; b,d) on the induction of I_{KCO} by P1060 (1 μM; a,b) and aprikalim (3 μM; c,d). (□) Control currents; (◇) currents in the presence of the respective K-channel opener; (○) currents in the presence of the K-channel opener and respective antagonist. Each point represents the mean value derived from 3 observations ± s.e.mean.

analogue) and aprikalim (a thioformamide) generate a non-activating K-current, I_{KCO}. In addition, P1060 and aprikalim each inhibited I_{TO}, a slowly activating, time- and voltage-dependent transient outward current, the properties of which were similar to those of a delayed rectifier current (see Bolton & Beech, 1992). The generation of I_{KCO}, together with the simultaneous reduction in I_{TO} produced by these agents are properties identical to those exhibited in rat portal vein by the benzopyran, levcromakalim (Noack *et al.*, 1992a). Thus, three structurally-dissimilar prototype K-channel openers produce qualitatively similar effects on macro K-currents which strongly suggests that all share common sites and mechanisms of action.

Such a conclusion is supported by analysis of the current noise associated with I_{KCO} in the present study. Using this procedure, a value of 0.4 pA was obtained for the single channel current from which the unitary conductance underlying I_{KCO} was calculated to be 10.5 pS at 0 mV under the quasiphysiological conditions of the present experiments. These values correlate well with the data obtained by Noack *et al.* (1992a,c) for levcromakalim and indicate that the same K-channel underlies the current I_{KCO} generated by the three types of K-channel opener. However, the results of the present study, together with those of previous investigations (Noack *et al.*, 1992a,c) give no support to the view (Standen *et al.*, 1989) that a K-channel of unitary conductance 146 pS represents the target of agents like P1060, aprikalim and levcromakalim.

Modulation of I_{KCO} by ATP: site of action of the K-channel openers

In the present study, inclusion of MgATP in the recording pipette inhibited the induction of I_{KCO} by P1060 suggesting that the K-channel underlying this current was ATP-sensitive. Such a view is consistent with that expressed in an earlier study (Noack *et al.*, 1992c) which also concluded that an ATP-sensitive K-channel (K_{ATP}; broadly similar to the Type 1 K_{ATP} described by Ashcroft & Ashcroft, 1990) carried I_{KCO}. In this investigation, Noack and coworkers (1992c) found that removal of substrates for the carboxylic acid cycle from the pipette solution (thus reducing the intracellular ATP concentration, $[ATP]_i$) induced a current (I_{met}) which declined with time and which had an underlying unitary conductance similar to that of I_{KCO} induced by levcromakalim. Furthermore, after 'run-down' of I_{met}, effects of levcromakalim could not be observed.

ATP is thought to regulate K_{ATP} by an interaction with two independent sites. At one of these, ATP binds and inhibits channel opening, whereas phosphorylation of the other site is a prerequisite for the flow of K-current (see Ashcroft, 1988; Edwards & Weston, 1993). The ability of K-channel openers to induce I_{KCO} (present study; Noack *et al.*, 1992a,c), the inhibition of this effect by inclusion of ATP in the recording pipette (present study) and the production of a current, I_{met}, with characteristics identical to those of I_{KCO}, by reduction of $[ATP]_i$ (Noack *et al.*, 1992c) combine to suggest that the K-channel openers compete with ATP for access to the ATP binding sites on K_{ATP} and that this is the basic mechanism

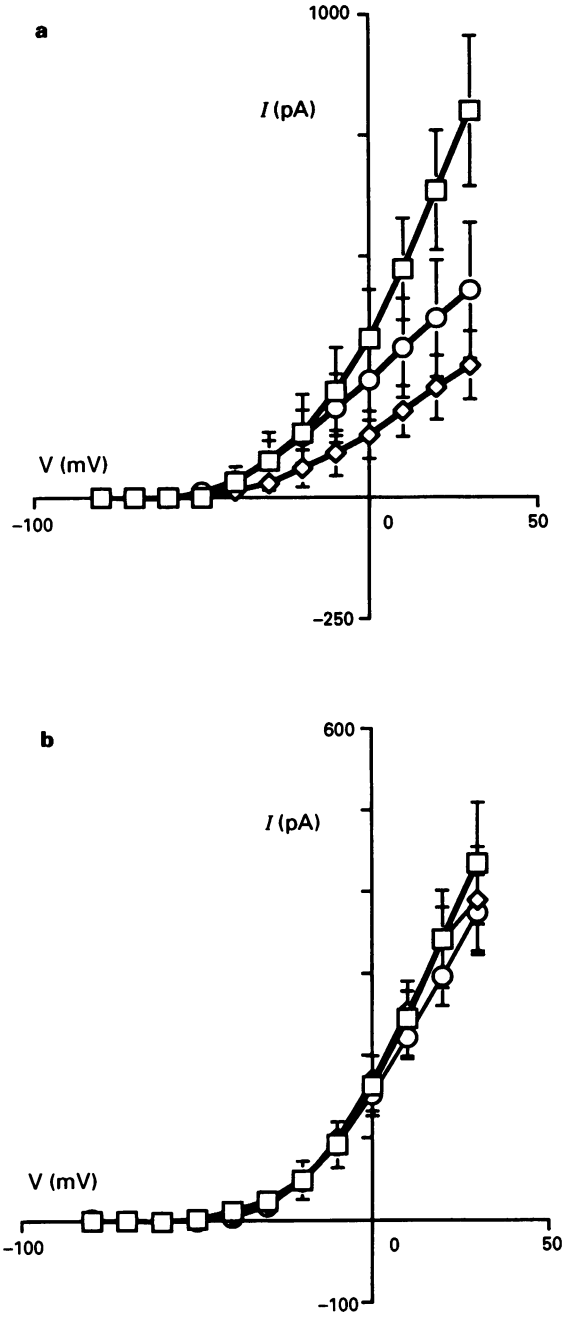


Figure 7 Effect of glibenclamide (1 μM) on the aprikalim (3 μM)-induced inhibition of I_{TO}. (a) Partial reversal of the aprikalim-induced inhibition of I_{TO} by glibenclamide. (b) Inhibition of the effect of aprikalim on I_{TO} by pre-exposure to glibenclamide. (□) Control current; (◇) current in the presence of aprikalim (a) or glibenclamide (b) alone; (○) current in the presence of both glibenclamide and aprikalim. Each point represents the mean value derived from 3 observations ± s.e.mean.

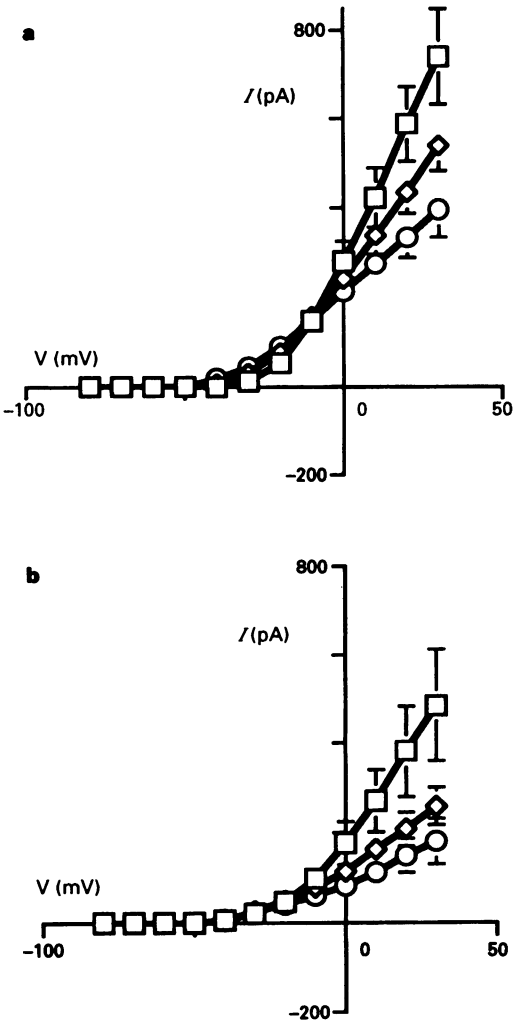


Figure 8 Effect of phentolamine (30 μM) on the inhibition of I_{TO} by P1060 (1 μM; a) and aprikalim (3 μM; b). (□) Control current; (◇) current in the presence of the respective K-channel opener; (○) current in the presence of the respective opener and phentolamine (30 μM). Each point represents the mean value derived from 3 observations ± s.e.mean.

which underlies the ability of these agents to open K_{ATP}. This suggestion, originally proposed by Thuringer & Escande (1989) following studies on the action of RP49356 in cardiac muscle, has received further support from an investigation of the effects of K-channel openers on ATP-sensitive chloride (Cl) currents (Sheppard & Welsh, 1992). These workers showed that levcromakalim, diazoxide and minoxidil sulphate closed the Cl-channels which underlie these currents. Since these Cl_{ATP} channels, in direct contrast to K_{ATP} in smooth muscle, close as ATP binding decreases, these data strongly support the view that the K-channel openers compete with ATP for the ATP-binding sites which modulate channel opening. The results further suggest that the (so-called) K-channel openers could exert effects wherever ATP binding sites exist with characteristics similar to those on K_{ATP} and Cl_{ATP}.

Inhibition of the delayed rectifier current, I_{TO}: possible link with I_{KCO}

Like K_{ATP}, the K-channels (K_V) responsible for carrying delayed rectifier currents must also be phosphorylated before they can be opened (see Peroso & Bezanilla, 1990). Thus, the ability of both P1060 and aprikalim (present study) and of levcromakalim (Noack *et al.*, 1990a) to inhibit the delayed rectifier current, I_{TO}, could be explained if these agents were able to prevent phosphorylation of K_V. This would induce a state of channel 'run-down' which would manifest itself as a reduction in the whole-cell current, I_{TO}. If the K-channel openers can indeed reduce the degree of phosphorylation of the delayed rectifier channel (K_V) with consequent reduction of I_{TO}, such an action could also be exerted at other ion channels which require phosphorylation prior to opening. One such channel is K_{ATP} itself (see Ashcroft, 1988) and it is possible that reduced phosphorylation and run-down could

account for the previously-reported anomalous closure of K_{ATP} by the K-channel openers (insulinoma cell, Dunne, 1990; cardiac ventricle, Faivre & Findlay, 1989) under certain conditions. In both the present study and in an earlier investigation (Noack *et al.*, 1992a), P1060, aprikalim and levcromakalim were each unable to increase the open probability of K_{ATP} to values greater than the range of 0.5–0.6. This suggests that the ability of all three agents to open K_{ATP} by decreasing ATP binding to this channel could be limited by the simultaneous induction of channel run-down.

Inhibitory effects of glibenclamide on I_{KCO}

As previously reported for levcromakalim (Noack *et al.*, 1992a), the induction of I_{KCO} by P1060 or aprikalim was inhibited by glibenclamide (1–10 μM). This effect could be observed either by application of glibenclamide after I_{KCO} had become established or by inclusion of glibenclamide in the bath solution prior to exposure to the K-channel opener. However, in the same concentration-range, glibenclamide itself had no effect on either I_{TO} or on the complex non-inactivating currents designated I_{NI}. This is the first clear demonstration in smooth muscle that even in micromolar concentrations, glibenclamide is a selective inhibitor of I_{KCO}, with essentially no such effect on the other calcium-independent outward currents, I_{TO} and I_{NI}. In whole portal veins bathed in normal, calcium-containing physiological salt solutions, glibenclamide (up to 10 μM) did not significantly modify spontaneous mechanical activity (Noack *et al.*, 1992b). Such an observation is consistent with the lack of effect of glibenclamide on I_{TO} and I_{NI} observed in the present study and further suggests that any effect of this sulphonylurea on calcium-dependent K-currrents is negligible.

An important objective of the present investigation was to determine the effect of glibenclamide on the inhibition of I_{TO} produced by P1060 and aprikalim since in an earlier study, Noack *et al.* (1992a) had reported that this phenomenon was glibenclamide-insensitive. Initial experiments were performed by first exposing the cells to either P1060 or aprikalim and allowing the inhibition of I_{TO} to develop followed by superfusion with glibenclamide in the continuing presence of the K-channel opener. Using this approach, identical to the one adopted by Noack *et al.* (1992a), reversal of the opener-induced inhibition of I_{TO} was seen in some cells, but not in others. Thus, in an attempt to clarify the position, further experiments were carried out by first exposing cells to glibenclamide and then to aprikalim, the opener which produced a greater inhibition of I_{TO} compared with P1060. Under these conditions in the continuing presence of glibenclamide, the ability of aprikalim to reduce the size of I_{TO} was abolished.

From these studies it is not possible to explain definitively why the relative timing of exposure to glibenclamide and aprikalim was so critical before consistent reversal of the aprikalim-induced inhibition of I_{TO} could be observed. It was suggested by Noack *et al.* (1992a) that the inhibition of I_{TO} caused by levcromakalim could be due to a decrease in the number of K_V channels in the phosphorylated state (i.e. an increase in the run-down of K_V). Antagonism of the K-channel openers by glibenclamide could involve inhibition of an A-kinase involved at a critical point in the glycolytic cascade (see Caro, 1990). This would shift this pathway in the direction of glycolysis and result in a net synthesis of ATP. If a reduction in the size of I_{TO} by the K-channel openers involves prevention of the phosphorylation of the delayed rectifier channel as already discussed, then a glibenclamide-driven increase in $[ATP]_i$ (under conditions in which there was a critical balance between ATP concentration and phosphorylation of the channel) could serve to reverse or prevent the inhibition of I_{TO} seen in the absence of glibenclamide. Thus, if such functional antagonism underlies glibenclamide-induced reversal of the inhibition of I_{TO} by aprikalim (present study), the relative contact times of glibenclamide and opener could be critical in determining the degree of observed antagonism.

Inhibitory effects of phentolamine on I_{KCO} *and* I_{TO}

In the present investigation, micromolar concentrations of phentolamine, like glibenclamide, had no effect on I_{NI} but inhibited the generation of I_{KCO} by both P1060 and aprikalim. Although phentolamine has previously been shown to inhibit K_{ATP} in insulin-secreting cells (Dunne, 1991; Jonas *et al.*, 1992), this is the first demonstration of its ability to inhibit a K-current in isolated smooth muscle cells and it explains the basis of the phentolamine-induced reversal of the mechano-inhibitory actions of K-channel openers in a variety of tissues (McPherson & Angus, 1989; Murray *et al.*, 1989).

In addition to its ability to inhibit I_{KCO}, phentolamine also produced a decrease in the magnitude of I_{TO}, especially at the upper end of the tested concentration-range. These effects of phentolamine were thus similar to those of the imidazoisoindole derivative, ciclazindol, which also inhibited both I_{KCO} and the delayed rectifier current, I_{TO} (Noack *et al.*, 1992b). Both ciclazindol (Noack *et al.*, 1992b) and phentolamine (Schwietert *et al.*, 1992; Weston, unpublished) stimulate the spontaneous mechanical activity of the rat portal vein and phentolamine induces marked rhythmic contractions in guinea-pig bladder detrusor muscle (Satake *et al.*, 1984). We thus suggest that it is the inhibition of the smooth muscle delayed rectifier which underlies the ability of ciclazindol, phentolamine and of other agents like antazoline (see Schwietert *et al.*, 1992) to increase spontaneous activity in these tissues. Such a view is supported by the finding that this mechanical effect is shared by the sotalol derivative E-4031 (Schwietert *et al.*, 1992), a compound known to be a potent inhibitor of the delayed rectifier channel in cardiac tissue (Wettwer *et al.*, 1991).

In contrast to its ability to inhibit I_{KCO}, phentolamine did not reverse the opener-induced inhibition of I_{TO}. Indeed, as already detailed, phentolamine itself inhibited I_{TO}, and there was a trend for this inhibition to increase in the joint presence of both phentolamine and the openers. Further experiments are clearly required to determine not only the mechanism which underlies antagonism of the K-channel openers by phentolamine but also that by which phentolamine inhibits I_{TO}.

Conclusions

The results of the present study in rat portal vein cells have shown that both P1060 and aprikalim induced I_{KCO}, a non-inactivating K-current and simultaneously inhibited I_{TO}, a current with the characteristics of a delayed rectifier. This pattern of K-current modulation was identical to that described in an earlier investigation with levcromakalim (Noack *et al.*, 1992a). It suggests that all three structurally-diverse K-channel openers share common mechanistic features and induce the current, I_{KCO}, by an action on a small conductance, glibenclamide- and ATP-sensitive K-channel. In the absence of the K-channel openers, glibenclamide itself had no effect on the control inactivating and non-inactivating currents. In addition, P1060 and aprikalim also inhibited the delayed rectifier current, I_{TO}, an effect which in the case of aprikalim was reversed by glibenclamide but not by phentolamine. It was found that phentolamine was itself able to inhibit I_{TO} in these cells, but that glibenclamide alone exerted no significant effect on this delayed rectifier current. Further studies are in progress to determine in more detail the mechanisms which underlie the actions of these K-channel modulators.

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References

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

ASHCROFT, S.J.H. & ASHCROFT, F.M. (1990). Properties and functions of ATP-sensitive K-channels. *Cell Signalling*, **2**, 197–214.

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

CARO, J.F. (1990). Effects of glyburide on carbohydrate metabolism and insulin action in the liver. *Am. J. Med.*, **89** (Suppl 2A), 17S–25S.

CHAN, S.L.F., DUNNE, M.J., STILLINGS, M.R. & MORGAN, N.G. (1991). The α_2-adrenoceptor antagonist efaroxan modulates K_{ATP} channels in insulin secreting cells. *Eur. J. Pharmacol.*, **204**, 41–48.

DUNNE, M.J. (1990). Effects of pinacidil, RP 49356 and nicorandil on ATP-sensitive potassium channels in insulin-secreting cells. *Br. J. Pharmacol.*, **99**, 487–492.

DUNNE, M.J. (1991). Block of ATP-regulated potassium channels by phentolamine and other α-adrenoceptor antagonists. *Br. J. Pharmacol.*, **103**, 1847–1850.

EDWARDS, G. & WESTON, A.H. (1990). Structure activity relationships of K-channel openers. *Trends Pharmacol. Sci.*, **11**, 417–422.

EDWARDS, G. & WESTON, A.H. (1993). The pharmacology of ATP-sensitive potassium channels. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 597–637.

FAIVRE, J.-F. & FINDLAY, I. (1989). Effects of tolbutamide, glibenclamide and diazoxide upon action potentials recorded from rat ventricular muscle. *Biochem. Biophys. Acta.*, **984**, 1–5.

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.

HU, S., KIM, H.S., OKOLIE, P. & WEISS, G.B. (1990). Alterations by glyburide of effects of BRL 34915 and P1060 on contraction, ^{86}Rb efflux and the maxi K^+ channel in rat portal vein. *J. Pharmacol. Exp. Ther.*, **253**, 771–777.

JONAS, J.C., PLANT, T.D. & HENQUIN, J.C. (1992). Imidazoline antagonists of α_2-adrenoceptors increase insulin release *in vitro* by inhibiting ATP-sensitive K^+ channels in pancreatic β-cells. *Br. J. Pharmacol.*, **107**, 8–14.

KLÖCKNER, U. & ISENBERG, G. (1985). Action potentials and net membrane currents of isolated smooth muscle cells (urinary bladder of the guinea-pig). *Pflügers Arch.*, **405**, 329–339.

MCPHERSON, G.A. & ANGUS, J.A. (1989). Phentolamine and structurally related compounds selectively antagonise the vascular actions of the K^+ channel opener, cromakalim. *Br. J. Pharmacol.*, **97**, 941–949.

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.

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. & WESTON, A.H. (1992b). The involvement of potassium channels in the action of ciclazindol in rat portal vein. *Br. J. Pharmacol.*, **106**, 17–24.

NOACK, TH., EDWARDS, G., DEITMER, P. & WESTON, A.H. (1992c). Potassium channel modulation in rat portal vein by ATP depletion; a comparison with the effects of levcromakalim (BRL 38227). *Br. J. Pharmacol.*, **107**, 945–955.

PEROSO, E. & BEZANILLA, F. (1990). Phosphorylation affects voltage-gating of the delayed rectifier K^+ channel by electrostatic interactions. *Neuron*, **5**, 685–690.

PLANT, T.D. & HENQUIN, J.C. (1990). Phentolamine and yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic β-cells. *Br. J. Pharmacol.*, **101**, 115–120.

SATAKE, N., SHIBATA, S. & UEDA, S. (1990). Phentolamine-induced rhythmic contractions in bladder detrusor muscle of guinea-pig. *Br. J. Pharmacol.*, **83**, 965–971.

SCHWIETERT, R., WILHELM, D., WILLFERT, B. & VAN ZWIETEN, P.A. (1992). The effect of some α-adrenoceptor antagonists on spontaneous myogenic activity in the rat portal vein and the putative involvement of ATP-sensitive K^+ channels. *Eur. J. Pharmacol.*, **211**, 87–95.

SHEPPARD, D.N. & WELSH, M.J. (1992). Effect of ATP-sensitive K^+ channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents. *J. Gen. Physiol.*, **100**, 573–591.

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.*, **172**, 517–522.

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.

THURINGER, D. & ESCANDE, D. (1989). Apparent competition between ATP and the potassium channel opener RP 49356 on ATP-sensitive K^+ channels of cardiac myocytes. *Mol. Pharmacol.*, **36**, 897–902.

WETTWER, E., SCHOLTYSIK, G., SCHAAD, A., HIMMEL, H. & RAVENS, U. (1991). Effects of the new class III antiarrhythmic drug E-4031 on myocardial contractility and electrophysiological parameters. *J. Cardiovasc. Pharmacol.*, **17**, 480–487.

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Pharmacological characteristics of the positive inotropic effect of angiotensin II in the rabbit ventricular myocardium

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1 In order to elucidate the mechanism underlying the positive inotropic effect (PIE) of angiotensin II (AII), we measured changes in phosphoinositide hydrolysis and contractile force induced by AII in the rabbit ventricular myocardium.

2 AII (1.0 nM–3 μM) produced a PIE in a concentration-dependent manner in the presence of bupranolol (0.3 μM) and prazosin (0.1 μM), the maximal response being about 40% of that to isoprenaline and the EC_{50} 30 nM.

3 The PIE of AII was associated with a concentration-dependent increase in the total duration of contraction; the time to peak force and the relaxation time were prolonged.

4 AII (10 nM–30 μM) elicited an accumulation of [^{3}H]-inositol monophosphate in a concentration-dependent manner in rabbit ventricular slices prelabelled with *myo*-[^{3}H]-inositol.

5 The PIE and the accumulation of [^{3}H]-inositol monophosphate induced by AII were inhibited by a non-selective AII receptor antagonist, saralasin (10 nM–1 μM) and by a selective AT_1 receptor antagonist, losartan (10 nM–1 μM), but not a selective AT_2 receptor antagonist, PD 123319 (1 μM).

6 A tumour-promoting phorbol ester, phorbol 12,13-dibutyrate (PDBu, 10–100 nM), inhibited the AII-induced PIE and [^{3}H]-inositol monophosphate accumulation in a concentration-dependent manner.

7 These results suggest that AII exerts a PIE through activation of AT_1 receptors and subsequent acceleration of phosphoinositide hydrolysis. Activation of protein kinase C by PDBu may inhibit the AII-induced stimulation of phosphoinositide hydrolysis and thereby the PIE of AII in the rabbit ventricular myocardium.

Keywords: Angiotensin II; saralasin; losartan; PD 123319; AT_1 receptor; positive inotropic effect; phosphoinositide hydrolysis; inositol phosphates; phorbol 12,13-dibutyrate; rabbit ventricular myocardium

Introduction

Angiotensin II (AII) plays an important role in the control of cardiovascular haemodynamic homeostasis through activation of the renin-angiotensin system. In addition to its well-established constrictor effect on the vascular smooth muscle, AII produces a positive inotropic effect (PIE) in mammalian ventricular myocardium including that of man (Schomisch *et al.*, 1990), dog (Kobayashi *et al.*, 1978) and rabbit (Dempsey *et al.*, 1971) and in the chick heart (Baker & Aceto, 1989). In whole animal experiments, AII modulates the myocardial contractility via a direct pathway and by an indirect pathway which is mediated by a cardiovascular reflex. Increased release of catecholamines may contribute to the indirect inotropic effect of AII (Ross & White, 1966). The mechanism of the direct PIE caused by AII has been postulated to be due to activation of specific AII receptors in myocardial cells (Dempsey *et al.*, 1971). Various pathways of intracellular signal transduction have been implicated as possible subcellular mechanisms of the action of AII: the activation of the slow Ca^{2+} channel (Freer *et al.*, 1976), acceleration of phosphoinositide hydrolysis (Baker & Aceto, 1989) and inhibition of adenylate cyclase (Anand-Srivastava, 1989). Furthermore, since AII stimulates protein synthesis in the myocardium, it has been suggested that AII may be involved in the pathogenesis of cardiac hypertrophy (Aceto & Baker, 1990). Recently, type 1 and type 2 AII receptor subtypes have been distinguished. Type 1 AII receptors (AT_1 receptors) have high affinity for the nonpeptide AT_1 receptor selective antagonist, losartan (also known as DuP 753 or MK 954) (Whitebread *et al.*, 1989). Type 2 angiotensin II receptors (AT_2 receptors) have a low affinity for losartan and a high affinity for the nonpeptide AT_2 receptor selective antagonists, PD 121981 (also known as WL-19) (Whitebread *et al.*, 1989) and PD 123319 (Dudley *et al.*, 1990). Although it has been shown that both subtypes of AII receptors exist in the rabbit ventricular myocardium (Chiu *et al.*, 1989), the role of receptor subtypes and subcellular mechanism of inotropic effects of AII have not yet been fully clarified.

In order to elucidate the mechanism of PIE of AII, experiments were designed to investigate the relationship between the PIE and acceleration of phosphoinositide hydrolysis induced by AII in the rabbit ventricular myocardium.

Methods

Isolation of rabbit papillary muscles and experimental procedures

Male albino rabbits weighing 1.5–2.2 kg were used for these experiments. The animals were anaesthetized with i.v. administration of sodium pentobarbitone (35 mg kg^{-1}; Tokyo Kasei, Tokyo, Japan) into a marginal ear vein. One thousand units per kg of heparin sodium was also administered simultaneously. After thoracotomy, the heart was removed immediately. Two or three papillary muscles were excised from the right ventricle of the rabbit and mounted in 20 ml organ baths containing Krebs-Henseleit solution with 0.057 mM ascorbic acid and 0.027 mM disodium EDTA. The solution was bubbled with 95% O_2 and 5% CO_2 at 37°C. The pH of the solution was 7.4 when bubbled with the gas mixture. The composition of the solution was as follows (mM): Na^+ 142.9, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 24.9, SO_4^{2-} 1.2, Cl^- 127.8 and glucose 11.1. Papillary muscles were electrically stimulated by square wave pulses of 5 ms duration

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and a voltage approximately 20% above threshold at a frequency of 1 Hz. The developed tension was recorded on a thermal pen writing oscillograph (Recti-Horiz-8K, NEC-San-ei Instrument, Tokyo, Japan) by means of strain gauge transducers (Shinkoh UL 10 GR, Minebea, Tokyo, Japan). During the equilibration period (60 min) papillary muscles were stretched initially under a tension of 5 mN and the length was later adjusted to give 90% of the maximal contractile force. (±)-Bupranolol (0.3 μM) and prazosin (0.1 μM) were allowed to act for 20 min before the administration of AII and were present in the organ bath throughout the experiments to avoid any interference with α- and β-adrenoceptor stimulation by catecholamines (Dempsey *et al.*, 1971). In a series of experiments with AII antagonists, saralasin, losartan or PD 123319 were allowed to act for 20 min before the administration of AII. At the end of each experiment, the maximal contractile force was determined in each muscle by administration of isoprenaline (1–10 μM) after washout of other drugs for at least 30 min until the basal force of contraction returned to the control level. The concentration of isoprenaline was elevated stepwise until the maximal response was achieved. The maximal response to isoprenaline comparable to that achieved by an elevation of the extracellular Ca^{2+} to 9–12 mM was attained by isoprenaline 30 min after washout of the competitive β-adrenoceptor antagonist, (±)-bupranolol. Concentration-response curves for AII were determined by cumulative administration of AII dissolved in 0.1 ml of 0.9% NaCl. When the inotropic responses reached a steady level (usually 5–15 min), the next higher concentration was added. The response to AII was expressed as a percentage of the maximal response to isoprenaline. Concentration-response curves for AII were determined only once in each preparation to avoid possible occurrence of desensitization. This also applies to studies in which antagonists were administered. Therefore, for the calculation of the dose-ratio for AII in the absence and presence of various concentrations of losartan, the mean value for AII in the control experiment (in the absence of losartan) was used as the denominator.

Determination of [^{3}H]-inositol phosphates

The hearts were quickly removed from rabbits, and placed in Krebs-Henseleit solution that was bubbled with 95% O_2:5% CO_2 at 4°C. The experimental procedure was the same as that used in the previous study (Takanashi & Endoh, 1991). Briefly, ventricular slices (0.5 mm thick) were prepared with a Thomas tissue slicer (Thomas, Philadelphia, PA, U.S.A.) and were weighed and equilibrated in Krebs-Henseleit solution for 30 min. The experiment was carried out in duplicate. Each tube contained one ventricular slice cut and trimmed to appropriate size. The slice in each tube was preincubated with 24 μCi *myo*-[^{3}H]-inositol in 4 ml Krebs-Henseleit solution for 120 min. Then the solution was changed to fresh solution containing 5 mM *myo*-inositol and 10 mM LiCl, and all the experiments were performed in the Li^+-containing solution. (±)-Bupranolol (0.3 μM) and prazosin (0.1 μM) were added to the solution 20 min before agonist administration to avoid any interference induced by adrenoceptor activation. At the end of the incubation, the slices were quickly blotted and put into 1.0 ml of chloroform-methanol-12 N HCl (100:200:1 by volume) to terminate the reaction. After the addition of 0.2 ml of 5 mM EDTA, the tissue was homogenized (Polytron PT-10). The tip of the homogenizer was rinsed with 0.5 ml of chloroform-methanol-12 N HCl-5 mM EDTA (100:200:1:80 by volume), and the rinsing fluid was added to the original solution. Chloroform (0.4 ml) and 5 mM EDTA (0.5 ml) were added sequentially and the samples were centrifuged at 1,400 *g* for 20 min to separate the aqueous and organic phases. An aliquot of the aqueous layer was removed, neutralized with 1 M KOH and applied to columns containing a 50% slurry of AG 1-X8 (anion exchange resin, 100–200 mesh, formate form, Bio-Rad, Richmond, CA, U.S.A.). Each column was washed first with 20 ml of distilled water, and the resulting glycerophosphoryl esters were eluted with 8 ml of 5 mM sodium tetraborate-60 mM sodium formate as described previously (Berridge *et al.*, 1983; Takanashi & Endoh, 1991). Then [^{3}H]-inositol phosphates were eluted by addition of 0.2 M ammonium formate in 0.1 M formate as described by Berridge *et al.* (1983). Aliquots of the eluate were counted for radioactivity in a scintillation mixture (ACS-II, Amersham, Arlington Heights, IL, U.S.A.) by use of a Tri-Carb 1500 scintillation counter (Packard, Downers Grove, IL, U.S.A.) at an efficiency of 66%. Since it was found in the preliminary experiment with this method that the radioactivity of [^{3}H]-inositol trisphosphate (IP_3) or [^{3}H]-inositol bisphosphate was low, probably due to their rapid metabolism to [^{3}H]inositol monophosphate (IP_1), IP_1 was used as an indicator of phosphoinositide hydrolysis (Takanashi & Endoh, 1991).

Drugs used

The drugs used were obtained from the following sources: prazosin HCl (Pfizer Taito, Tokyo); (±)-bupranolol HCl (Kaken Kagaku, Tokyo); AII (Peptide Institute, Osaka, Japan); saralasin ([Sar^1, Val^5, Ala^8]angiotensin II, Sigma Chemical, St. Louis, MO, U.S.A.); losartan (2-n-butyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl) methyl]imidazol, potassium salt, Du Pont, Wilmington, DE, U.S.A.); phorbol 12,13-dibutyrate (PDBu), (−)-isoprenaline hydrochloride (Sigmal Chemical, St. Louis, MO, U.S.A.); *myo*-[^{3}H]-inositol (specific activity 115.5 Ci mmol^{-1}) (Amersham, Buckinghamshire, U.K.); PD 123319-121b (Warner-Lambert Co., Ann Arbor, MI, U.S.A.). PDBu was dissolved in 100% DMSO, and the stock solution (20 mM) was diluted with 0.9% NaCl to the desired concentration. The stock solution of isoprenaline was prepared in 1% ascorbic acid, further diluted with 0.9% NaCl solution and kept ice-cold.

Statistics

Experimental values are presented as means ± s.e.mean. Significant differences between mean values were estimated by means of Student's *t* test. A *P* value of smaller than 0.05 was considered to indicate significance.

Results

Positive inotropic effect of angiotensin II on the rabbit ventricular myocardium

AII (1 nM–3 μM), in the presence of (±)-bupranolol (0.3 μM) and prazosin (0.1 μM), produced a PIE in a concentration-dependent manner on rabbit isolated papillary muscles (Figure 1). The pD_2 value [−log 50% effective concentration (EC_{50})] was 7.50 ± 0.24, and the maximum response to AII was achieved at 1–3 μM of AII and was 40.85 ± 4.57% of the maximal response to isprenaline ($n = 7$). The slope of Hill plot for the concentration-response curve for AII was 0.88 ± 0.04, which was not significantly different from 1.0 ($P > 0.05$).

The PIE of AII was associated with characteristic changes in the time course of isometric contractions. Typical tracings of isometric contraction are shown in Figure 2a. The total duration of contraction was prolonged in a concentration-dependent manner to 281 ± 8.0 ms from 236 ± 7.5 ms by AII (0.3 μM) (Figure 2b). The time to peak force was prolonged by AII (0.3 μM) to 125 ± 4.6 ms from 105 ± 5.8 ms and the half relaxation time to 63.6 ± 2.4 ms from 54.3 ± 2.4 ms, respectively (Figure 2b).

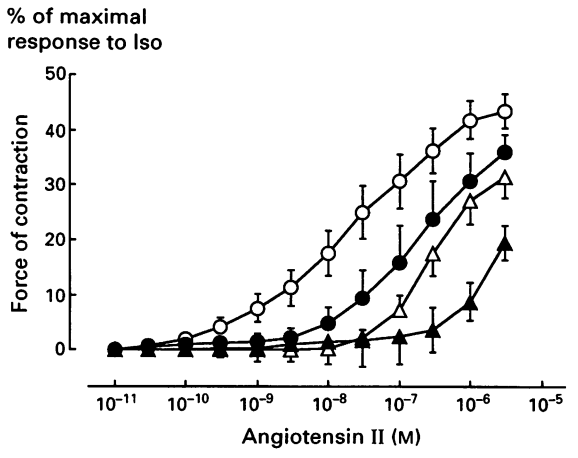


Figure 1 The influence of saralasin on the positive inotropic effect of angiotensin II (AII) in the rabbit isolated papillary muscle in the presence of (±)-bupranolol (0.3 μM) and prazosin (0.1 μM). Saralasin was allowed to act for 20 min before the determination of concentration-response curves for AII and experiments were carried out in its presence. (○) Control ($n = 14$); (●) saralasin 10 nM ($n = 6$); (△) saralasin 100 nM ($n = 6$); (▲) saralasin 1 μM ($n = 6$). Saralasin (10 nM–1 μM) did not significantly affect the basal force of contraction. All values represent means ± s.e.mean. The basal force and maximal response to isoprenaline (Iso) in this series of experiments were 2.85 ± 0.29 and 12.4 ± 0.9 mN ($n = 32$), respectively.

Influence of saralasin, losartan and PD 123319 on the positive inotropic effect of angiotensin II

Figure 1 shows the influence of a nonselective AII receptor antagonist, saralasin on the PIE of AII in the presence of (±)-bupranolol and prazosin. Saralasin up to a concentration of 1 μM did not affect the basal force of contraction. Saralasin, at a concentration of 10 nM, partially inhibited the PIE induced by AII, and at a higher concentration of 1 μM, shifted further the concentration-response curve for AII. Figure 3a shows the influence of the nonpeptide AT_1 receptor selective antagonist, losartan on the PIE of AII in the presence of (±)-bupranolol and prazosin. Losartan (10 nM–1 μM) did not affect the basal force of contraction, but inhibited the PIE of AII concentration-dependently (Figure 3a). Schild analysis using the dose ratio at the AII concentration eliciting 20% of the maximal response to isoprenaline indicated that the slope was not different from unity (0.98 ± 0.11) and the pA_2 value was 9.00 ± 0.84 (inset of Figure 3a). It was not shown whether the maximal response to AII in the presence of losartan (0.1–1 μM) could reach that of control, because of the availability of AII. On the other hand, the nonpeptide AT_2 receptor selective antagonist, PD 123319 (1 μM) did not influence the PIE of AII (Figure 3b).

Effects of angiotensin II on phosphoinositide hydrolysis

After administration of 10 μM AII, the accumulation of [^{3}H]-IP_1 in rabbit ventricular slices in the presence of 10 mM LiCl, (±)-bupranolol (0.3 μM) and prazosin (0.1 μM) reached a significant level at 4 min and then increased time-dependently to reach about 200% of the control level at 30 min (Figure 4a).

The concentration-response curve for the effect of AII on the [^{3}H]-IP_1 accumulation was determined in ventricular slices incubated with various concentrations of AII for 30 min. The accumulation of [^{3}H]-IP_1 increased with AII in a concentration-dependent manner (10 nM–30 μM) and the maximal response reached about 200% of the basal level at 30 μM (Figure 4b).

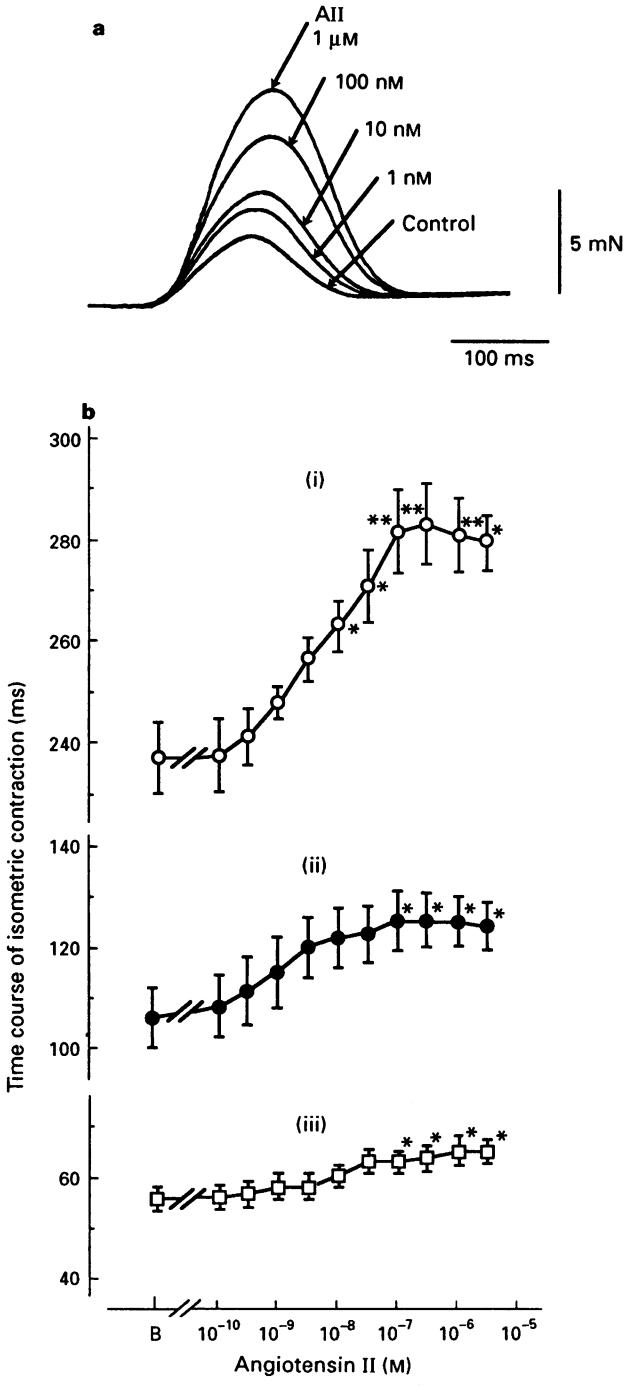


Figure 2 (a) Representative tracings showing the change in time course of isometric contractions induced by angiotensin II (AII). (b) Concentration-response curve for AII in the rabbit isolated papillary muscle in the presence of (±)-bupranolol (0.3 μM) and prazosin (0.1 μM). Total duration of contraction (i), time to peak force (ii) and half relaxation time (iii) are plotted against the concentration of AII. (B) Basal values. All values represent means ± s.e.mean ($n = 7$). $^*P < 0.05$, $^{**}P < 0.01$ vs. the corresponding control values.

Influence of saralasin, losartan, PD 123319 and PDBu on the accumulation of [^{3}H]-IP_1 induced by angiotensin II

Figure 5a shows the influence of saralasin on the [^{3}H]-IP_1 accumulation induced by AII (10 μM) in rabbit ventricular slices. AII at a concentration of 10 μM caused an increase in the [^{3}H]-IP_1 level to 183.2 ± 15.1% ($n = 8$) of the control. Saralasin at a concentration of 10 nM attenuated the AII-induced accumulation of [^{3}H]-IP_1, but the reduction was not

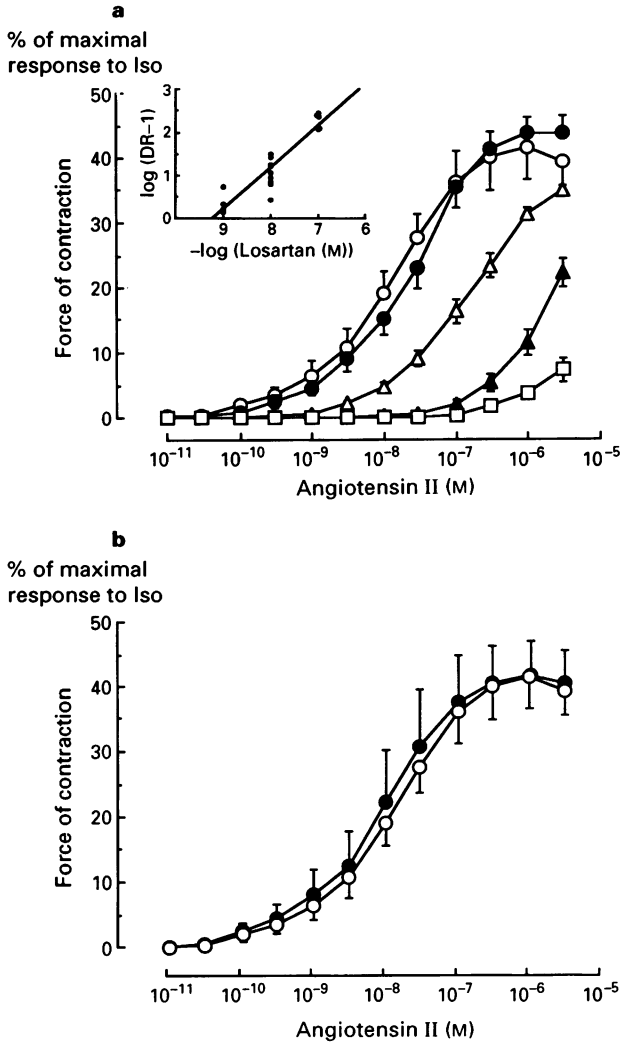


Figure 3 Effects of losartan and PD 123319 on the positive inotropic effect of angiotensin II (AII) in the rabbit isolated ventricular papillary muscle in the presence of (±)-bupranolol (0.3 μM) and prazosin (0.1 μM). Losartan (1 nM–1 μM) or PD 123319 (1 μM) was allowed to act for 20 min before the determination of concentration-response curves for AII and experiments were carried out in its presence. (a) (○) Control ($n = 6$); (●) losartan 1 nM ($n = 8$); (△) losartan 10 nM ($n = 9$); (▲) losartan 100 nM ($n = 5$); (□) losartan 1 μM ($n = 5$). Losartan (1 μM) did not significantly affect the basal force of contraction. All values represent means ± s.e.mean. The basal force and maximal response to isoprenaline (Iso) in this series were 2.9 ± 0.3 and 12.0 ± 1.0 mN ($n = 33$), respectively. Inset: Schild plot calculated with the dose-ratio at the level of 20% in (a). DR; AII dose-ratio. (b) (○) Control ($n = 6$); (●), PD 123319 1 μM ($n = 6$). PD123319 1 μM did not significantly affect the basal force of contraction. The basal force and maximal response to isoprenaline in this series were 4.3 ± 0.3 and 13.9 ± 1.3 mN ($n = 12$), respectively.

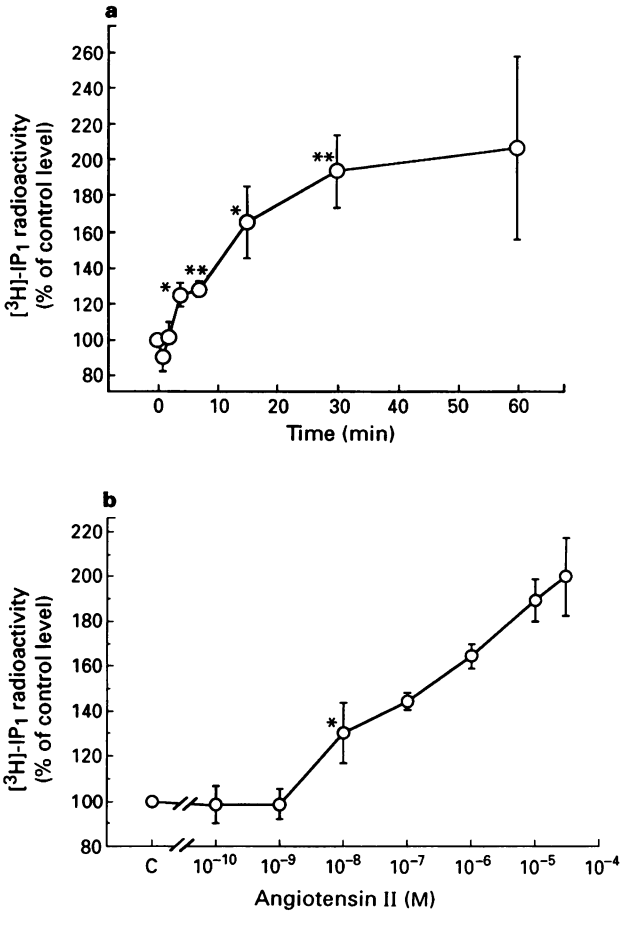


Figure 4 The angiotensin II (AII)-induced accumulation of [^{3}H]-inositol monophosphate ([^{3}H]-IP$_1$) in rabbit ventricular slices prelabelled with *myo*-[^{3}H]-inositol. (a) Time-response curve for [^{3}H]-IP$_1$ accumulation. AII (10 μM) was allowed to act for periods of time from 0 to 60 min and then [^{3}H]-IP$_1$ levels were determined. Actual radioactivity of 100% was 40.6 ± 6.8 d.p.m. mg^{-1} of wet weight of the tissue ($n = 6$). Values represent means ± s.e.mean ($n = 6$ for each determination). $^*P < 0.05$, $^{**}P < 0.01$ vs. the corresponding control values. Average wet weight of slices was 84.69 ± 4.03 mg ($n = 48$). (b) Concentration-response curve for the accumulation of [^{3}H]-IP$_1$. Accumulation of [^{3}H]-IP$_1$ was assessed 30 min after the addition of AII in the presence of 10 mM LiCl. Actual radioactivity of 100% was 28.3 ± 3.8 d.p.m. mg^{-1} of wet weight of the tissue ($n = 4$). Values represent means ± s.e.mean ($n = 4$ in duplicate for each concentration). Asterisk represents the threshold concentration ($P < 0.05$). Average wet weight of slices was 53.0 ± 2.2 mg ($n = 32$).

statistically significant. Saralasin at 100 nM significantly ($P < 0.01$, $n = 8$) decreased the accumulation of [^{3}H]-IP$_1$ induced by AII (Figure 5a). The concentration-dependent inhibition by saralasin of the AII-induced [^{3}H]-IP$_1$ accumulation indicates that the AII-induced acceleration of phosphoinositide hydrolysis is caused through activation of AII receptors in the rabbit ventricular myocardium.

The selective AT$_1$ receptor antagonist, losartan, inhibited significantly the AII-induced accumulation of [^{3}H]-IP$_1$ at concentrations of 100 nM and higher ($n = 8$ each, Figure 5b). On the other hand, a selective AT$_2$ receptor antagonist, PD 123319 (0.1–1.0 μM) did not affect the AII-induced accumulation of [^{3}H]-IP$_1$ ($n = 6$ each, Figure 5c).

Influence of PDBu on the positive inotropic effect and phosphoinositide hydrolysis induced by angiotensin II

Previously we have found that the phorbol esters, phorbol 12-myristate 13-acetate and PDBu, antagonize selectively the PIE mediated by myocardial α_1-adrenoceptors or endothelin receptors that was elicited in association with acceleration of phosphoinositide hydrolysis in the rabbit papillary muscle (Kushida *et al.*, 1988; Endoh *et al.*, 1991; Takanashi & Endoh, 1991). Therefore, we examined the influence of PDBu on the PIE of AII. The PIE of AII was antagonized by PDBu in a concentration-dependent manner (Figure 6a) over a similar concentration-range that inhibited the PIE of phenylephrine and endothelin-1 (Takanashi & Endoh, 1991). PDBu (10 nM) inhibited the PIE of AII by approximately 65% without affecting the basal force of contraction. At a concentration of 100 nM, PDBu decreased the basal force of contraction ca. 20% and abolished the PIE of AII.

The effects of PDBu on the accumulation of [^{3}H]-IP$_1$

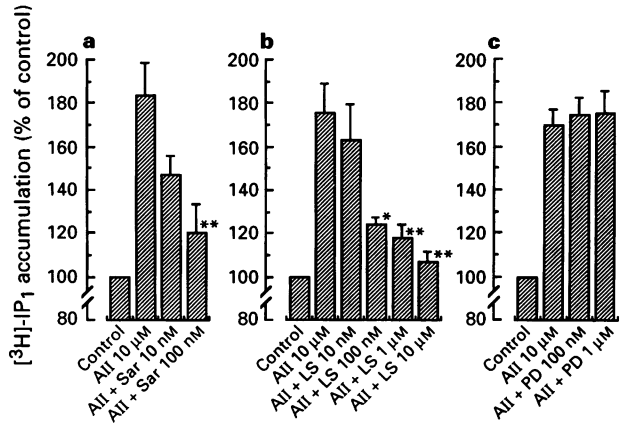


Figure 5 The influence of saralasin (Sar), losartan (LS) and PD 123319 (PD) on the angiotensin II (AII)-induced accumulation of [^{3}H]-inositol monophosphate ([^{3}H]-IP$_1$) in rabbit ventricular slices prelabelled with *myo*-[^{3}H]-inositol. Sar, LS, PD or 0.9% NaCl was added 20 min before the administration of AII and experiments were carried out in their presence. AII (10 μM) was allowed to act for 30 min in the absence (control) or presence of Sar (a), LS (b) or PD (c) and then the [^{3}H]-IP$_1$ level was determined. Actual radioactivities of 100% were 54.3 ± 4.3 (a, $n = 8$), 45.1 ± 5.3 (b, $n = 8$) and 61.4 ± 4.8 (c, $n = 6$) d.p.m. mg^{-1} of wet weight of the tissue, respectively. All experiments were determined in duplicate. Values represent means ± s.e.mean. The effect of Sar, LS or PD on the response to AII was compared to the corresponding value with AII alone. $^*P<0.05$, $^{**}P<0.01$ vs. the value with AII alone. Average wet weight of slices was 70.9 ± 5.2 mg (a, $n = 32$), 82.2 ± 3.4 mg (b, $n = 40$), 39.8 ± 1.9 mg (c, $n = 24$).

induced by AII are shown in Figure 6b. Pretreatment with 10 nM PDBu for 20 min, the concentration at which it inhibited significantly the PIE of AII, significantly reduced the accumulation of [^{3}H]-IP$_1$ caused by AII ($P<0.001$, $n = 8$). Pretreatment with a higher concentration of PDBu (100 nM), which abolished the PIE of AII, further attenuated the accumulation of [^{3}H]-IP$_1$. The level of [^{3}H]-IP$_1$ in the presence of 100 nM PDBu was not significantly different from the control.

Discussion

In the rabbit isolated papillary muscle, AII elicited a concentration-dependent PIE in the presence of α- and β-adrenoceptor blockade. The inotropic effect of AII was antagonized by saralasin, a nonselective angiotensin II receptor antagonist (Figure 1). It has been demonstrated that angiotensin receptors exist in myocardium of various experimental animals (Wright *et al.*, 1983; Rogers, 1984; Aceto & Baker, 1990). AII has been shown to produce a PIE in some species including man, cat, and rabbit, but not in guinea-pig and ferret (Dempsey *et al.*, 1971; Baker & Aceto, 1989; Schomisch *et al.*, 1990). This implies that AII shows a wide range of species-dependent difference in its inotropic effect. In this respect, rabbit is characteristic in the mammalian species in that its cardiac muscle responds to AII with a pronounced positive inotropy. The selective AT$_1$ receptor antagonist, losartan, inhibited concentration-dependently the PIE of AII, whereas the selective AT$_2$ receptor antagonist, PD 123319, did not influence the PIE (Figure 3), indicating that the AT$_1$ receptor subtype may be involved in the PIE of AII in the rabbit ventricular myocardium.

A possible subcellular mechanism for the intropic action of AII is an increase in phosphoinositide hydrolysis catalyzed via AT$_1$ receptor activation (Figures 4 and 5). This view is supported by previous findings showing that AII stimulates phosphoinositide hydrolysis in various tissues including vascular smooth muscle (Smith *et al.*, 1984), liver (Burgess *et al.*, 1991), kidney (Kurtz & Penner, 1989) and chick cardiac muscle (Baker & Aceto, 1989).

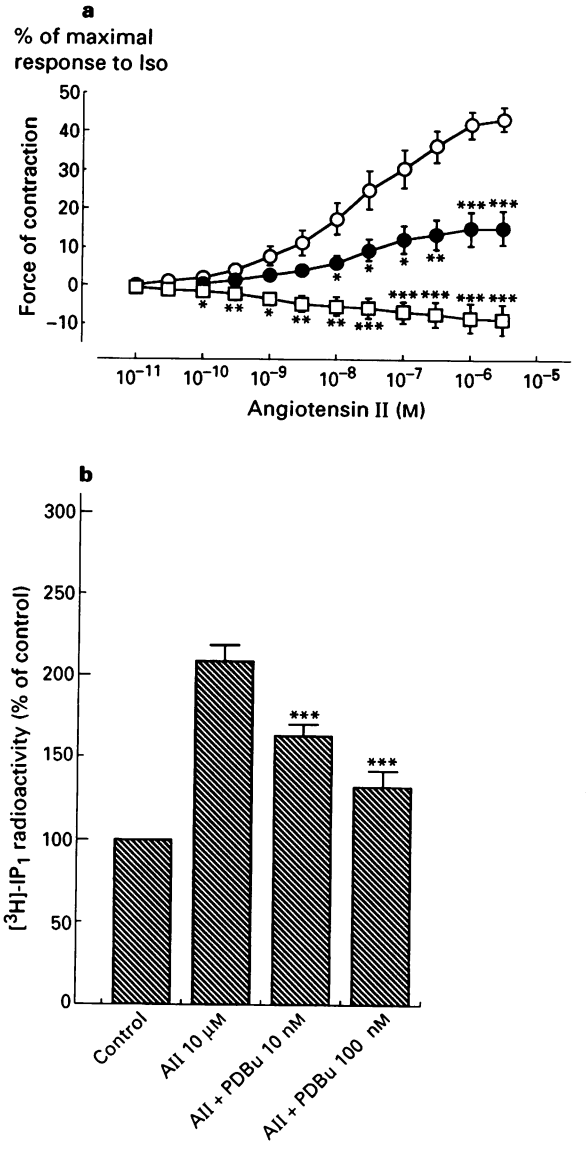


Figure 6 (a) The influence of phorbol 12, 13-dibutyrate (PDBu) on the positive inotropic effect of angiotensin II (AII) in the rabbit isolated ventricular papillary muscle in the presence of (±)-bupranolol (0.3 μM) and prazosin (0.1 μM). PDBu was applied 20 min before the determination of concentration-response curves for addition of AII and experiments carried out in its presence. (○) Control ($n = 14$); (●) PDBu 10 nM ($n = 6$); (□) PDBu 100 nM ($n = 6$). All values represent means ± s.e.mean. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. the corresponding control values. At 20 min after the administration of 10 or 100 nM PDBu, the contractile force was 90.9 ± 3.3% ($n = 6$) or 78.6 ± 6.3% ($n = 6$; $P<0.05$) of the control basal force of contraction. The basal force and maximal response to isoprenaline (Iso) in this series were 3.35 ± 0.62 and 12.4 ± 0.9 mN ($n = 26$), respectively. (b) The influence of PDBu on the AII (10 μM)-induced accumulation of [^{3}H]-inositol monophosphate ([^{3}H]-IP$_1$) in rabbit ventricular slices prelabelled with *myo*-[^{3}H]-inositol. PDBu or 0.9% NaCl was added 20 min before the administration of AII and experiments were carried out in their presence. AII (10 μM) was allowed to act for 30 min in the absence (control) or presence of 10 or 100 nM PDBu, and then the [^{3}H]-IP$_1$ level was determined. Actual radioactivity of 100% was 38.1 ± 4.5 d.p.m. mg^{-1} of wet weight of the tissue ($n = 8$). Values represent means ± s.e.mean ($n = 8$ in duplicate for each column). The effect of PDBu on the response to AII was compared to the value with AII alone. $^{***}P<0.001$ vs. the value with AII alone. Average wet weight of slices was 46.5 ± 2.6 mg ($n = 32$).

In the rabbit ventricular myocardium, striking similarities have been noted between the inotropic effect of AII and myocardial α_1-adrenoceptor and endothelin receptor stimulation, all of which stimulate phosphoinositide hydrolysis. Firstly, characteristics of contractile regulation through these classes of receptors are very similar. Figure 2 shows the changes in time course of isometric contractions induced by AII, which are akin to those produced by α_1- or endothelin receptor activation in the rabbit papillary muscle (Takanashi & Endoh, 1991). Stimulation of myocardial α_1-, endothelin and AII receptors consistently results in prolongation of action potential duration (Dempsey *et al.*, 1971; Argentieri *et al.*, 1990; Dirksen & Sheu, 1990). These changes were in strong contrast to those produced by β-adrenoceptor stimulation (Endoh & Blinks, 1988). The maxial response to α_1- or endothelin receptor agonists was about 50–60% of that to isoprenaline (Takanashi & Endoh, 1991), which was slightly higher than that of AII.

Secondly, a tumour-promoting phorbol ester, PDBu (10–100 nM) inhibited the PIE and phosphoinositide hydrolysis induced by AII in rabbit ventricular muscle (Figure 6). The effects of α_1-adrenoceptor agonists and endothelin-1 were also inhibited by PDBu or PMA (Kushida *et al.*, 1988; Endoh *et al.*, 1991; Takanashi & Endoh, 1991). It is noteworthy that the inhibitory action of PDBu was selective for the inotropic effect associated with phosphoinositide hydrolysis: the basal force of contraction, and the inotropic effects of isoprenaline and Bay K 8644 were scarcely affected by PDBu.

Inhibition by PDBu of the agonist-induced phosphoinositide hydrolysis and functional changes have been demonstrated in a variety of cells, including smooth muscle cells (Brock *et al.*, 1985), platelets (Kagaya *et al.*, 1990) and myocardial cells (Yuan *et al.*, 1987). In addition, in chick embryo myocytes, PDBu decreased Ca^{2+} transients increased by AII (Baker *et al.*, 1989) and PMA inhibited the AII-mediated increase in Ca^{2+} transients in rat myocytes (Kem *et al.*, 1991). These observations indicate that the externally administered tumour-promoting phorbol esters may inhibit the receptor-mediated stimulation of phosphoinositide hydrolysis and thereby antagonize the inotropic effect. Phosphorylation of receptors (Resink *et al.*, 1990), and/or of GTP binding proteins (Katada *et al.*, 1985) due to protein kinase C activation have been postulated to be responsible for the subcellular mechanism for uncoupling of the GTP binding protein to phospholipase C activation in noncardiac cells.

A wide range of species-dependent variation of the positive inotropic effects of α_1-adrenoceptor agonists (Endoh, 1982), endothelin-1 (Takanashi & Endoh, 1991) and AII is seen among mammalian species. The species-dependent variation may be largely due to difference in coupling processes, because the presence of receptors has been demonstrated in the species in which the inotropic effect was not induced by these receptor agonists (Baker & Singer, 1988; Endoh *et al.*, 1991; Takanashi & Endoh, 1991).

Finally, activation of AII receptors as well as α_1- and endothelin receptors may be involved in the development of myocardial hypertrophy (Simpson, 1985; Aceto & Baker, 1990; Ito *et al.*, 1991). Thus, receptors that stimulate phosphoinositide hydrolysis may be coupled to divergent physiological and pathophysiological signal transduction in cardiac muscle.

In summary, while the subcellular mechanism of the PIE of AII is not yet fully clarified, we have noted marked similarities among the AII (AT_1), α_1-adrenoceptor and endothelin receptor-mediated modulation of myocardial contractility and phosphoinositide hydrolysis. The present findings together with earlier experimental evidence strongly suggest that acceleration of phosphoinositide hydrolysis induced by AT_1 receptor activation may contribute to the receptor-mediated regulation of myocardial contractility.

Note added in proof

After our manuscript had been submitted, a pertinent paper by Scott *et al.*, appeared, in which it was shown that in the rabbit papillary muscle the PIE of angiotensin II was antagonized by losartan in a competitive manner (Scott, A.L., Chang, R.L.S., Lotti, V.J. & Siegl, P.K.S. (1992). Cardiac angiotensin receptors: effects of selective angiotensin II receptor antagonists, DUP 753 and PD 121981, in rabbit heart. *J. Pharmacol. Exp. Ther.*, **261**, 931–935).

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References

ACETO, J.F. & BAKER, K.M. (1990). [Sar^1]angiotensin II receptor-mediated stimulation of protein synthesis in chick heart cells. *Am. J. Physiol.*, **258**, H806–H813.

ANAND-SRIVASTAVA, M.B. (1989). Angiotensin II receptors negatively coupled to adenylate cyclase in rat myocardial sarcolemma. Involvement of inhibitory guanine nucleotide regulatory protein. *Biochem. Pharmacol.*, **38**, 489–496.

ARGENTIERI, T.M., CARROLL, M.S., TROY, H.H., RUBANYI, G.M. & SULLIVAN, M.E. (1990). Endothelin-1 increases action potential duration in isolated canine cardiac tissues. *Circulation*, **82**, Suppl. III, III–227.

BAKER, K.M. & ACETO, J.A. (1989). Characterization of avian angiotensin II cardiac receptors: coupling to mechanical activity and phosphoinositide metabolism. *J. Mol. Cell. Cardiol.*, **21**, 375–382.

BAKER, K.M. & SINGER, A. (1988). Identification and characterization of guinea-pig angiotensin II ventricular and atrial receptors: coupling to inositol phosphate production. *Circ. Res.*, **62**, 896–904.

BAKER, K.M., SINGER, H.A. & ACETO, J.F. (1989). Angiotensin II receptor mediated stimulation of cytosolic free calcium and inositol phosphates in chick myocytes. *J. Pharmacol. Exp. Ther.*, **251** 578–585.

BERRIDGE, M.J., DAWSON, R.M.C., DOWNNES, C.P., HESLOP, J.P. & IRVINE, R.F. (1983). Changes in the levels of inositol phosphate after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.*, **212**, 473–482.

BROCK, T.A., RITTENHOUSE, S.E., POWERS, C.W., EKSTEIN, L.S., GIMBRONE, M.A. Jr & ALEXANDER, R.W. (1985). Phorbol ester and 1-oleoyl-2-acetylglycerol inhibit angiotensin activation of phospholipase C in cultured vascular smooth muscle. *J. Biol. Chem.*, **260**, 14156–14162.

BURGESS, G., BIRD, G. ST. J., OBIE, J.F. & PUTNEY, J.W. Jr. (1991) The mechanism for synergism between phospholipase C- and adenylylcyclase-linked hormones in liver. *J. Biol. Chem.*, **266**, 4772–4781.

CHIU, A.T., HERBLIN, W.F., MCCALL, D.E., ARDECKY, R.J., CARINI, D.J., DUNCIA, J.V., PEASE, L.J., WONG, P.C., WEXLER, R.R., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1989). Identification of angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.*, **165**, 196–203.

DEMPSEY, P., MCCALLUM, Z., KENT, K. & COOPER, T. (1971). Direct myocardial effects of angiotensin II. *Am. J. Physiol.*, **220**, 477–481.

DIRKSEN, R.T. & SHEU, S.-S. (1990). Modulation of ventricular action potential by α_1-adrenoceptors and protein kinase C. *Am. J. Physiol.*, **258**, H907–H911.

DUDLEY, D.T., PANEK, R.L., MAJOR, T.C., LU, G.H., BRUNS, R.F., KLINKEFUS, B.A., HODGES, J.C. & WEISHAAR, R.E. (1990). Subclasses of angiotensin II binding sites and their functional significance. *Mol. Pharmacol.*, **38**, 370–377.

ENDOH, M. (1982). Adrenoceptors and the myocardial inotropic response: do alpha and beta receptor sites functionally coexist? In *Trends in Autonomic Pharmacology*, vol. 2. ed. Kalsner, S., pp. 303–322. Baltimore-Munich: Urban & Schwarzenberg.

ENDOH, M. & BLINKS, J.R. (1988). Actions of sympathomimetic amines on the Ca^{2+} transients and contractions of rabbit myocardium: reciprocal changes in myofibrillar responsiveness to Ca^{2+} mediated through α- and β-adrenoceptors. *Circ. Res.*, **62**, 247–265.

ENDOH, M., HIRAMOTO, T., ISHIHATA, A., TAKANASHI, M. & INUI, J. (1991). Myocardial α_1-adrenoceptors mediate positive inotropic effect and changes in phosphatidylinositol metabolism. Species differences in receptor distribution and the intracellular coupling process in mammalian ventricular myocardium. *Circ. Res.*, **68**, 1179–1190.

FREER, R.J., PAPPANO, A.J., PEACH, M.J., BING, K.T., MCLEAN, M.J., VOGEL, S. & SPERELAKIS, N. (1976). Mechanism for the positive inotropic effect of angiotensin II on isolated cardiac muscle. *Circ. Res.*, **39**, 178–183.

ITO, H., HIRATA, Y., HIROE, M., TSUJINO, M., ADACHI, S., TAKAMOTO, T., NITTA, M., TANIGUCHI, K. & MARUMO, F. (1991). Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ. Res.*, **69**, 209–215.

KAGAYA, A., MIKUNI, M., KUSUMI, I., YAMAMOTO, H. & TAKAHASHI, K. (1990). Serotonin-induced acute desensitization of serotonin$_2$ receptors in human platelets via a mechanism involving protein kinase C. *J. Pharmacol. Exp. Ther.*, **255**, 305–311.

KATADA, T., GILMAN, A.G., WATANABE, Y., BAUER, S. & JAKOBS, K.H. (1985). Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppressed its function in humoral inhibition of adenylate cyclase. *Eur. J. Biochem.*, **151**, 431–437.

KEM, D.C., JOHNSON, E.I.M., CAPPONI, A.M., CHARDONNENS, D., LANG, U., BLONDEL, B., KOSHIDA, H. & VALLOTTON, M.B. (1991). Effect of angiotensin II on cytosolic free calcium in neonatal rat cardiomyocytes. *Am. J. Physiol.*, **261**, C77–C85.

KOBAYASHI, M., FURUKAWA, Y. & CHIBA, S. (1978). Positive chronotropic and inotropic effects of angiotensin II in the dog heart. *Eur. J. Pharmacol.*, **50**, 17–25.

KURTZ, A. & PENNER, R. (1989). Angiotensin II induces oscillations of intracellular calcium and blocks anomalous inward rectifying potassium current in mouse renal juxtaglomerular cells. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3423–3427.

KUSHIDA, H., HIRAMOTO, T., SATOH, H. & ENDOH, M. (1988). Phorbol ester does not mimic, but antagonizes the alpha-adrenoceptor-mediated positive inotropic effect in the rabbit papillary muscle. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, 169–176.

RESINK, T.J., SCOTT-BURDEN, T., WEBER, E. & BUHRLE, F.R. (1990). Phorbol ester promotes a sustained down-regulation of endothelin receptors and cellular responses to endothelin in human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.*, **166**, 1213–1219.

ROGERS, T.B. (1984). High affinity angiotensin II receptors in myocardial sarcolemmal membranes. *J. Biol. Chem.*, **259**, 8106–8114.

ROSS, G. & WHITE, F. (1966). Role of catecholamine release in cardiovascular responses to angiotensin. *Am. J. Physiol.*, **211**, 1419–1423.

SCHOMISCH, M.C., SCHLUCHTER, M.D., PARANANDI, L., CZERSKA, B., STEWART, R.W., ROSENKRANZ, E. & BOND, M. (1990). Inotropic effects of angiotensin II on human cardiac muscle in vitro. *Circulation*, **82**, 1973–1984.

SIMPSON, P. (1985). Stimulation of hypertrophy of cultured neonatal rat heart cells through an α_1-adrenergic receptor and induction of beating through an α_1- and β_1-adrenergic receptor interaction. Evidence for independent regulation of growth and beating. *Circ. Res.*, **56**, 884–894.

SMITH, J.B., SMITH, L., BROWN, E.R., BARNES, D., SABIR, M.A., DAVIS, J.S. & FARESE, R.V. (1984). Angiotensin II rapidly increases phosphatidate-phosphoinositide synthesis and phosphoinositide hydrolysis and mobilizes intracellular calcium in cultured arterial muscle cells. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 7812–7816.

TAKANASHI, M. & ENDOH, M. (1991). Characterization of positive inotropic effect of endothelin on mammalian ventricular myocardium. *Am. J. Physiol.*, **261**, H611–H619.

WHITEBREAD, S., MELE, M., KAMBER, B. & DE GASPARO, M. (1989). Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.*, **163**, 284–291.

WRIGHT, G.B., ALEXANDER, R.W., EKSTEIN, L.S. & GIMBRONE, M.A. Jr (1983). Characterization of the rabbit ventricular myocardial receptor for angiotensin II. *Mol. Pharmacol.*, **24**, 213–221.

YUAN, S., SUNAHARA, F.A. & SEN, A.K. (1987). Tumor-promoting phorbol esters inhibit cardiac functions and induce redistribution of protein kinase C in perfused beating rat heart. *Circ. Res.*, **61**, 372–378.

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Ion channel blockade by oximes and recovery of diaphragm muscle from soman poisoning *in vitro*

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1 The actions of oximes and related compounds on the nicotinic acetylcholine receptor ion channel at the adult mouse muscle endplate were investigated by use of single-channel recording techniques. The aim of the study was to determine whether the channel-blocking properties of the compounds could contribute to their therapeutic effectiveness against soman poisoning *in vitro*.

2 Therapeutic effectiveness was assessed in guinea-pig phrenic nerve-hemidiaphragm preparations by measuring the degree of recovery of neuromuscular function produced by the compounds following poisoning by soman. A number of the compounds, including some which lacked the oxime group, produced a significant recovery of neuromuscular function which was unrelated to acetylcholinesterase (AChE) reactivation; this was reversed by washing off the compound, and was therefore attributed to a direct pharmacological action on the muscle.

3 Single channel recordings showed that some of the compounds blocked open nicotinic receptor ion channels in preparations of mouse muscle fibres. The compounds which showed the greatest direct pharmacological actions in diaphragms produce a very fast, flickering blockade of the channels. Several quantitative measures of channel-blocking activity correlated very well with the direct pharmacological action. Furthermore, for two compounds studied in greater detail, the direct action and channel-blocking showed similar concentration-response relationships.

4 The results of this study indicate that the direct pharmacological action of oximes and their analogues against neuromuscular blockade by soman *in vitro* is due to their channel-blocking activity. The direct action does not correlate well with protection against soman poisoning *in vivo*, however, which suggests that additional non-reactivating properties of these compounds, at sites other than the neuromuscular junction, may also be important for their therapeutic effectiveness.

Keywords: Acetylcholine; anticholinesterase; endplate; ion channel blockade; muscle, skeletal; neuromuscular junction; nicotinic receptor; organophosphate; oxime

Introduction

Oximes are used in the treatment of poisoning by organophosphorus anticholinesterases, and are generally believed to exert their therapeutic effect by reactivating the inhibited acetylcholinesterase (AChE). With some organophosphorus compounds, particularly soman, the inhibited enzyme rapidly undergoes a dealkylation reaction, called aging, to form a complex which cannot be reactivated by oximes (Loomis & Salafsky, 1963; Stares, 1976). Some oximes have been reported to be effective against soman poisoning, even after aging of the inhibited AChE has taken place (Stares, 1976; Smith & Muir, 1977; Hamilton & Lundy, 1989). Furthermore, similar protection against soman has been found with related compounds in which the oxime group required for reactivation is absent (Stares, 1976; Clement, 1981; Inns & Leadbeater, 1983). These observations indicate that such compounds have an additional therapeutic action which is not related to the regeneration of AChE.

The therapeutic effects of some oximes against soman poisoning can be demonstrated *in vitro* with the guinea-pig phrenic nerve-hemidiaphragm preparation (Wolthuis *et al.*, 1981; Smith *et al.*, 1981; French *et al.*, 1983). The neuromuscular recovery produced in these experiments is reversed by washing out the oxime and thus appears to be due to a direct pharmacological action. One possible mechanism of this effect is that the oximes may block the ion channel associated with the nicotinic acetylcholine receptor and thus counteract the effects of excessive cholinergic stimulation (Alkondon *et al.*, 1988; Alkondon & Albuquerque, 1989). In order to investigate this possibility, the actions of a range of oximes and related compounds were studied in guinea-pig diaphragms in which aging of the soman-inhibited AChE had taken place. The concentrations tested were similar to those achieved in blood plasma following therapeutic administration *in vivo*. These actions were then compared with the effects of the same concentrations of the drugs on nicotinic receptor ion channels in mouse muscle endplates, studied with patch clamp techniques.

Methods

Phrenic nerve-hemidiaphragm experiments

Male Dunkin-Hartley guinea-pigs weighing between 300 and 600 g were used. They were stunned and killed by exsanguination, and the left and right hemidiaphragms were removed and prepared for recording in a similar manner to that described by Bülbring (1946). The preparations were suspended in modified Tyrode solution with the following composition (mM): NaCl 137, $NaHCO_3$ 12, NaH_2PO_4 1, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2 and glucose 25. They were stimulated via the phrenic nerve through bipolar platinum electrodes with rectangular pulses of 0.3 ms duration. The stimulus intensity was adjusted to produce supramaximal stimulation of the preparations. Muscle contractions were recorded from a baseline tension of 4 g using an isometric force transducer and a Lectromed MX216 recorder.

Single twitch responses, elicited every 10 s, were recorded throughout the experiment. Every 15 min, a 50 Hz tetanus was elicited for a duration of 3 s. Following control tetani, soman (10^{-7} M) was added to the tissue bath for 30 min. The soman was then removed by washing and the preparation was left for 30 min to allow the inhibited AChE to age before addition of the test compound. The test compound was

added to the bath for 30 min. This was then washed off, and 30 min later a second dose of soman (10^{-7} M) was added to the tissue bath to inhibit any reactivated AChE. All compounds were tested initially at a concentration of 200 μM, this being the highest blood plasma concentration which is usually achieved during studies of oxime therapy *in vivo* (Benschop *et al.*, 1976; Stares, 1976; Wolthuis *et al.*, 1976; Green *et al.*, 1986; Klimmek & Eyer, 1986; Hamilton & Lundy, 1989; Ecobichon *et al.*, 1990). If a compound significantly blocked twitch contractions at this concentration, it was then tested at lower concentrations.

Neuromuscular function was assessed by measuring the tetanic tension 1 s into the tetanus. This was expressed as a percentage of the mean of three tetani recorded before the application of soman. Three aspects of neuromuscular recovery following oxime administration were measured as follows: (a) recovery due to direct pharmacological actions of the oxime on the muscle was measured by subtracting the response after washing out the oxime from the response before washing; (b) oxime-induced reactivaton of acetylcholinesterase was calculated by subtracting the response after reinhibition by the second dose of soman from the response after washing out the oxime but before the second application of soman; (c) the remaining recovery following the second dose of soman was termed residual recovery, since it was due neither to direct action of the oxime nor to reactivation of acetylcholinesterase. The residual recovery has been termed adaptation by Smith *et al.* (1981).

The statistical significance of each of these types of recovery was assessed in two ways. Firstly, paired *t* tests (two-tailed) were used to evaluate the changes following washing out of each test compound and application of the second dose of soman. Secondly, the degree of direct action, reactivation and residual recovery were compared with control experiments using unpaired *t* tests (two-tailed). Values of $P<0.05$ were considered statistically significant.

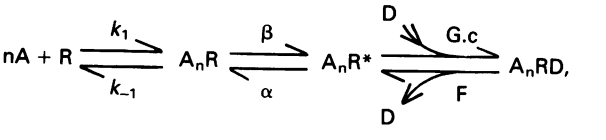
Single channel recordings

Flexor digitorum brevis muscles were dissected from the hind limbs of adult mice (1.5–3 months old) which had been stunned and killed by cervical dislocation. The muscles were dissociated into single fibres by an enzymatic method previously described by Brehm & Kullberg (1987). Briefly, the muscles were incubated for 2 h at 37°C in Dulbecco's modification of Eagle's medium (Flow Laboratories) containing 3 mg ml^{-1} collagenase (Sigma, Type I). They were then washed and incubated for a further 15 min at 37°C in Ca^{2+}-free minimum essential medium (MEMS, Flow Laboratories) containing 0.5 mg ml^{-1} trypsin (Sigma, Type XI) and 0.2 mg ml^{-1} EDTA. The tissue was dissociated into single fibres by gentle trituration through a Pasteur pipette in saline which had the following composition (mM): NaCl 120, KCl 1, $CaCl_2$ 1; HEPES 10 pH 7.2 with NaOH. All experiments were performed at room temperature (20°C).

Patch pipettes were pulled from borosilicate glass to a tip diameter of 1–2 μm and filled with physiological saline containing acetylcholine (ACh, 300–500 nM), either alone or in combination with a test compound. Single channel currents were recorded in cell-attached patches at or near the endplate region with an Axopatch-1B amplifier (Axon Instruments, Burlingame, CA, U.S.A.). The unfiltered signals were sampled continuously at 44 kHz by a pulse code modulator and stored on videotape. The signals were played back through a low-pass filter (4-pole Bessel, −3dB cutoff 5 kHz), and digitised at 20 kHz by a Labmaster interface (Scientific Solutions, Solon, OH, U.S.A.) for subsequent computer analysis by the programmes pCLAMP, IPROC and LPROC (Axon Instruments). Sections of data in which more than one channel was open were excluded from the analysis. The threshold for detection was set at 50% of the estimated single channel amplitude, and a burst was considered valid when the average current was greater than 80% of this value. A closing was counted when the current fell below 50% of the estimated single channel amplitude. The rise time of the recording system was approximately 70 μs, which corresponds to the shortest event which could be accurately detected (Colquhoun & Sigworth, 1983). Histograms of open and closed durations were fitted with one or more exponential functions by a least squares method using a Levenberg-Marquardt algorithm. Most measurements were made at a membrane potential of −80 mV, but the conductance was calculated from measurements of single channel amplitudes at a number of different membrane potentials. At the end of each recording, the resting potential of the muscle fibre was measured by breaking the membrane patch to record in the whole cell mode. This value was later confirmed from the current-voltage curve for the channel, assuming the reversal potential to be 0 mV. Patches were only accepted for analysis if the two values agreed within 10 mV.

Analysis of burst durations was performed in patches with relatively low open probabilities, i.e. those in which the mean interburst closed interval was greater than 100 ms. In these patches, the bursts of openings were well separated and readily indistinguishable. The burst terminator value (the threshold level used to discriminate between intra- and interburst closed durations) used during analysis was at least 7 times the mean intraburst closed time determined from exponential fits of closed duration histograms (Alkondon & Albuquerque, 1989). The burst open probability was measured as the fraction of time spent in the open state during that burst. The total open time per burst was therefore calculated by multiplying the duration of each burst by the open probability during that burst. Histograms of burst durations and total open time per burst were fitted with exponential functions using the least squares method.

A simple sequential model of channel block was used to interpret the data. This assumed that the blocking drug binds reversibly to the conducting state of the channel to form a species with little or no conductance (Adams, 1976; Adler *et al.*, 1978; Neher & Steinbach, 1978). The model may be represented by the following reaction scheme:



where A is the agonist molecule, R is the nicotinic receptor, n is the number (usually considered to be two) of agonist molecules bound per receptor, A_nR is the agonist-bound closed state, A_nR^* is the open state and A_nRD is the blocked state. The closed and blocked states are usually indistinguishable on the basis of conductance, but may be distinguished by their temporal characteristics.

According to this model, the mean open time is equal to $1/(\alpha + G.c)$, where G is the forward rate constant for the blocking reaction and c is the concentration of the blocking drug. Thus, a plot of the reciprocal of the open time versus the concentration of the blocking drug should yield a straight line, the slope of which is equal to G and the intercept of which is equal to α. As the unblocking reaction is the only step leading away from the blocked state, the unblocking rate constant, F, may be estimated as the inverse of the mean duration of the blocked state (Neher & Steinbach, 1978).

With some of the compounds tested, the channel block was too rapid for individual events to be adequately resolved by the recording system. To overcome this problem, the method of charge integral analysis was used (Neher, 1983). Integrals were calculated by point-to-point summation of each digitised burst. The burst open probability (P_o) was then calculated as

$$P_o = \frac{\text{charge integral}}{\text{burst length} \times \text{full channel amplitude}} \quad (1)$$

This method offers at least three advantages over the kinetic analysis described above: (a) it does not require the measurement of individual open and blocked intervals; (b) it is independent of the exact form of the open and closed time distributions and (c) the area under the responses is not affected by the limited frequency response of the recording system.

The burst open probability so obtained can then be used to characterize the blocking reaction in a more model-independent manner. If there is only one open state, and if the individual openings are short relative to the burst duration, an apparent dissociation constant K'_D can be defined (Neher, 1983):

$$K'_D = c.T'/(T-T'), \quad (2)$$

where T is the burst duration and T' is the total conducting time. Substituting burst open probability, this becomes:

$$K'_D = c.P_o/(1-P_o). \quad (3)$$

and hence

$$1/P_o = (c/K'_D) + 1 \quad (4)$$

This assumes that the burst open probability in control conditions (P_c) is equal to one. An expression containing the true dissociation constant, K_D, can be obtained by substituting the true P_c:

$$1/P_o = (c/K_D) + (1/P_c) \quad (5)$$

Thus, a plot of the reciprocal of the burst open probability against blocker concentration will give a straight line with a slope of $1/K_D$. Furthermore, K_D can be estimated using a single concentration of the blocker, from the expression:

$$K_D = \frac{c}{(1/P_o) - (1/P_c)} \quad (6)$$

Statistics

Data are expressed as mean ± s.e.mean. Groups of data were compared by Student's *t* test (two-tailed). A difference between means was considered significant if the probability of it being due to random variation, *P*, was less than 0.05.

Drugs

Acetylcholine chloride, pyridostigmine bromide and physostigmine salicylate were purchased from Sigma. Pyrimidoxime (PMX) was kindly supplied by H. Garrigue (Centre d'Etudes du Bouchet, Paris, France); toxogonin and HI-6 were gifts from M.G. Hamilton (Defence Research Establishment Suffield, Alberta, Canada). All other pyridinium compounds (>98% pure) and soman (> 95% pure) were synthesized at this Establishment. Soman was stored as a 5 mg ml^{-1} solution in isopropanol and diluted to its final concentration with physiological saline. The structures of the pyridinium compounds used are shown in Figure 1. Since all recordings were made in the cell-attached mode, only one drug concentration was applied to each patch.

Results

Diaphragms

Application of soman (100 nM) to the diaphragm preparation blocked the sustained phase of the tetanus, so that all that remained was a brief, rapidly decaying contraction (Figure 2). The tension 1 s after the start of the tetanus was reduced to less than 5% of its control value by soman. In control experiments, in which no oxime was applied, the blockade was not removed by washing off the soman, although there was a gradual increase in the tetanic response over time (Figure 2a). This recovery was not due to spontaneous regeneration of active AChE, since it was not blocked by a second application of soman.

A number of the oximes tested, such as PMX, produced a recovery of up to 50% of the tetanic response, by lengthening the decay of the tetanus (Figure 2b). Most of this recovery was reversed by washing off the oxime, and was therefore considered a direct action of the oxime on the muscle. The remaining response was not due to reactivation of AChE, as it was resistant to blockade by a second dose of soman.

Similar results were obtained with some of the compounds which lacked the oxime group, such as SAD-128. These also caused recovery of the tetanic response, most of which was abolished by washing off the drug (Figure 2c). Once again, the remaining response was not significantly reduced by the second dose of soman.

The results for all 12 compounds tested are summarized in Figure 3. LA54, P65 and SAD-128 significantly blocked muscle twitch at a concentration of 200 μM, and so were tested at lower concentrations. None of the drugs caused significant reactivation of AChE, and only one of them, P2S, produced a significant increase in the residual recovery remaining after the second dose of soman. There was, however, a wide variation between compounds in the degree of direct action on the diaphragm. The monopyridinium compounds had no significant direct effect, but many of the bispyridinium drugs and PMX produced a very marked direct recovery of tetanic tension, up to 30% of the control response. These measurements of the direct therapeutic effects of the compounds were subsequently used for comparison with their channel-blocking activities.

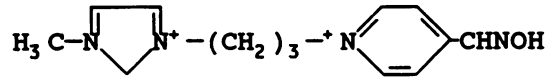
Single channels

The drugs were tested on single nicotinic receptor ion channels at the same concentrations which had been used in the diaphragm recovery experiments. Typical recordings are shown in Figure 4. The left hand trace in each panel is a representative channel opening. Relatively long duration bursts are shown in each case, as they illustrate more effectively the open channel blocking actions of each compound. (Bursts are defined as single openings or groups of openings separated from similar groups by a closed interval greater than or equal to the burst terminator value: see Methods). The right hand trace in each panel shows an ensemble current produced by averaging 100 single channel bursts. The leading edge of each burst was used to trigger the averaging. These averaged traces closely approximate macroscopic endplate currents, and show the decline in the mean current following channel activation.

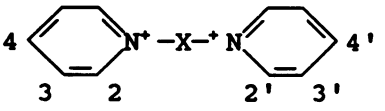
The records are arranged in order of increasing direct pharmacological action measured for each compound in the diaphragm. In control patches and in the presence of drugs which had no significant direct action, the channel openings were simple square wave events interrupted by only a few brief closings, and separated by long silent periods ranging from tens of milliseconds to several seconds. Closed duration histograms could be fitted by single exponential functions, and there was no evidence of a second, faster exponential function in the closed time distributions. The noise level of the open state remained similar to that during the closed state of the channel. The ensemble currents in these conditions decayed according to a single exponential function.

Compounds which had pronounced direct actions in the diaphragm produced a rapid, flickering block of the channels, so that the openings took the form of bursts of short events. Many of the events were too brief to be completely resolved by the 5 kHz bandwidth of the recording equipment, and the apparent amplitude of the single channel current was smaller than in control patches. The noise level of the open state was often greater than that during the closed state, probably reflecting the presence of unresolved brief events. With these compounds, closed duration histograms showed

Imidazopyridinium compound

PM 

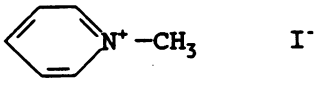
Bispyridinium compounds



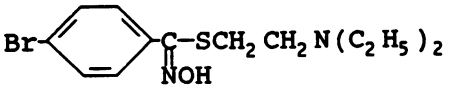
Compound	4	2	X	3′	4′
TMB-4	-CHNOH	H	$-(CH_2)_3-$	H	-CHNOH
SAD-128	$-C(CH_3)_3$	H	$-CH_2\ OCH_2-$	H	$-C(CH_3)_3$
Toxogonin	-CHNOH	H	$-CH_2\ OCH_2-$	H	-CHNOH
HI-6	H	-CHNOH	$-CH_2\ OCH_2-$	H	$-CONH_2$
P65	$-C(CH_3)_3$	H	$-(CH_2)_6-$	H	$-C(CH_3)_3$
BPT	H	H	$-(CH_2)_3-$	H	H
BPE	H	H	$-CH_2\ OCH_2-$	H	H
HS-6	H	-CHNOH	$-CH_2\ OCH_2-$	$-CONH_2$	H

Monopyridinium compounds and LA54

Pyridine methiodide



LA54



P2S

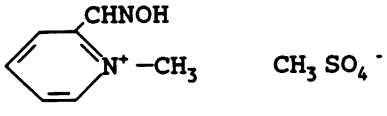


Figure 1 Structural formulae of oximes and analogues tested.

an additional fast exponential component compared with controls, which was related to the intraburst closures. The ensemble currents obtained in the presence of these drugs showed dual exponential decays and smaller peak amplitudes than in control patches.

In order to quantify the channel-blocking properties of the compounds for correlation with their direct actions, several parameters of channel block were measured. These were conductance, mean open duration, mean burst duration, mean open probability within a burst and mean number of openings per burst. In the presence of many of the compounds, the burst duration histogram showed a double exponential distribution: the time constant of the slower exponential was used to estimate the mean burst duration. Considerable error was introduced into the measurements for some compounds by the very rapid nature of their blocking action, which led to incomplete resoluton of many of the events. An attempt was made to improve the resolution by increasing the bandwidth to 10 kHz, but this produced a large increase in the number of false detections due to the rise in background noise. Charge integral analysis, which is largely unaffected by the limited bandwidth of the recording system, was used to estimate burst open probability (see Methods).

Figure 5 shows scatter plots of parameters of channel block at − 80 mV membrane potential versus the degree of direct recovery for the range of 12 compounds. There is considerable scatter in the data, but this is to be expected in view of the errors in measurement described above. Despite this scatter, however, there were strong correlations between the parameters of channel block and the direct action measured in diaphragms. Compounds with large direct actions reduced the conductance, mean open time and and burst open probability of the channels, and increased the mean burst duration and the mean number of events in a burst.

Concentration-response relationships

If the direct action seen in diaphragms was indeed related to channel blocking by the oximes, the two effects would be expected to show similar concentration-dependence. Two of the compounds which showed the greatest effects, PMX and SAD-128, were therefore studied further by investigating the actions of different concentrations on soman-poisoned dia-

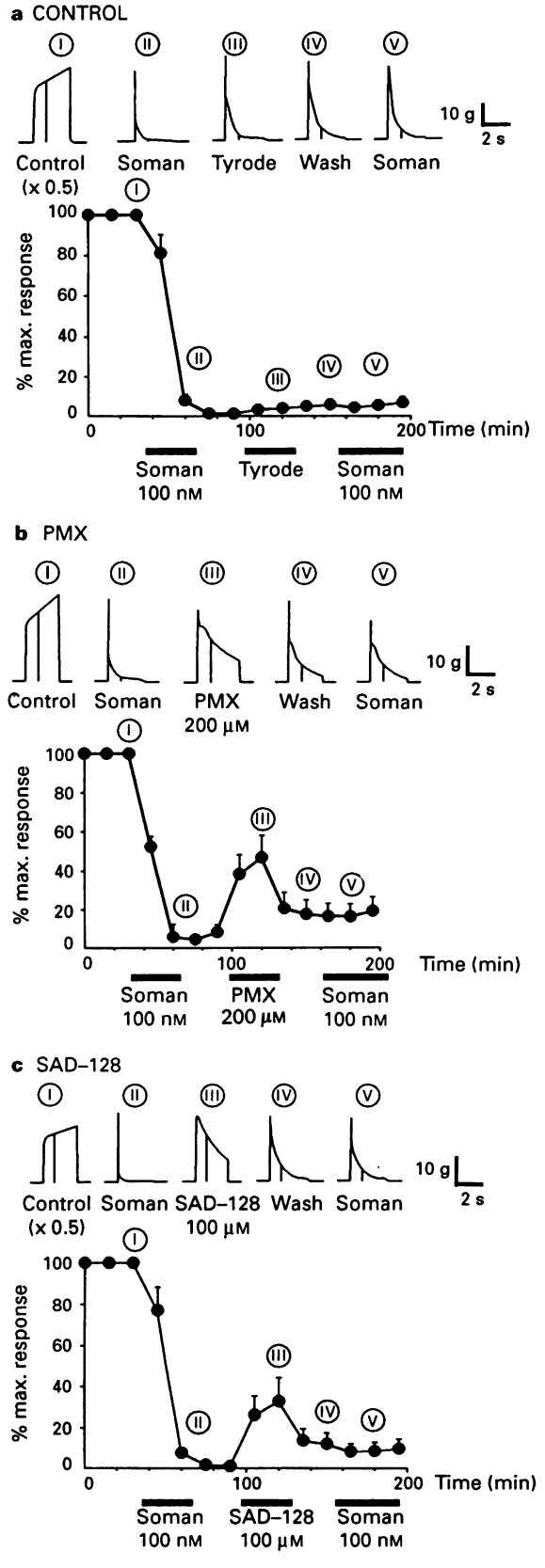


Figure 2 Recovery of tetanic contractions following soman poisoning (a) in control diaphragms, (b) after pyrimidoxime (PMX) and (c) after SAD-128. Graphs show mean ± s.e.mean for 4 preparations in each group, insets are representative contractions at the points indicated. The vertical lines indicate the tension 1 s after the start of each tetanus. Note the change in gain from control traces in (a) and (c). Three components of recovery could be measured in these experiments: direct action (III-IV), reactivation (IV-V) and residual recovery (V).

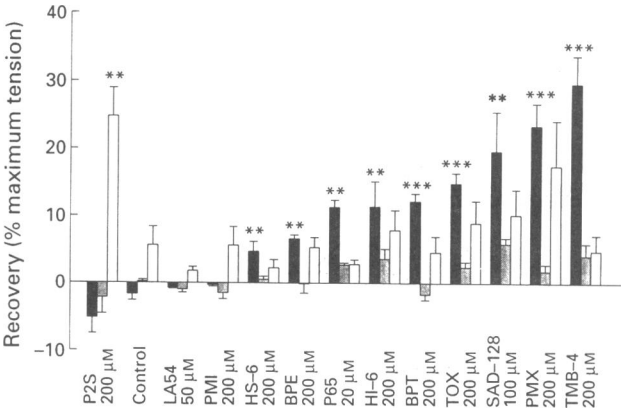


Figure 3 Recovery produced by oximes and analogues in guinea-pig diaphragm following soman poisoning. Each column shows the mean ± s.e.mean of 4 preparations. Solid columns, recovery due to direct action; hatched columns, recovery due to reactivation; open columns, residual recovery. Significant differences from control values are indicated: $*P<0.05$; $**P<0.01$; $***P<0.001$.

phragms and on single channels. Figure 6 shows concentration-response relationships obtained with PMX. Significant recovery of the diaphragm due to direct action of the oxime appeared at a concentration of 100 μM. In single channel experiments, the threshold concentration for significant effects on mean open time and conductance was also 100 μM. Significant effects on burst open probability, burst duration and number of events per burst were found at the slightly lower concentration of 50 μM.

With the non-oxime compound, SAD-128, significant direct recovery appeared at a lower concentration than for PMX, 20 μM, but the concentration-response curve was less steep than the one for PMX (Figure 7). Significant effects of SAD-128 on all of the single channel parameters measured appeared at a similar concentration, between 5 and 10 μM. The relationships obtained for burst duration and number of events per burst showed an apparent decrease in the effects of SAD-128 at higher concentrations, but this was probably a reflection of the errors incurred in these measurements by the incomplete resolution of very brief events, as described previously.

As predicted by the simple sequential model, the inverse of the mean open time was linearly related to the concentration of PMX or SAD-128 for all concentrations tested (Figure 8a), and the slope of this relationship was used to determine the forward rate constant of the channel-blocking step, G. According to the model, the fast exponential which appeared in the closed time distribution in the presence of PMX or SAD-128 described the duration of the blocked state of the channel. The time constants of these fast exponentials were used to estimate F, the unblocking rate constant, which was independent of the concentration of PMX or SAD-128. The dissociation constant for channel block, K_D, was then calculated from G and F. The values obtained for G, F and K_D are given in Table 1.

Charge integral analysis was used to obtain independent estimates of the dissociation constant for channel block, K_D. As predicted from equation (4) in the Methods, the reciprocal of burst open probability was linearly related to the concentration of SAD-128 or PMX for all concentrations tested (Figure 8b), and the slope of this relationship was used to calculate K_D, shown in Table 1. Values of burst open probabilities measured for other bispyridinium compounds, which were each tested at only a single concentration, were used to estimate K_D for these compounds from equation (3). These estimates are listed in Table 2: no values were calculated for P2S or PMI, because the burst open probabilities were not significantly different from control values.

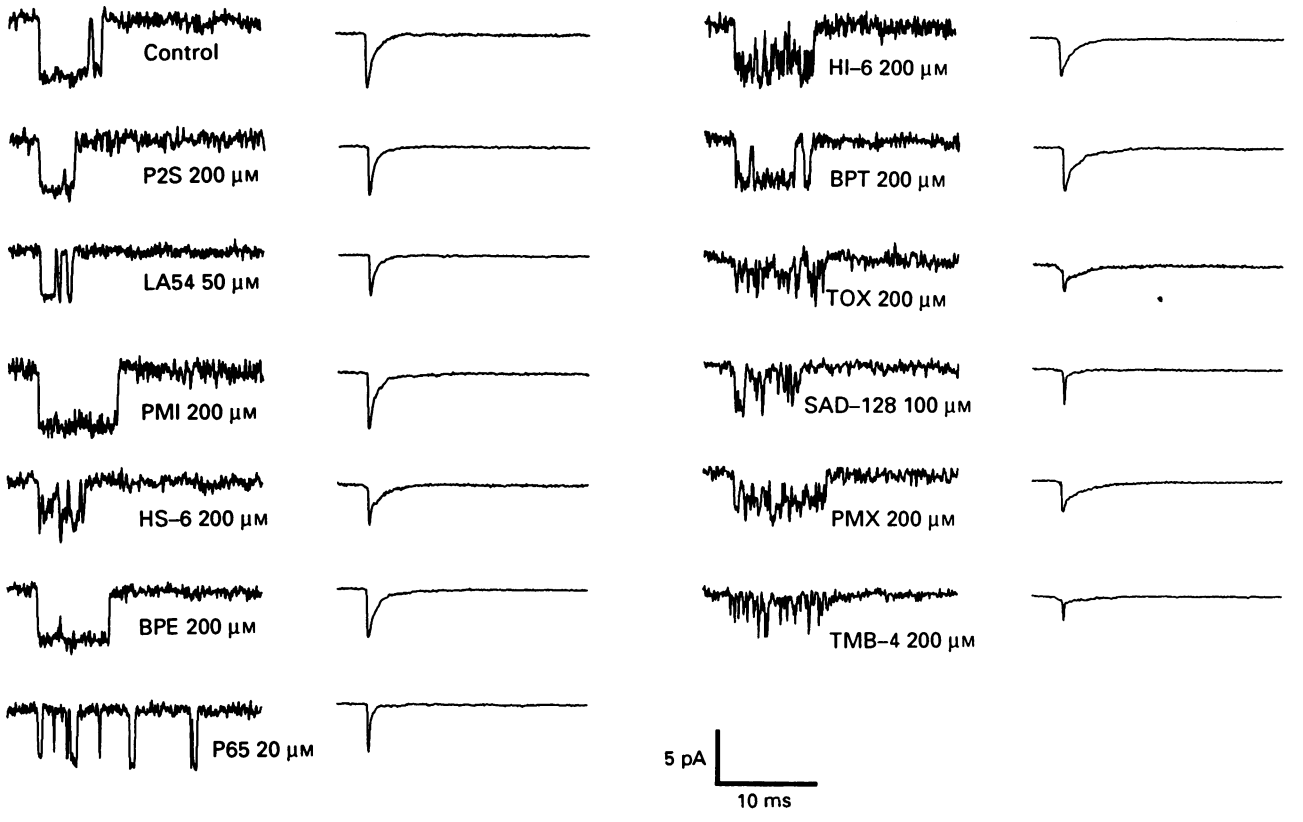
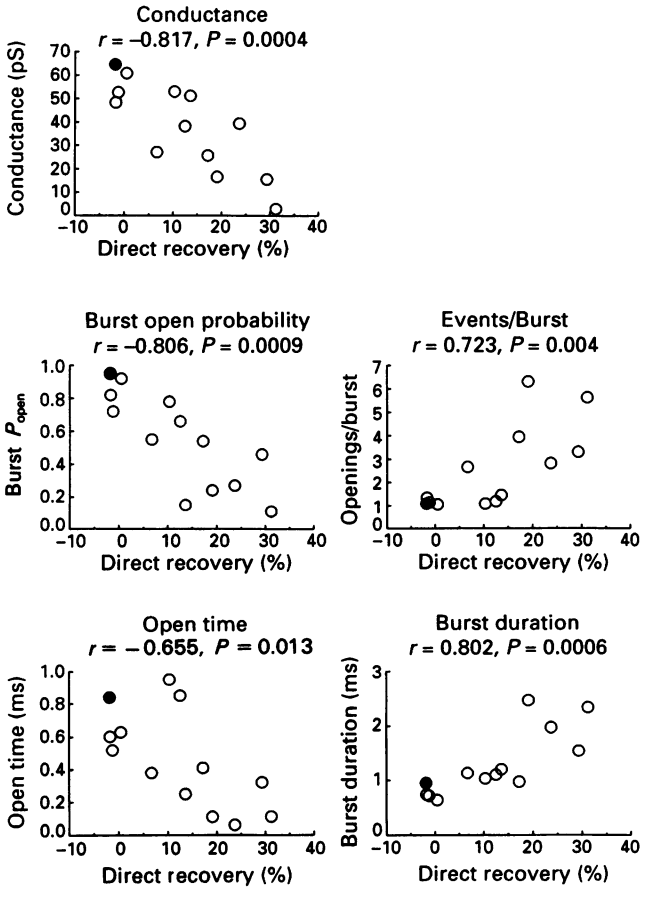


Figure 4 Representative single channel currents (left panels) and ensemble currents produced by averaging 100 single channel openings (right panels) in the presence of oximes and analogues. The membrane potential was − 80 mV in each patch. Compounds are arranged in order of increasing direct action, measured in diaphragm experiments.



Discussion

The results obtained in this study demonstrate that there is a very significant correlation between the channel-blocking activity of pyridinium compounds and their direct pharmacological actions on soman-poisoned diaphragm muscle *in vitro*. Furthermore, the threshold concentration of drug which causes channel-blocking is similar to that required for direct recovery. These results strongly indicate that the direct recovery produced *in vitro* is due to blocking of open nicotinic receptor ion channels at the neuromuscular junction. Following inhibition of AChE by soman, the channel-blocking action of the pyridinium drugs could counteract the effects of excessive buildup of ACh at the neuromuscular junction, by reducing the current flowing through nicotinic channels.

Diaphragm recovery

The experiments with the *in vitro* diaphragm preparation confirmed that a range of pyridinium oximes and analogues can produce neuromuscular recovery from soman poisoning *in vitro*, which is not related to reactivation of AChE. The recovery is reversed by removal of the oxime, and therefore appears to be due to a direct pharmacological action on the muscle. Furthermore, this direct recovery can be produced by concentrations of oximes which are likely to be produced in

Figure 5 Scatter plots of 5 parameters of channel opening (at a membrane potential of − 80 mV) versus direct recovery measured in diaphragm preparations: (●) control values; (○) values in the presence of oximes and analogues. The correlation coefficient (r) and its statistical significance (P) are given for each plot.

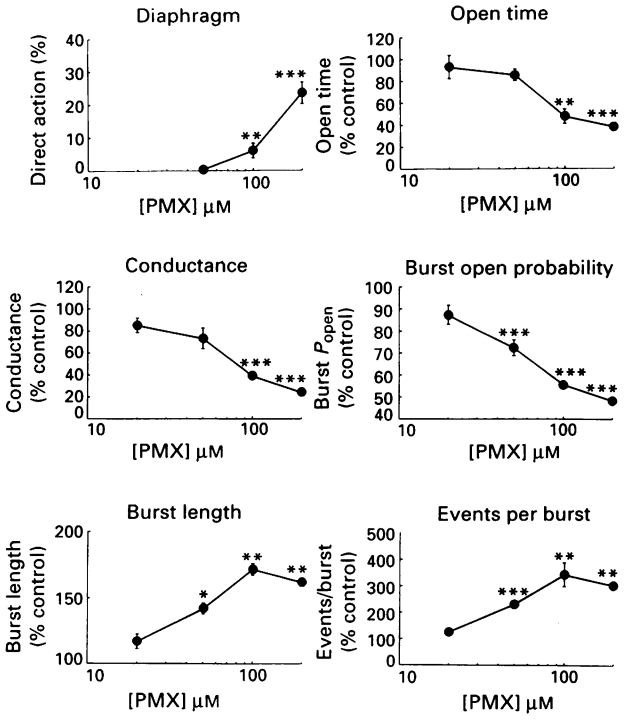


Figure 6 Concentration-dependence of the direct effect of pyrimidoxime (PMX) on soman-poisoned guinea-pig diaphragm and of its effects on single channel currents at a membrane potential of − 80 mV. Each point shows mean ± s.e.mean for at least three patches. Significant differences from control values are indicated: $*P<0.05$; $**P<0.01$; $***P<0.001$.

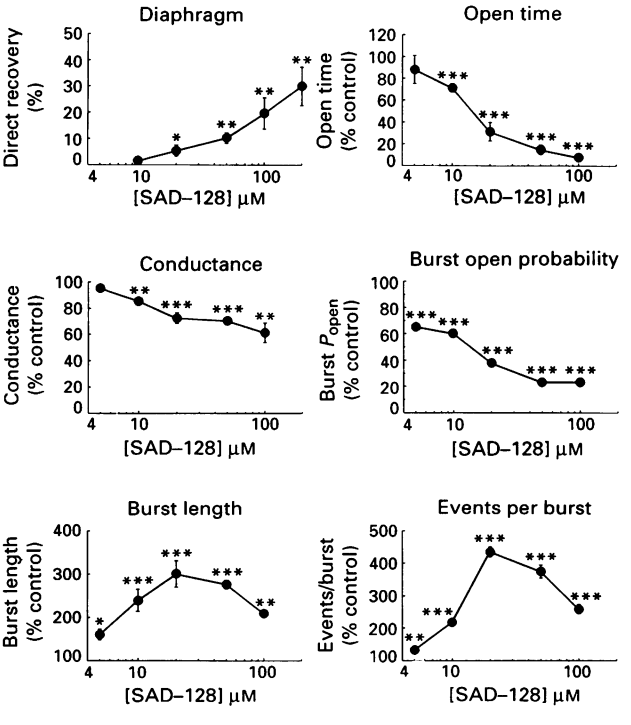


Figure 7 Concentration-dependence of the direct effect of SAD-128 on soman-poisoned guinea-pig diaphragm and of its effects on single channel currents at a membrane potential of − 80 mV. Each point shows mean ± s.e.mean for at least three patches. Significant differences from control values are indicated: $*P<0.05$; $**P<0.01$; $***P<0.001$.

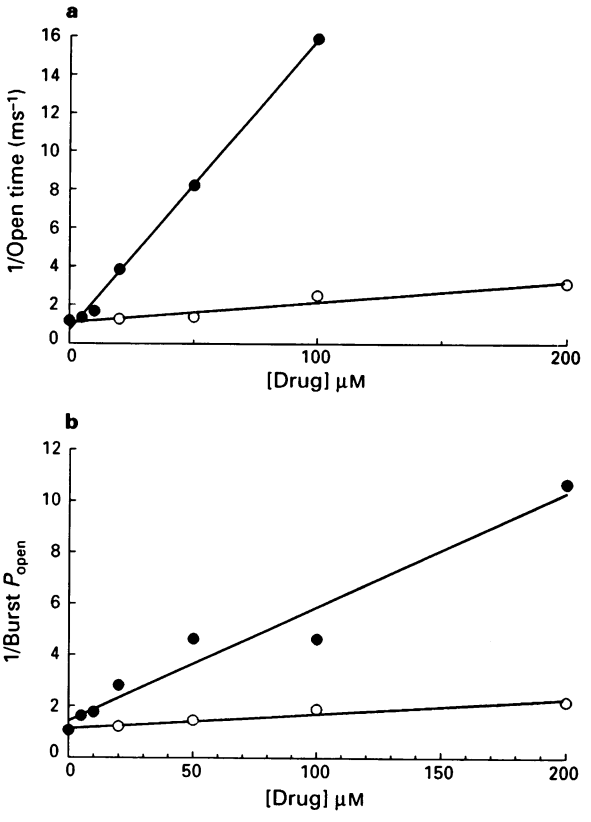


Figure 8 Relationship between the reciprocal of the mean channel open time (a) or the reciprocal of the mean burst open probability (b) and the concentration of pyrimidoxime (PMX) (○) or SAD-128 (●) at a membrane potential of − 80 mV. Lines were fitted by linear regression. The slope of the relationship in (a) is equal to G, the rate constant for channel blocking. The slope of the relationship in (b) is equal to $1/K_D$, the dissociation constant for channel blocking.

Table 1 Constants for open channel-block by pyrimidoxime (PMX) and SAD-128 at − 80 mV membrane potential

	PMX	*SAD-128*
G, ms^{-1} μM^{-1}	0.0105	0.1512
F, ms^{-1}	15.20	2.50
K_D (= F/G), μM	1447.62	16.53
K_D, μM (from charge integral analysis)	120.0	22.49

the blood plasma by administration of therapeutic doses of the drugs. None of the compounds caused significant reactivation of AChE, indicating that the inhibited enzyme had almost completely aged before the compounds were applied. This was expected, since the half time of aging for soman-inhibited AChE in the guinea-pig is 6–8 min (Harris *et al.*, 1971; Talbot *et al.*, 1988).

Previous studies on guinea-pig muscle *in vitro* have also demonstrated direct therapeutic actions of oximes, but generally at higher concentrations than those used here (Smith *et al.*, 1981; French *et al.*, 1983). French *et al.* (1983) used the compounds at previously-determined optimum concentrations, which were usually higher than those tested in the present study. Their ranking of the compounds by the magnitude of direct action is in general agreement with the results presented here, with P2S having the smallest direct

Table 2 Dissociation constants for open channel-block by oximes at −80 mV membrane potential, estimated from burst open probability at a single concentration of drug

	Conc (μM)	K_D (μM)
LA-54	50	154.7 ± 19.4
HS-6	200	264.5 ± 24.8
BPE	200	844.7 ± 19.1
P65	20	3.6 ± 0.6
HI-6	200	251.9 ± 11.5
BPT	200	457.6 ± 57.6
Tox	200	63.9 ± 3.6
SAD-128	100	27.5 ± 0.3
PMX	200	169.7 ± 8.3
TMB-4	200	25.6 ± 2.5

Values are mean ± s.e.mean for 4 patches.
See Figure 1 for structure of drugs.

action, and TMB-4 and SAD-128 the largest. French *et al.* (1983), however, reported a higher ranking for BPE and HS-6, and a lower ranking for BPT, than was found in the present experiments. These discrepancies are probably due to the much higher concentrations of the drugs which they used and the time of application, which was only 1 min after soman in their study.

Single channels

Single channel recordings showed that many of the compounds blocked open nicotinic receptor ion channels, and the degree of this blockade could be related to the direct pharmacological action against soman poisoning. The compounds which showed the greatest direct action produced a very rapid flickering blockade of the channels, accompanied by an apparent decrease in the single channel conductance. It is likely that the apparent decrease in conductance was due to inadequate resolution of very brief openings by the recording system, and was therefore an indication of the rapid kinetics of the block. With the exception of P65, which contains a hexamethonium-like bridge between the two pyridinium moieties, none of the compounds produced significant competitive antagonism of ACh or desensitization of the nicotinic receptor, as measured by the frequency of channel opening (inverse of mean interburst closed duration) and its decrease over time.

As predicted by the simple sequential model, the reciprocal of mean channel open time increased linearly with the concentration of PMX or SAD-128. The model also predicts that the mean burst duration increases with concentration of the blocking drug, and this was found to be true for PMX at concentrations up to 100 μM and for SAD-128 up to 20 μM. At higher concentrations, however, the mean burst duration began to decrease, suggesting that the model may not adequately describe blocking under these conditions. A further deviation from model was revealed by estimates of the total open time per burst. The model predicts that this should be unaffected by the presence of a channel blocker, but both PMX and SAD-128 caused an apparent increase in the total open time per burst, which fell again at higher concentrations of the two compounds. It is conceivable that this apparent increase was at least partly due to an inability of the recording system to detect very brief closings within bursts, leading to an overestimate of the total open time, rather than to an inadequacy of the sequential model. The results for SAD-128 contrast with those reported by Alkondon & Albuquerque (1989), who found that this compound reduced the total open time per burst of channels from frog muscle. Their study was conducted at a lower temperature, 10°C, at which the kinetics of channel block may have been slower (see below) and possibly easier to follow.

The estimates of the channel-blocking rate constants, G and F, for SAD-128 were both approximately three times greater than those reported by Alkondon & Albuquerque (1989) in the frog, but the dissociation constant, K_D, was very similar in the two preparations. The slower rate constants for the frog preparation may be due to the lower temperature at which these were measured (10°C). In the mouse preparation, G was nearly fifteen times smaller, and F approximately six times greater, for PMX than for SAD-128. K_D was therefore two orders of magnitude greater for PMX, and was similar to values reported for HI-6 and 2-PAM in the frog (Alkondon *et al.*, 1988).

The dissociation constant derived for SAD-128 from charge integral analysis was very similar to the value of K_D calculated using the sequential model. The value of K_D obtained for PMX by charge integral analysis, however, was an order of magnitude smaller than the value calculated from kinetic analysis, suggesting that the limited bandwidth of the recording system introduced large errors in the estimates of G and F. Both methods showed that SAD-128 had a much higher affinity for the channel than did PMX, consistent with its significant channel-blocking activity (and direct action in the diaphragm experiments) at lower concentrations.

The K_D for HI-6 obtained by charge integral analysis was an order of magnitude lower than the value calculated by Alkondon *et al.* (1988) from kinetic analysis. This difference could once again be a result of errors due to the limited recording bandwidth (their records were filtered at 3 kHz), or it may possibly reflect species differences between frog and mouse preparations. No previously published values are available for the other compounds listed in Table 2.

Relevance to therapeutic and toxic effects

It is becoming increasingly apparent that mechanisms of action of pyridinium compounds other than reactivation of inhibited AChE can be beneficial in cases of organophosphorus poisoning (Rousseaux & Dua, 1989). Evidence from a number of studies suggests that these alternative mechanisms are more important in the treatment of soman poisoning than against other organophosphates, presumably due to the rapid aging of soman-inhibited AChE (Broomfield, 1981; van Helden *et al.*, 1991). It has been suggested that nicotinic receptors are an important target site (Clement, 1981; Broomfield, 1981; Su *et al.*, 1983), and that the channel-blocking action of oximes may be related to their antidotal properties against organophosphates (Alkondon *et al.*, 1988; Alkondon & Albuquerque, 1989).

The present study provides strong evidence that the channel-blocking activity of oximes and analogues does indeed underlie their ability to produce recovery of neuromuscular transmission following soman poisoning *in vitro*. As pointed out by French *et al.* (1983), however, these *in vitro* effects of oximes on neuromuscular function cannot easily be translated to *in vivo* effects, since the neuromuscular recovery does not correlate well with *in vivo* protection against soman poisoning. Thus, whilst the channel-blocking activity of oximes appears to underlie their antidotal effects in the *in vitro* muscle preparation, it cannot fully explain their actions against soman in whole animal protection studies. Other effects may be important, such as ganglion-blocking and antimuscarinic activities (Kuhnen-Clausen, 1972; Lundy & Tremblay, 1978; Clement, 1981), and it has been suggested that some of the oximes may cross the blood-brain barrier to have effects on the central nervous system (Klimmek & Eyer, 1986; Rousseaux & Dua, 1989). It is also possible that harmful side effects of some compounds, such as those described for some pyridinium-4-oximes (Erdmann, 1976; Schoene, 1976), may diminish their beneficial actions.

Although the therapeutic activity of bispyridinium compounds against soman poisoning is still not fully understood, the results presented here demonstrate how one mechanism of action of these compounds, open channel blocking, could

contribute to their overall antidotal effectiveness. Unlike competitive antogonists, which prevent channel activation, channel blockers are able to modulate rather than eliminate nicotinic receptor function. A competitive antagonist, such as tubocurarine, would counteract the effects of ACh by preventing activation of a proportion of nicotinic receptors, but the antagonism could be overcome by increased concentrations of ACh accumulating at the neuromuscular junction and competing with the antagonist. Furthermore, there is a danger that high concentrations of the competitive antagonist would block neuromuscular transmission completely.

In contrast to a competitive antagonist, a channel blocker with rapid kinetics, such as many of the bispyridinium compounds tested here, would reduce the mean current flowing through a channel during a burst without greatly altering the burst length or affecting channel activation. Such an action would provide an effective counter to the overstimulation of nicotinic receptors at an endplate subjected to ACh accumulation following inhibition of AChE. Furthermore, open channel blockade is use-dependent, so that the antagonism would become stronger as more channels were activated. In addition, the rapid cycling of the nicotinic receptor ion channel between blocked and open states could serve as an alternative pathway to protect the receptor from desensitization (Alkondon & Albuquerque, 1989). A further advantage over competitive antagonists is that high concentrations of such channel blockers would be less likely to block transmission completely. By selecting oximes with optimal channel-blocking properties in addition to their other beneficial activities, it may be possible to improve the effectiveness of current treatments against soman poisoning.

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References

ADAMS, P.R. (1976). Drug blockade of open end-plate channels. *J Physiol.*, **260**, 531–552.

ADLER, M., ALBUQUERQUE, E.X. & LEBEDA, F.J. (1978). Kinetic analysis of endplate currents altered by atropine and scopolamine. *Mol. Pharmacol,*. **14**, 514–529.

ALKONDON, M. & ALBUQUERQUE, E.X. (1989). The nonoxime bispyridinium compound SAD-128 alters the kinetic properties of the nicotinic acetylcholine receptor ion channel – a possible mechanism for antidotal effects. *J. Pharmacol. Exp. Ther.*, **250**, 842–852.

ALKONDON, M., RAO, K.S. & ALBUQUERQUE, E.X. (1988). Acetylcholinesterase reactivators modify the functional properties of the nicotinic acetylcholine receptor ion channel. *J. Pharmacol. Exp. Ther.*, **245**, 543–556.

BENSCHOP, H.P., DE JONG, L.P.A., VINK, J.A.J., KIENHUIS, H., BERENDS, F., ELSKAMP, D.M.W., KEPNER, L.A., MEETER, E. & VISSER, R.P.L.S. (1976). The prophylactic value of oximes against organophosphate poisoning. In *Medical Protection Against Chemical-Warfare Agents*. ed. Stares, J.E.S. pp. 120–133. Stockholm: Almqvist & Wirksell International.

BREHM, P. & KULLBERG, R. (1987). Acetylcholine receptor channels on adult mouse skeletal muscle are functionally identical in synaptic and nonsynaptic membrane. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 2550–2554.

BROOMFIELD, C.A. (1981). Some proposals for the mechanism of action of the H-oximes. In *Evaluation of H-Series Oximes. Proceedings of a Symposium*. Biomedical Laboratory Technical Report USABML-SP-81-001. ed. Broomfield, C.A., Hackley, B.E., Hahn, F.E., Lenz, D.E. & Maxwell, D.M. pp. 35–53. Aberdeen Proving Ground, MD, USA: US Army Medical Research and Development Command, Biomedical Laboratory.

BULBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation. *Br. J. Pharmacol. Chemother.*, **1**, 38–61.

CLEMENT, J.G. (1981). Toxicology and pharmacology of bispyridinium oximes – insight into the mechanism of action vs. soman poisoning in vivo. *Fundam. Appl. Toxicol.*, **1**, 193–202.

COLQUHOUN, D. & SIGWORTH, F.J. (1983). Fitting and statistical analysis of single-channel records. In *Single Channel Recording*. ed. Sakmann, B. & Neher, E. pp. 191–263. New York: Plenum Press.

ECOBICHON, D.J., COMEAU, A.M., O'NEIL, W.M. & MARSHALL, W.D. (1990). Kinetics, distribution, and biotransformation of the chemical HI-6 in the rat, dog, and rhesus monkey. *Can. J. Physiol. Pharmacol.*, **68**, 614–621.

ERDMANN, W.D. (1976). Success and failure of oxime therapy in acute poisoning by organophosphorus compounds. In *Medical Protection Against Chemical-Warfare Agents*. ed. Stares, J.E.S. pp. 46–52. Stockholm: Almqvist & Wirksell International.

FRENCH, M.C., WETHERELL, J.R. & WHITE, P.D.T. (1983). The reversal by oximes and their deoximinomethyl analogues of neuromuscular block produced by soman. *Eur. J. Pharmacol.*, **91**, 399–409.

GREEN, M.D., TALBOT, B.G. & CLARK, C.R. (1986). Pharmacokinetics of pralidoxime chloride in the rat. *Life Sci.*, **39**, 2263–2269.

HAMILTON, M.G. & LUNDY, P.M. (1989). HI-6 therapy of soman and tabun poisoning in primates and rodents. *Arch. Toxicol.*, **63**, 144–149.

HARRIS, L.W., YAMAMURA, H.I. & FLEISHER, J.H. (1971). De novo synthesis of acetylcholinesterase in guinea-pig retina after inhibition by pinacolyl methylphosphonofluoridate. *Biochem. Pharmacol.*, **20**, 2927–2930.

INNS, R.H. & LEADBEATER, L. (1983). The efficacy of bispyridinium derivatives in the treatment of organophosphate poisoning in the guinea pig. *J. Pharm. Pharmacol.*, **35**, 427–433.

KLIMMEK, R. & EYER, P. (1986). Pharmacokinetics and pharmacodynamics of the oxime HI6 in dogs. *Arch. Toxicol.*, **59**, 272–278.

KUHNEN-CLAUSEN, D. (1972). Structure-activity relationship of mono- and bisquaternary pyridines in regard to their parasympathetic effects. *Toxicol. Appl. Pharmacol.*, **23**, 443–454.

LOOMIS, T.A. & SALAFSKY, B. (1963). Antidotal action of pyridinium oximes in anticholinesterase poisoning, comparative effects of soman, sarin, and neostigmine on neuromuscular function. *Toxicol. Appl. Pharmacol.*, **5**, 685–690

LUNDY, P.M. & TREMBLAY, K.P. (1979). Ganglion blocking properties of some bispyridinium soman antagonists. *Eur. J. Pharmacol.*, **60**, 47–53.

NEHER, E. (1983). The charge carried by single-channel currents of rat cultured muscle cells in the presence of local anaesthetics. *J. Physiol.*, **339**, 663–678.

NEHER, E. & STEINBACH, J.H. (1978). Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol.*, **277**, 153–176.

ROUSSEAUX, C.G. & DUA, A.K. (1989). Pharmacology of HI-6, an H-series oxime. *Can. J. Physiol. Pharmacol.*, **67**, 1183–1189.

SCHOENE, K. (1976). Kinetic studies on chemical reactions between acetylcholinesterase, toxic organophosphates and pyridinium oximes. In *Medical Protection Against Chemical-Warfare Agents*. ed. Stares, J.E.S. pp. 88–100. Stockholm: Almqvist & Wirksell International.

SMITH, A.P. & MUIR, A.W. (1977). Antidotal action of the oxime HS6 at the soman poisoned neuromuscular junction of the rat and guinea-pig. *J. Pharm. Pharmacol.*, **29**, 762–764.

SMITH, A.P., VAN DER WIEL, H.J. & WOLTHUIS, O.L. (1981). Analysis of oxime-induced neuromuscular recovery in guinea pig, rat and man following soman poisoning in vitro. *Eur. J. Pharmacol.*, **70**, 371–379.

STARES, J.E.S. (1976). Medical protection against chemical-warfare agents. In *Medical Protection Against Chemical-Warfare Agents*. ed. Stares, J.E.S. pp. 157–166. Stockholm: Almqvist & Wirksell International.

SU, C.-T., TANG, C.P., MA, C., SHIH, Y.S., LIU, C.Y. & WU, M.T. (1983). Quantitative structure-activity relationships and possible mechanisms of action of bispyridinium oximes as antidotes against pinacolyl methylphosphonofluoridate. *Fundam. Appl. Toxicol.*, **3**, 271–277.

TALBOT, B.G., ANDERSON, D.R., HARRIS, L.W., YARBROUGH, L.W. & LENNOX, W.J. (1988). A comparison of in vivo and in vitro rates of aging of soman-inhibited erythrocyte acetylcholinesterase in different animal species. *Drug Chem. Toxicol.*, **11**, 289–305.

VAN HELDEN, H.P.M., DE LANGE, J., BUSKER, R.W. & MELCHERS, B.P.C. (1991). Therapy of organophosphate poisoning in the rat by direct effects of oximes unrelated to ChE reactivation. *Arch. Toxicol.*, **65**, 586–593.

WOLTHUIS, O.L., CLASON-VAN DER WIEL, H.J. & VISSER, R.P.L.S. (1976). The dependence of the blood level of the oxime HS-6 on the severity of organophosphate poisoning. *Eur. J. Pharmacol.*, **39**, 417–421.

WOLTHUIS, O.L., VAN WERSCH, R.A.P. & VAN DER WIEL, H.J. (1981). The efficacy of some bis-pyridinium oximes as antidotes to soman in isolated muscles of several species including man. *Eur. J. Pharmacol.*, **70**, 355–369.

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Enhancement of airway reactivity to histamine by isoprenaline and related β-adrenoceptor agonists in the guinea-pig

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1 The effect of isoprenaline, adrenaline and salbutamol on airway reactivity to histamine was observed in anaesthetized, ventilated guinea-pigs. Airway reactivity was determined before and 20 min and 90 min after a 30-min i.v. infusion of each agonist by constructing cumulative dose-response curves from breath-by-breath measurements of the effect of different rates of i.v. infusion of histamine on lung resistance (R_L) and dynamic compliance (C_{dyn}).

2 (±)-Isoprenaline infused i.v. for 30 min at a rate of 0.4 μmol h^{-1} kg^{-1} caused bronchodilatation and a fall in blood pressure. Recovery to starting values of R_L and C_{dyn} occurred within 20 min of stopping the infusion.

3 Reactivity to histamine was greatly enhanced when measured 20 min and 90 min after stopping the infusion of (±)-isoprenaline. This was not an effect of the prior infusion of histamine or of the dissolving solution.

4 Infusion of (−)-isoprenaline for 30 min at a rate of 0.2 μmol h^{-1} kg^{-1} also enhanced reactivity to histamine. However, enhancement of reactivity to histamine was not demonstrable after infusion of (+)-isoprenaline at equal or higher dose rates.

5 Infusions of bronchodilator concentrations of adrenaline and salbutamol also enhanced airway reactivity to histamine, but the bronchodilator effect of salbutamol lasted longer than that of isoprenaline or adrenaline and the development of hyperreactivity was delayed.

6 After acute bilateral vagotomy, infusion of (±)-isoprenaline enhanced airway reactivity but only at the highest dose of histamine.

7 (±)-Isoprenaline did not enhance contractile responses to histamine in isolated preparations of first branch bronchi.

8 We conclude that the bronchodilator effect of activating β-adrenoceptors in the airways of guinea-pigs is followed by a more persistent state of hyperreactivity to histamine.

Keywords: β-Agonists; isoprenaline; adrenaline; salbutamol; histamine; airway hyperreactivity; lung resistance, isolated bronchi

Introduction

A number of investigators have reported indirect evidence of enhanced reactivity to bronchoconstrictor stimuli in animals treated with isoprenaline and related agonists. Thus, Conolly *et al.* (1971) noted increased mortality from histamine-induced bronchospasm in guinea-pigs pretreated with i.m. isoprenaline, terbutaline or salbutamol and Bouhuys *et al.* (1972) reported that i.m. isoprenaline increased mortality to histamine given i.p. to guinea-pigs. Also, Izard *et al.* (1971) found that mortality from antigen given i.v. to sensitized guinea-pigs was increased after treatment with i.p. adrenaline.

Recently, Morley and co-workers (Mazzoni *et al.*, 1987; Morley & Sanjar, 1987; Sanjar *et al.*, 1990) have reported that an i.v. infusion of (±)-isoprenaline in the anaesthetized guinea-pig enhanced the bronchoconstrictor effect of a fixed dose of bombesin, an effect prevented by bilateral vagotomy. They also reported that the (+)-isomer, which at similar concentrations lacks β-adrenoceptor agonist activity, was as effective as the racemate. Noradrenaline and dopamine also enhanced responses to bombesin, but adrenaline did not. Most recently, Morley *et al.* (1991) have presented evidence that the two optical isomers of salbutamol also enhance airway reactivity in the guinea-pig. The authors conclude that these compounds induce airway hyperreactivity by a mechanism not involving stereoselective activation of β-adrenoceptors but dependent on an intact vagal innervation. Since an important aim of treatment of asthma is to reduce the airway hyperreactivity characteristic of the disease, these unexpected findings are both cause for concern and worthy of further study, for they provide an experimental basis for clinical reports (as discussed later) that β-agonist bronchodilators can enhance airway reactivity in asthmatic patients and make control of their symptoms more difficult.

Prompted by the original reports of Morley and co-workers, we explored this effect of β-agonists on airway reactivity in the anaesthetized guinea-pig by using cumulative dose-response curves rather than responses to single doses of the bronchoconstrictor agent as a measure of reactivity. Our results, derived from breath-by-breath measurements of lung resistance and dynamic compliance, confirm the primary observation of Morley and co-workers, but differ in some other respects. Preliminary accounts of this work have been published (Galland & Blackman, 1988; 1989).

Methods

For the experiments *in vivo*, guinea-pigs (weight 467 ± 8 g, mean ± s.e.mean, $n = 42$) were anaesthetized with pentobarbitone (40 mg kg^{-1}, i.p.), paralyzed with suxamethonium (0.5 mg kg^{-1}, i.v.) and ventilated at 1 Hz through a tracheal cannula with a constant volume positive pressure pump set to deliver 7.0 ml kg^{-1} body wt. Rectal temperature was maintained at 38°C with a thermostatically-controlled heating pad. The chest of each animal was opened and end-expiratory pressure was maintained at 200–250 Pa. The right carotid artery was cannulated for measuring blood pressure and the left jugular vein cannulated for injecting or infusing solutions of drugs.

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Measurement of R_L and C_{dyn}^{-1}

Animals were placed in a body plethysmograph and lung resistance (R_L) and the reciprocal of dynamic compliance (C_{dyn}^{-1}) were computed breath-by-breath from transpulmonary pressure, tidal volume and flow rate by the isovolume technique described by Amdur & Mead (1958). Transpulmonary pressure was measured as the difference between the pressure in the side arm of the tracheal cannula and the pressure in the plethysmograph, which, because the chest was open, gave a direct measure of pressure in the pleural space. Tidal volume excursions were recorded by calibrating the pressure change measured in the plethysmograph with a differential pressure transducer. Flow rates were obtained from the slopes of lines fitted to the tidal volume curve at the mid-inspiratory and mid-expiratory points (isovolume points). The on-line computations were performed with a programme written by Dr R.D. Purves for an Apple IIe computer equipped with a 12-bit analogue-to-digital converter (Galland *et al.*, 1986). Baseline values of R_L and C_{dyn}^{-1} were reliably recorded by the method, but during infusion of histamine, particularly at the higher dose rates, occasional irregularities in the tidal volume curve produced discrepant estimates of R_L and C_{dyn}^{-1}. These were readily detected and were deleted before the results were analysed.

Infusion of β-adrenoceptor agonists

Racemic isoprenaline, its (+)- and (−)-isomers, and salbutamol, were dissolved in 0.9% NaCl immediately before infusion into the jugular vein at a rate of 0.05 ml min^{-1} for 30 min with a constant velocity pump. Immediately before infusion of adrenaline at the same rate, the stock solution of adrenaline containing Na metabisulphite (0.1% w/v) was diluted with 0.9% NaCl to the required concentration. The 42 animals used in these experiments were allocated to 8 experimental groups for infusion of the different drugs as follows: (±)-isoprenaline ($n = 4$); (±)-isoprenaline after vagotomy ($n = 6$); (+)-isoprenaline ($n = 7$); (−)-isoprenaline ($n = 5$); saline control ($n = 6$); salbutamol ($n = 4$); adrenaline ($n = 6$); Na metabisulphite control ($n = 4$).

Histamine infusion

As a measure of airway reactivity, dose-response curves to histamine were determined 20 min before, and 20 and 90 min after the 30-min infusion of isoprenaline (or other agonist) by measuring the peak changes in R_L and C_{dyn}^{-1} produced by infusing a fixed concentration of histamine into the jugular vein at increasing rates equivalent to 12, 24 and 48 μg min^{-1} kg^{-1}, and in some experiments equivalent to 96 μg min^{-1} kg^{-1}, each in succession for 90 s. The flow of solution at the lowest infusion rate of histamine was 0.03 ml min^{-1} so that the total volume infused was either 0.6 ml or 1.4 ml delivered over 4.5 or 6 min respectively. Anaesthesia and paralysis were maintained during the 3 h of each experiment with at least 3 supplementary injections of pentobarbitone and suxamethonium made at appropriate intervals.

Vagotomy

For vagotomy, the nerves were identified in 6 guinea-pigs and freed ready for transection at least 20 min before the first determination of airway reactivity to histamine. Both vagus nerves were then cut and 50 min later reactivity was determined again. The solution of (±)-isoprenaline was then infused for 30 min. Twenty min after stopping the infusion, reactivity to histamine was determined a third time.

Isolated bronchial smooth muscle preparations

For the experiments *in vitro*, four guinea-pigs (434 ± 11 g, mean ± s.e.mean) were anaesthetized with pentobarbitone (50 mg kg^{-1}). The trachea and attached lungs were quickly removed and placed in ice-cold Krebs solution of the following composition (mmol l^{-1}): NaCl 120, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7. Both left and right first branch bronchial rings from each guinea-pig were dissected and prepared for isometric recording according to the method of Hooker *et al.* (1977). Each preparation was mounted in a 50 ml organ bath filled with Krebs solution maintained at 37°C and bubbled with a 95% O_2, 5% CO_2 gas mixture. The resting muscle tension was adjusted to 1 g wt and the tissue allowed to equilibrate for 60 min. During this time the solution was frequently replaced. Each bronchial ring was exposed to cumulative increases in the concentration of histamine given every 4 min until a maximal response was reached. The histamine was then washed out and the tissue allowed to return to baseline tension for 1 h. Racemic isoprenaline (1 μmol l^{-1}) was added to the organ bath for 30 min, washed out, and responses to cumulative increases in the concentration of histamine were again recorded 20 min later. The sensitivity of the bronchial smooth muscle preparations was estimated from the cumulative concentration-response curves as the effective concentration producing 50% of the maximal response (EC_{50}).

Statistical analyses

Results are expressed as mean values ± standard error of the mean (s.e.mean). The significance of differences between dose-response curves was determined by two-way analysis of variance (CLR ANOVA programme for Apple Macintosh). For the *in vitro* experiments, the significance of differences between EC_{50} values was calculated by Student's *t* test.

Drugs

Use in the text of the name of a β-agonist without qualification implies that the (±)-isomer was used. The drugs and suppliers were as follows: histamine diphosphate (Sigma); (±)-isoprenaline hydrochloride (Sigma); (+)-isoprenaline (+)-bitartrate (Sigma); (−)-isoprenaline (+)-bitartrate (Sigma); salbutamol hemisulphate (Sigma); adrenaline hydrogen tartrate (adrenaline injection B.P.); sodium pentobarbitone (Abbott); succinylcholine chloride (Sigma). The identity of the sample of (+)-isoprenaline (+)-bitartrate was confirmed by determination of the specific optical rotation ($[\alpha]_D^{25} = +38°$) and the infra-red spectrum (Keller, 1986).

Results

Time course of bronchodilator effect of i.v. infusions of agonists

Figure 1 shows that an i.v. infusion for 30 min of each of the three β-agonists, isoprenaline, adrenaline and salbutamol caused a progressive fall in lung resistance (R_L) and in the reciprocal of the dynamic compliance (C_{dyn}^{-1}). The bronchodilator response was usually rapid in onset and approached steady-state within 20 to 30 min of starting the infusion. With isoprenaline and adrenaline, recovery on stopping the infusion was rapid: both R_L and C_{dyn}^{-1} approached baseline values within 20 min. With salbutamol, the value of C_{dyn}^{-1} recovered rapidly also (Figure 1b), but the value of R_L remained low for at least 20 min (Figure 1a), recovering, however, within 90 min. In three separate experiments with isoprenaline, we found that the same degree of bronchodilatation was produced by a second infusion 30 min later, indicating that tachyphylaxis did not occur.

Cardiovascular effects of β-agonists and histamine

The i.v. infusion of (±)-isoprenaline, (−)-isoprenaline, adrenaline and salbutamol, all caused arterial blood pressure to

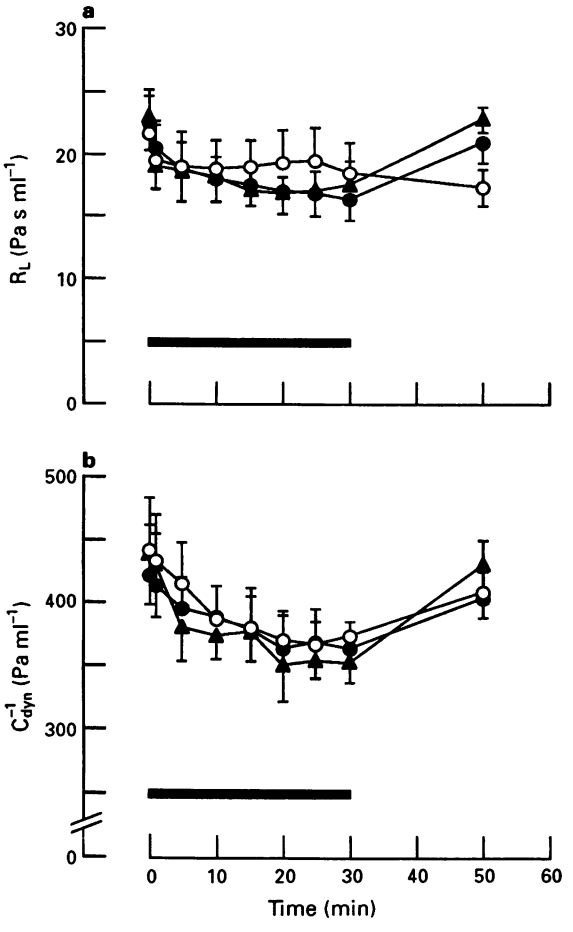


Figure 1 Changes in mean absolute values of R_L (a) and C_{dyn}^{-1} (b) produced by infusions of 0.4 μmol h^{-1} kg^{-1} isoprenaline (●), 0.4 μmol h^{-1} kg^{-1} salbutamol (○), and 0.3 μmol h^{-1} kg^{-1} adrenaline (▲). Data from 4, 4, and 6 guinea-pigs respectively; s.e.mean shown by vertical bars. Horizontal bar shows duration of infusion.

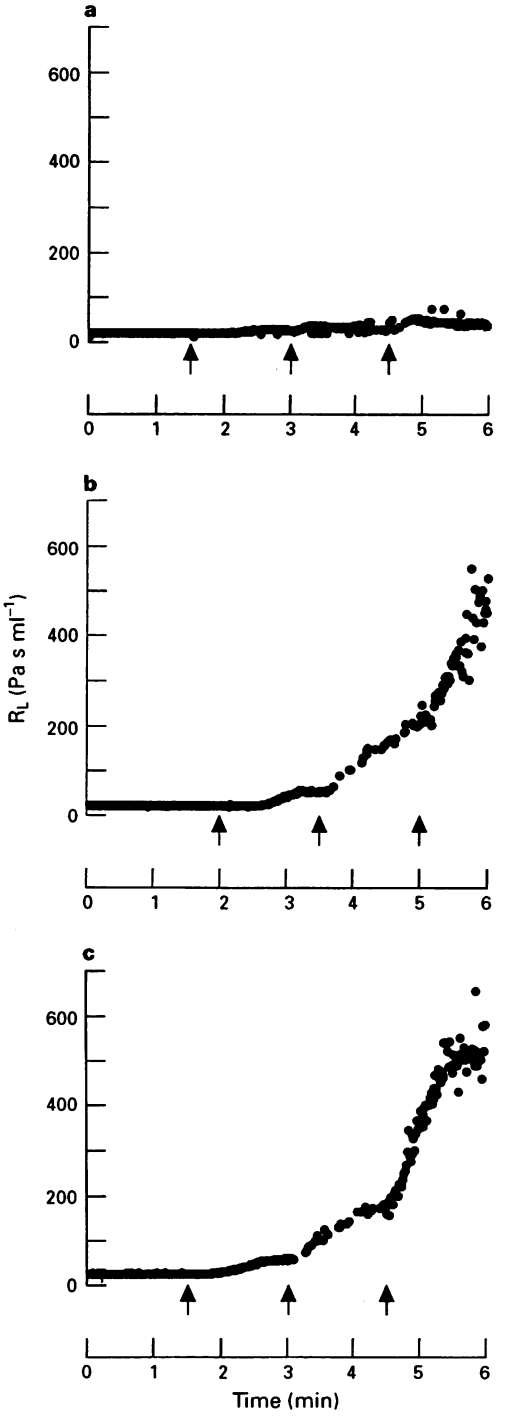


Figure 2 Enhancement by (±)-isoprenaline of responses to histamine. Breath-by-breath values of R_L recorded in response to successively increased rates of i.v. infusion of histamine (12, 24 and 48 μg min^{-1} kg^{-1} at arrows) in one guinea-pig 20 min before (a), and 20 min (b) and 90 min (c) after a 30-min infusion of (±)-isoprenaline (0.4 μmol h^{-1} kg^{-1}).

fall. For example, the mean fall produced by isoprenaline in 10 animals was 17.43 ± 1.71 mmHg. Falls in blood pressure were maximal during the first few min of infusion but partly recovered as the infusion continued. With each drug, blood pressure returned to pre-infusion levels within 20 min of stopping the infusion. Infusion of (+)-isoprenaline had no effect.

The i.v. infusions of histamine used to determine airway reactivity consistently lowered arterial blood pressure. Mean falls in blood pressure were not related to the histamine infusion rate. They were, however, dependent on the initial blood pressure. In 12 animals, the mean fall producd by the lowest histamine infusion rate before infusion of the β-agonists was 12.4 ± 3.17 mmHg.

Effect of (±)-isoprenaline infusion on bronchial reactivity to histamine

Figure 2 shows the effect on bronchial reactivity to histamine of infusing (±)-isoprenaline at 0.4 μmol h^{-1} kg^{-1} for 30 min in one guinea-pig. Breath-by-breath changes in R_L produced by cumulative increases in histamine dose rate recorded 20 and 90 min after stopping the infusion of (±)-isoprenaline were greatly enhanced, particularly at the higher dose rates of histamine (Figures 2b and 2c).

Figure 3a shows the significant enhancement ($P<0.05$) of the mean % increase in R_L produced by histamine measured in 4 animals 20 and 90 min after stopping the 30-min infusion of (±)-isoprenaline. This confirmed a similar observation made in a preliminary study in 6 animals (Galland & Blackman, 1988). In both series of experiments, the enhancement at 90 min did not differ significantly ($P>0.05$) from that observed at 20 min. Enhancement was observed whether measurements were of R_L or C_{dyn}^{-1}.

The infusion of (±)-isoprenaline did not enhance effects of histamine on blood pressure and heart rate. Infusions of saline in 6 control animals had no effect on bronchoconstrictor responses to histamine (Figure 3b).

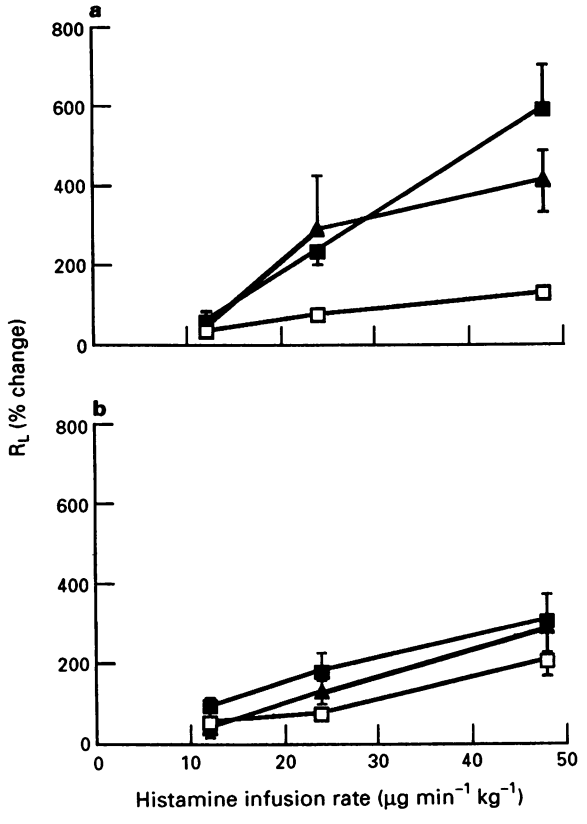


Figure 3 Changes in R_L in response to successively increased rates of i.v. infusion of histamine 20 min before (□), and 20 min (■) and 90 min (▲) after an infusion of either 0.4 μmol h^{-1} kg^{-1} (±)-isoprenaline in 4 animals (a) or saline in 6 animals (b); s.e.mean shown by vertical bars. Resting values of R_L (Pa s ml^{-1}) for the 3 periods of measurement in (a) were: 23.5 ± 2.7, 21.0 ± 1.9 and 21.5 ± 2.2 respectively, and for (b): 24.0 ± 2.0, 20.7 ± 0.7 and 22.5 ± 1.3 respectively.

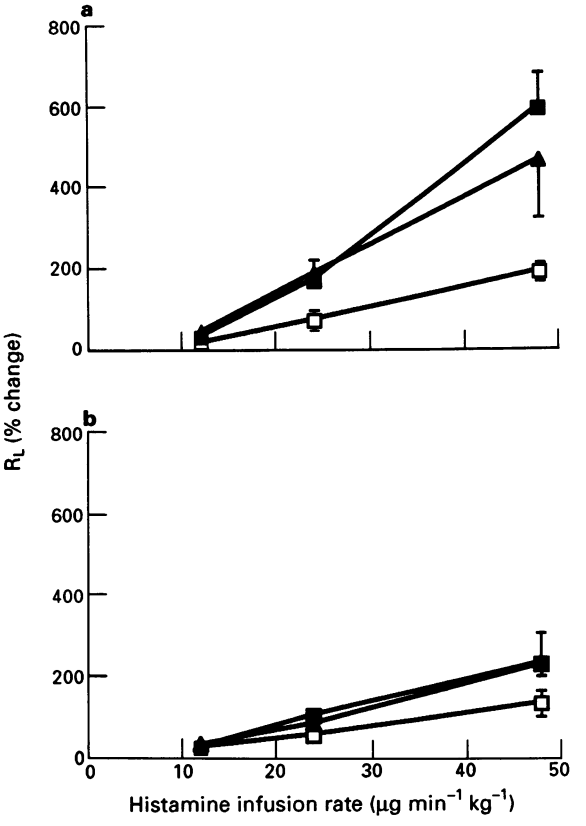


Figure 4 Comparison of the effects of the two optical isomers of isoprenaline. (a) Changes in R_L in response to successively increased rates of i.v. infusion of histamine 20 min before (□), and 20 min (■) and 90 min (▲) after an infusion of (−)-isoprenaline (0.2 μmol h^{-1} kg^{-1}) in 5 animals; s.e.mean shown by vertical bars. Resting values of R_L (Pa s ml^{-1}) for the 3 periods of measurement were: 18.5 ± 0.6, 18.2 ± 0.4 and 19.7 ± 0.9 respectively. (b) The changes in R_L in response to successively increased rates of i.v. infusion of histamine 20 min before (□), and 20 min (■) and 90 min (▲) after infusion of (+)-isoprenaline (0.28 μmol h^{-1} kg^{-1}) in 4 animals; s.e.mean shown by vertical bars. Resting values of R_L (Pa s ml^{-1}) for the 3 periods of measurement were: 20.8 ± 2.1, 20.3 ± 2.0 and 20.8 ± 1.7 respectively.

Effect of the (−)- and (+)-isomers of isoprenaline

Infusion of (−)-isoprenaline for 30 min at a dose rate of 0.2 μmol h^{-1} kg^{-1} in 5 animals significantly enhanced ($P<0.01$) airway responses to histamine (Figure 4a). Infusions at 0.4 and 0.8 μmol h^{-1} kg^{-1}, each in 4 animals, also increased reactivity (no figure shown), but not to a greater extent than at the low dose rate. The enhancement of reactivity at the dose rate of 0.2 μmol h^{-1} kg^{-1} appeared therefore to be maximal.

In contrast, infusion of (+)-isoprenaline for 30 min at a dose rate of 0.28 μmol h^{-1} kg^{-1} in 4 animals failed ($P>0.05$) to enhance responses to histamine (Figure 4b). Infusion at 0.4 μmol h^{-1} kg^{-1} in one animal and at 0.8 μmol h^{-1} kg^{-1} in two animals failed also to enhance responses to histamine.

Effects of adrenaline and salbutamol

Figure 5a shows the effect of adrenaline infused at 0.3 μmol h^{-1} kg^{-1} for 30 min on bronchial reactivity to histamine in one animal. As with (±)-isoprenaline, breath-by-breath changes in R_L in response to histamine were markedly enhanced (Figures 5b and 5c).

Figure 6a shows the mean % increase in R_L produced by histamine after infusion of adrenaline in 6 animals. Control infusions in 4 animals of 0.9% NaCl containing Na metabisulphite at the concentration present in the adrenaline used for infusion had no effect on reactivity to histamine.

Figure 6b shows that infusion of salbutamol at 0.4 μmol h^{-1} kg^{-1} for 30 min in 4 animals significantly enhanced ($P<0.05$) reactivity to histamine 90 min after stopping the infusion, but not 20 min after. At this time, the absolute value of R_L had not recovered to its pretreatment value (Figure 1a), indicating that the bronchodilator effect of salbutamol was still present 20 min after stopping the infusion.

Effect of vagotomy on isoprenaline-induced increase in reactivity to histamine

Acute bilateral vagotomy alone had no significant effect on baseline levels of R_L or C_{dyn}^{-1} or on responses to histamine measured 50 min after transection of the vagus nerves in 6 animals (Figure 7). This figure shows that after vagotomy, infusion of isoprenaline could still induce enhanced reactivity to histamine, but only at the highest dose rate ($P<0.01$); no enhancement occurred at the lower dose rates of histamine. However, comparison of the dose-response curves in Figure 7 and Figure 3a indicates that vagotomy had no significant effect on the degree of enhancement of reactivitiy to histamine ($P>0.05$).

Reactivity of bronchial smooth muscle

Isoprenaline (1 μmol l^{-1}) initially decreased resting tension in isolated preparations of first-branch bronchi. Tension recov-

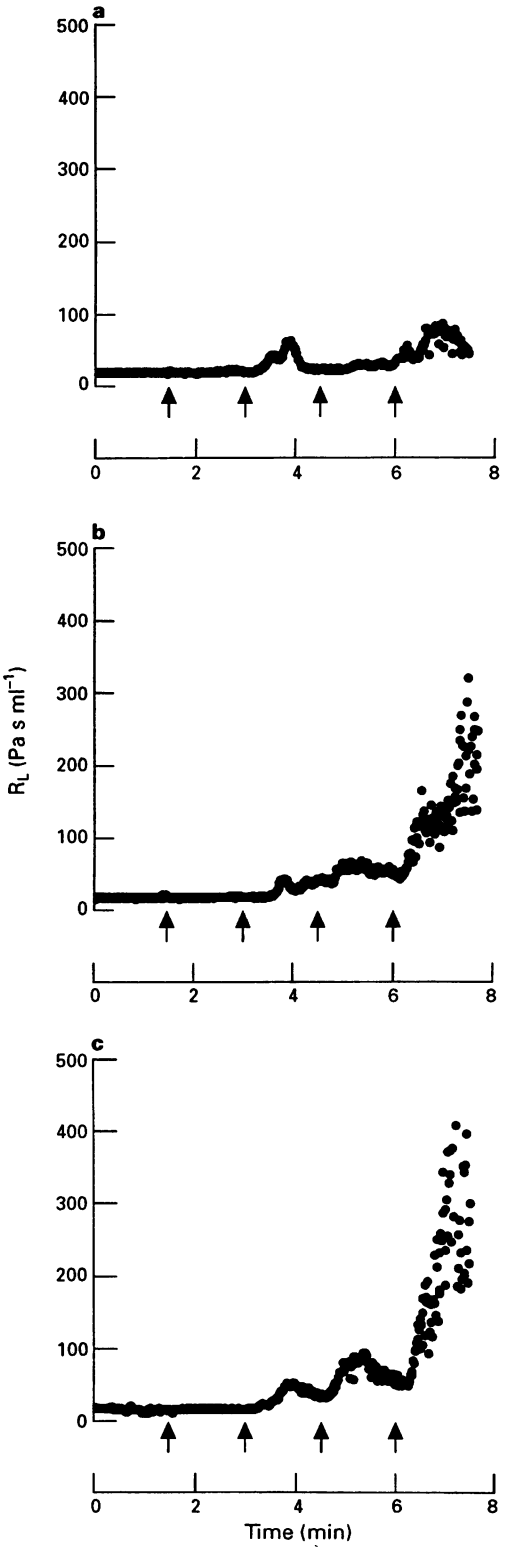


Figure 5 Enhancement by adrenaline of responses to histamine. Breath-by-breath values of R_L recorded in response to successively increased rates of i.v. infusion of histamine (12, 24, 48 and 96 μg min^{-1} kg^{-1} at arrows) in one guinea-pig 20 min before (a), and 20 min (b) and 90 min (c) after a 30-min infusion of adrenaline (0.3 μmol h^{-1} kg^{-1}).

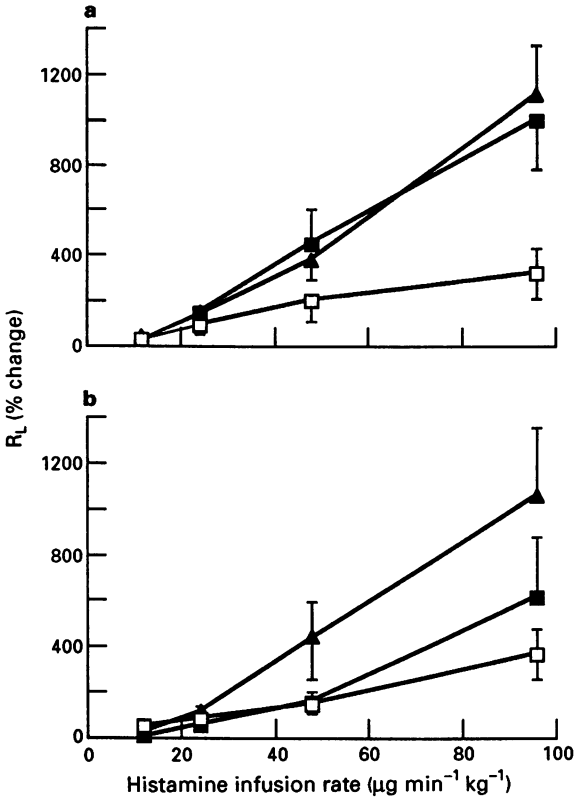


Figure 6 Changes in R_L in response to successively increased rates of i.v. infusion of histamine 20 min before (□), and 20 min (■) and 90 min (▲) after 30-min infusions of 0.3 μmol h^{-1} kg^{-1} adrenaline in 6 animals (a) and 0.4 μmol h^{-1} kg^{-1} salbutamol in 4 animals (b); s.e.mean shown by vertical bars. Resting values of R_L (Pa s ml^{-1}) for the 3 periods of measurement in (a) were: 21.4 ± 2.4, 19.6 ± 1.9 and 19.6 ± 1.3 respectively, and for (b); 23.5 ± 0.7, 17.3 ± 1.8 and 19.8 ± 2.7 respectively.

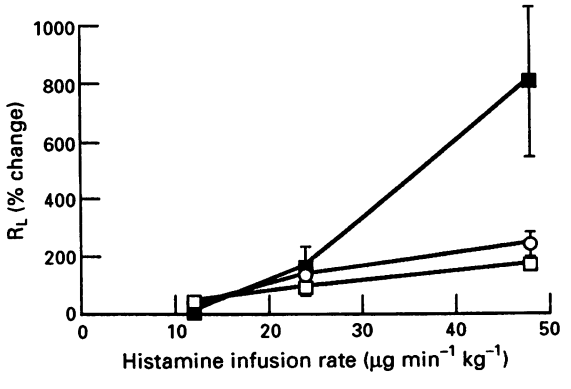


Figure 7 The change in R_L in response to successively increased rates of i.v. infusion of histamine 20 min before (□) and 50 min following bilateral vagotomy (○), and then 20 min after a 30-min infusion of 0.4 μmol h^{-1} kg^{-1} (±)-isoprenaline (■) in 6 animals; s.e.mean shown by vertical bars. Resting values of R_L (Pa s ml^{-1}) for the 3 periods of measurement were: 28.8 ± 5.3, 25.0 ± 3.2 and 22.8 ± 2.4 respectively.

ered to near normal during the 30 min exposure. Sensitivity to histamine did not change significantly after 30 min exposure to isoprenaline (Figure 8): values of EC_{50} before and after treatment were respectively 0.8 ± 0.1 and 1.5 ± 0.5 μmol l^{-1}. Maximal responses were not changed.

Discussion

Our observations confirm the principal findings reported by Morley and co-workers (Morley & Sanjar, 1987; Mazzoni *et al.*, 1987; Sanjar *et al.*, 1990; Morley *et al.*, 1991) that i.v. infusions of isoprenaline or salbutamol enhance airway reac-

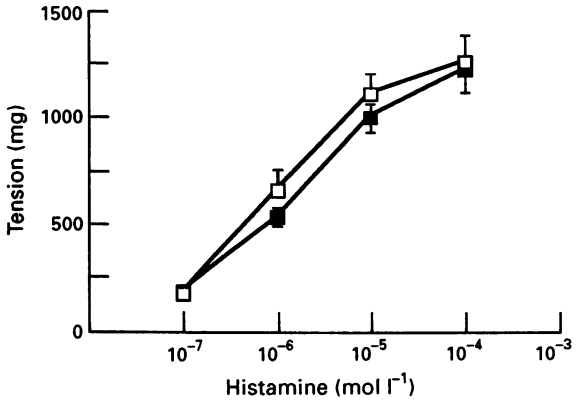


Figure 8 Responses of 8 isolated first-branch bronchi to cumulatively increased concentrations of histamine before (□) and after (■) exposure to (±)-isoprenaline (1 μmol l^{-1}); s.e.mean shown by vertical bars.

tivity in anaesthetized guinea-pigs. However, some of our results differ and require some discussion.

First, we found that adrenaline enhanced reactivity to histamine about as effectively as a similar concentration of isoprenaline. This is at variance with the observations of Sanjar *et al.* (1990) who observed no enhancement. The difference can perhaps be explained by inspection of our Figure 6a. In the absence of adrenaline, the two lower dose rates of histamine produced increases in R_L ranging from less than 150% to 250%. The bolus test doses of bombesin or histamine used by Sanjar *et al.* (1990) caused increases in R_L of not more than about 140%, which in our experiments would not have been enough to reveal significant hyperreactivity. Our observations with adrenaline (and also salbutamol) are consistent with reports that increased sensitivity to allergen in guinea-pigs occurs following pretreatment with these agonists (Izard *et al.*, 1971; Chapman *et al.*, 1990).

Secondly, our experience with vagotomy was different. Sanjar *et al.* (1990) reported that although bilateral vagotomy performed after infusion of isoprenaline was without effect on the development of hyperreactivity, vagotomy carried out before infusion of isoprenaline prevented enhancement of reactivity from which they concluded that development of hyperreactivity depended on intact innervation of the airways by the vagus. We also found that bilateral vagotomy carried out before infusion of isoprenaline prevented enhancement of reactivity, but only at the lower dose rates of histamine. At the highest dose rate of histamine, vagotomy did not prevent enhancement by isoprenaline (compare Figures 3 and 7). It thus appeared that vagotomy had shifted the histamine dose-response curve somewhat to the right, consistent with the removal of vagal tone. Our observation is also consistent with the finding of Sanjar *et al.* (1990) that treatment with atropine before infusion of isoprenaline did not prevent the development of hyperreactivity. Our finding that bronchial smooth muscle responses to histamine *in vitro* were not enhanced by isoprenaline, supports the conclusion that isoprenaline does not affect the reactivity of the smooth muscle directly. This evidence together with the results observed following vagotomy suggests that isoprenaline enhances reactivity at a peripheral site both independent of vagal innervation and not directly involving the smooth muscle. In the absence of further evidence, it is not possible to propose a more specific site of action.

Thirdly, we have concluded that the (−)-isomer of isoprenaline induced hyperreactivity in our preparations, evidently by stereoselective activation of β-adrenoceptors since the (+)-isomer had little if any activity (Galland & Blackman, 1988). Sanjar *et al.* (1990) found on the other hand that the isomers were equally effective at increasing airway reactivity and concluded that the enhancement of response was not mediated by steroselective activation of β-adrenoceptors. Because in our preparations (+)-isoprenaline did not enhance reactivity, we checked the identity of our sample. The specific optical rotation and infra-red spectrum were consistent with its being the (+)-isomer. Its lack of hypotensive effect was also consistent. We can offer no explanation for the different outcome in the two studies. Since ours is a negative finding, we hesitate without further study to contradict the conclusion that isoprenaline and related compounds produce hyperreactivity by a mechanism not involving stereoselective activation of β-adrenoceptors, particularly since the non-bronchodilator isomer (+)-salbutamol has been shown to enhance airway reactivity (Morley *et al.*, 1991).

We also found that (±)-salbutamol increased airway reactivity to histamine, and like Morley *et al.* (1991) noted that the longer bronchodilator action of this drug prevented observation of an early increase in reactivity. Perhaps because we infused salbutamol for a shorter time, we were able to observe hyperreactivity at 90 min.

The differences we have discussed possibly reflect differences in the methods used in the two studies to determine airway reactivity. In particular, we plotted cumulative dose-response curves to increasing i.v. infusion rates of a fixed concentration of histamine whereas Sanjar *et al.* (1990) measured peak responses to single i.v. bolus doses of bombesin or histamine. Although the normal dose-response relationship to histamine inferable from their published record (Sanjar *et al.*, 1990, Figure 3) is approximately the same as that found in our experiments (our Figure 2a), the difference in the method of determining reactivity was substantial.

A feature of the hyperreactivity produced by i.v. infusion of a β-agonist is that it is an acute response; it is observed as soon as the bronchodilator effect has waned. The effect is surprisingly long-lasting. In our experiments, hyperreactivity was unabated 90 min after stopping infusion of the β-agonist, and in one set of experiments by Morley and colleagues (Sanjar *et al.*, 1990), hyperreactivity was observed 4 h after stopping the infusion. Other evidence indicates that it may last even longer (Morley *et al.*, 1991).

Whatever the mechanism of this acute effect of β-agonists given i.v., an important question is whether β-agonists can be shown to increase airway reactivity acutely in man. Indirect evidence of an acute increase in reactivity following administration of β-agonists by inhalation has been described, but only in asthmatic subjects (Keighley, 1966; Eisenstadt & Nicholas, 1969; van Metre, 1969; Reisman, 1969; Paterson *et al.*, 1971). We are not aware of studies designed to test directly the possibility that the effect occurs in man with normal respiratory function following either i.v. administration or inhalation. Nor are we aware of experiments designed to see whether inhalation of β-agonists increases bronchial reactivity in guinea-pigs or other animals. Study of the effect of β-agonists in normal human subjects would parallel more strictly the observations in animals and would indicate that the effect could occur independently of the disease process.

Other studies indicate that enhanced airway reactivity can result from repeated exposure to conventional β-agonist bronchodilator drugs in asthmatic patients being treated with these agents prophylactically (Peel & Gibson, 1980; Kraan *et al.*, 1985; Kerribijn *et al.*, 1987; Vathenen *et al.*, 1988; van Schayck *et al.*, 1990; Sears *et al.*, 1990). Regular daily use of a β-agonist has been shown to worsen the disease and make control of symptoms difficult (Sears *et al.*, 1990) and to be associated with an increase in asthma mortality (Crane *et al.*, 1989; Spitzer *et al.*, 1992). Morley *et al.* (1990), reviewing reports of 'anomalous'or 'paradoxical bronchospasm' in asthma patients treated with β-agonists, considered five possible explanations, namely, reactive myogenic tone, metabolic products, tachyphylaxis to the β-agonist, increased inflammatory burden and induction of airway hyperreactivity and decided that only the last seemed able to explain the observations.

If β-agonists acutely enhance airway reactivity in man, why

has the effect not been more readily demonstrable in asthmatic patients being treated with these agents? A possible answer is that therapeutic doses in man do not achieve concentrations at the site of action equivalent to those reached in the guinea-pig. However, the evidence suggests otherwise. For example, in severe asthma, a typical bronchodilator infusion rate of salbutamol is 10 $\mu g\,min^{-1}$ i.v., which is equivalent to 0.035 $\mu mol\,h^{-1}\,kg^{-1}$, a little less than one tenth the rate used in our guinea-pigs. Allowing for the effect of scale (Gibaldi & Perrier, 1982), clearance of salbutamol in relation to body wt should be significantly greater in the guinea-pig than in man, so that the steady-state concentrations reached in the two species should be of the same order. Moreover, the concentrations needed to induce hyperreactivity may be much lower. Isoprenaline infused at 1 $\mu g\,h^{-1}\,kg^{-1}$ (approximately 0.004 $\mu mol\,h^{-1}\,kg^{-1}$) can significantly enhance reactivity in the guinea-pig (Sanjar *et al.*, 1990). It is reasonable, therefore, to suppose that the bronchodilator doses used in man are at least equivalent to those shown to induce hyperreactivity in the guinea-pig.

At least part of the answer as to why enhancement of reactivity by β-agonists has not been more readily demonstrable is that few studies with β-agonists have been designed to detect it, and when they have (as noted above), evidence of only a limited enhancement has been obtained. Another part of the answer, is that any acute increase in airway hyperreactivity will be detected only if it outlasts the bronchodilator or other beneficial effects of β-agonist treatment. In the guinea-pig, the induced hyperreactivity long outlasts the initial bronchodilator effect, but in man, the pharmacokinetic behaviour of the β-agonists appears to ensure that the bronchodilator effect is sustained longer. Also, and perhaps more importantly, since the airways in the more severely affected asthmatic patient already exhibit marked hyperresponsiveness, any additional effect of a β-agonist may represent only a small relative increase. Nevertheless, a small increase may be critical for the survival of a patient suffering a life-threatening asthmatic attack. Enhancement of reactivity is likely to be more obvious in the mild to moderate asthmatic patient, and indeed, it is in this group of patients that evidence of enhanced reactivity has been detected. Experiments directly showing that β-agonists can or cannot acutely enhance airway reactivity in normal and asthmatic human subjects are needed to settle such speculative argument.

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References

AMDUR, M.O. & MEAD, J.M. (1958). Mechanics of respiration in unanaesthetized guinea-pigs. *Am. J. Physiol.*, **192**, 364–368.

BOUHUYS, A., DOUGLAS, J.S. & LEWIS, A.J. (1972). Hypersensitivity to adrenoceptor agents in the guinea-pig *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **46**, 520–521P.

CHAPMAN, I., MAZZONI, L. & MORLEY, J. (1990). An anomalous effect of salbutamol in sensitised guinea-pigs. *Br. J. Pharmacol.*, **99**, 66P.

CONOLLY, M.E., DAVIES, D.S., DOLLERY, C.T. & GEORGE, C.F. (1971). Resistance to β-adrenoceptor stimulants (a possible explanation for the rise in asthma deaths). *Br. J. Pharmacol.*, **43**, 389–402.

CRANE, J., FLATT, A., JACKSON, R., BALL, M., PEARCE, N., BURGESS, C., KWONG, T. & BEASLEY, R. (1989). Prescribed fenoterol and death from asthma in New Zealand, 1981–1983: case-control study. *Lancet*, **i**, 917–922.

EISENSTADT, W.S. & NICHOLAS, S.S. (1969). The adverse effect of adrenergic aerosols in bronchial asthma. *Ann. Allergy*, **27**, 283–288.

GALLAND, B.C. (1989). Bronchial hyperreactivity in the guinea-pig. *Ph.D. thesis*. Dunedin: University of Otago Medical School.

GALLAND, B.C. & BLACKMAN, J.G. (1988). Enhancement of airways reactivity to histamine by (±)-isoprenaline and its isomers in guinea-pigs. *Proc. Univ. Otago Med. Sch.*, **66**, 25–26.

GALLAND, B.C. & BLACKMAN, J.G. (1989). Enhancement of airways reactivity to histamine in the guinea-pig by β-adrenoceptor agonists. *N.Z. Med. J.*, **102**, 226.

GALLAND, B.C., PURVES, R.D. & BLACKMAN, J.G. (1986). Bronchial hyper-reactivity in the immunologically sensitized guinea-pig. *Proc. Univ. Otago Med. Sch.*, **64**, 35–36.

GIBALDI, M. & PERRIER, D. (1982). *Pharmacokinetics*, 2nd ed. pp. 375–382. New York: Marcel Dekker.

HOOKER, C.S., CALKINS, P.J. & FLEISCH, J.H. (1977). On the measurement of vascular and respiratory smooth muscle responses *in vitro*. *Blood Vessels*, **14**, 1–11.

IZARD, S.R., HENSON, E.C., COLLINS, A.D. & BRUNSON, J.G. (1971). Increased sensitivity to anaphylactic shock in guinea-pigs induced by prolonged treatment with epinephrine prior to challenge. *J. Allergy*, **47**, 309–314.

KEIGHLEY, J.F. (1966). Iatrogenic asthma associated with adrenergic aerosols. *Ann. Intern. Med.*, **65**, 985–995.

KELLER, R.J. (1986). *Sigma Library of FTR Spectra*, Vol. 1, p. 673, spectrum A. St Louis: Sigma Chemical Co.

KERREBIJN, K.F., VAN ESSEN-ZANDVLIET, E.E.M. & NEIJENS, H.J. (1987). Effect of long-term treatment with inhaled corticosteroids and beta-agonists on the bronchial responsiveness in children with asthma. *J. Allergy Clin. Immunol.*, **79**, 653–659.

KRAAN, J., KOETER, G.H., VAN DER MARK, TH. W., SLUITER, H.J. & DE VRIES, K. (1985). Changes in bronchial hyperreactivity induced by 4 weeks of treatment with antiasthmatic drugs in patients with allergic asthma: a comparison between budenoside and terbutaline. *J. Allergy Clin. Immunol.*, **76**, 628–636.

MAZZONI, L., MORLEY, J., SANJAR, S. & SCHAEUBLIN, E. (1987). An anomalous interaction between propranolol and isoprenaline. *Br. J. Pharmacol.*, **91**, 326P.

MORLEY, J. & SANJAR, S. (1987). Isoprenaline induces increased airway reactivity in the guinea-pig. *J. Physiol.*, **390**, 180P.

MORLEY, J., SANJAR, S. & NEWTH, C. (1990). Viewpoint: untoward effects of beta-adrenoceptor agonists in asthma. *Eur. Resp. J.*, **3**, 228–233.

MORLEY, J., CHAPMAN, I.D., FOSTER, A., HOSHIKO, K. & MAZZONI, L. (1991). Effects of (+) and racemic salbutamol on airway responses in the guinea-pig. *Br. J. Pharmacol.*, **104**, 295P.

PATERSON, J.W., EVANS, R.J.C. & PRIME, F.J. (1971). Selectivity of bronchodilator action of salbutamol in asthmatic patients. *Br. J. Dis. Chest*, **65**, 21–38.

PEEL, E.T. & GIBSON, G.J. (1980). Effects of long term inhaled salbutamol therapy on the provocation of asthma by histamine. *Am. Rev. Respir. Dis.*, **121**, 317–321.

REISMAN, R.E. (1969). Asthma induced by adrenergic aerosols. *J. Allergy*, **46**, 162–177.

SANJAR, S., KRISTERSSON, A., MAZZONI, L., MORLEY, J. & SCHAEUBLIN, E. (1990). Increased airway reactivity in the guinea-pig follows exposure to intravenous isoprenaline. *J. Physiol.*, **425**, 43–54.

SEARS, M.R., TAYLOR, D.R., PRINT, C.G., LAKE, D.C., LI, Q., FLANNERY, E.M., YATES, D.M., LUCAS, M.K. & HERBISON, G.P. (1990). Regular inhaled beta-agonist treatment in asthma. *Lancet*, **336**, 1391–1396.

SPITZER, W.O., SUISSA, S., ERNST, P., HORWITZ, R.I., HABBICK, B., COCKCROFT, D., BOIVIN, J.-F., MCNUTT, M., BUIST, A.S. & REBUCK, A.S. (1992). The use of β-agonists and the risk of death and near death from asthma. *New Engl. J. Med.*, **326**, 501–506.

VAN METRE, T.E. (1969). Adverse effects of inhalation of excessive amounts of nebulized isoproterenol in status asthmaticus. *J. Allergy*, **43**, 101–113.

VAN SCHAYCK, C.P., GRAAFSMA, S.J., VISCH, M.B., DOMPELING, E., VAN WEEL, C. & VAN HERWAARDEN, C.L.A. (1990). Increased bronchial hyperresponsiveness after inhaling salbutamol during 1 year is not caused by subsensitization to salbutamol. *J. Allergy Clin. Immunol.*, **86**, 793–800.

VATHENEN, A.S., KNOX, A.J., HIGGINS, B.G., BRITTON, J.R. & TATTERSFIELD, A.E. (1988). Rebound increase in bronchial responsiveness after treatment with inhaled terbutaline. *Lancet*, **i**, 554–558.

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Regulation of neural responses in the canine pyloric sphincter by opioids

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1 Regulation of excitatory and inhibitory junction potentials (e.j.ps and i.j.ps) by opioid peptides was studied in isolated muscle strips from the pyloric sphincter of the dog.

2 Methionine enkephalin (MetEnk; 10^{-10} to 10^{-6} M) and [D-Ala^2, D-Leu^5] enkephalin (DADLE; 10^{-11} to 10^{-7} M), a δ-specific opioid agonist, inhibited i.j.ps and e.j.ps recorded from cells in the myenteric and submucosal regions of the circular muscle layer. These compounds had no effect on resting potential or slow wave activity suggesting that the effects on junction potentials were not due to direct effects on smooth muscle cells.

3 MetEnk and DADLE caused similar effects on junction potentials in preparations in which the myenteric plexus was removed, suggesting that opioids inhibit pre-junctional effects on nerve fibres within the muscularis externa.

4 Inhibition of junction potentials by MetEnk and DADLE was blocked by approximately the same extent by naloxone (10^{-6} M) and ICI 174,864 (10^{-6} M), a δ-specific antagonist.

5 MetEnk and DADLE blocked a portion of the i.j.p. that was sensitive to arginine analogues; after treatment with N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M), MetEnk and DADLE had no further effect on i.j.ps. These data suggest that opioids regulate nitric oxide-dependent neurotransmission.

6 Naloxone (10^{-6} M) alone had no effect on i.j.ps elicited by short trains of electrical field stimuli.

7 I.j.p. amplitude was reduced after a period of conditioning stimulation (2 min, 30 Hz, 30 V). Naloxone blocked the post-stimulation inhibition. Repetitive stimulation at high frequencies (30 Hz) resulted in sustained hyperpolarization. Naloxone increased the amplitude of the hyperpolarization responses elicited by high frequency stimulation.

8 These results show that e.j.ps and i.j.ps in the canine pylorus are inhibited by opioids. A portion of the inhibitory effects appears to be mediated via δ receptors.

9 Although pyloric muscles are richly innervated by nerves containing opioid peptides, brief trains of stimuli do not appear to release concentrations of opioids that are effective in regulating junction potentials. Higher frequency stimulation (or longer durations of stimulation) appear to be necessary to release concentrations of opioids that are effective in modulating the amplitude of junction potentials.

Keywords: Enkephalins; enteric nervous system; pylorus; gastric emptying; gastric smooth muscle; nitric oxide; gastrointestinal motility

Introduction

The major, naturally-occurring opioids are derived from three classes of precursor polypeptides that have all been found in gastrointestinal tissues (for review see Miller & Hirning, 1989). Much of the expression of these peptides has been localized to intrinsic neurones (e.g. Schultzberg *et al.*, 1980; Furness *et al.*, 1983), and several studies have shown that opioids can be released by stimuli such as electrical field stimulation (Glass *et al.*, 1986) and during peristalsis (Donnerer *et al.*, 1984). Opioid peptides affect gut function through a variety of specific receptors, and functional μ, δ and κ receptors have been found in neurones of the gastrointestinal tract (see Duggan & North, 1984).

Opioid peptides may regulate the contractile behaviour of gastrointestinal muscles by reducing the excitability of enteric neurones by hyperpolarization (i.e. enhancement of K conductance) or inhibition of voltage-dependent Ca^{2+} currents in cell bodies (Morita & North, 1981; Cherubini & North, 1985; Mihara & North, 1986; North *et al.*, 1987; McFadzean, 1988) and by reducing neurotransmitter release by pre-junctional effects at axonal varicosities (Cowie *et al.*, 1978; Vizi *et al.*, 1984). Support for the latter mechanisms is provided by recent studies in which several naturally-occurring and synthetic opioid agonists were shown to reduce the amplitude of inhibitory junction potentials in strips of intestinal muscle from which the myenteric and submucosaal ganglia were removed (Bauer & Szurszewski, 1991; Bauer *et al.*, 1991). The inhibitory effects of opioids appeared to be mediated by both μ and δ receptors. Opioids had no effect on the resting membrane potentials or slow wave activity of smooth muscle cells suggesting that these compounds do not directly affect smooth muscle cells in canine, human and baboon small intestines (Bauer & Szurszewski, 1991; Bauer *et al.*, 1991). Similar results were obtained in experiments on human colonic muscles (Hoyle *et al.*, 1990), but in these muscles the inhibitory effects of opioids appeared to be mediated predominantly by δ receptors.

Although exogenous opioids can clearly reduce junction potentials, regulation of neural transmission by endogenous opioids has been difficult to demonstrate. For example, the opioid receptor antagonist, naloxone, did not affect junction potentials elicited by single pulses or brief trains of electrical field stimulation in the dog intestine (Bauer & Szurszewski, 1991). It may be that the concentrations of opioids released during brief periods of stimulation are ineffective in regulating transmitter release. Mechanical studies suggest that relatively high frequencies of stimulation may be needed to elicit naloxone-sensitive responses (e.g. Puig *et al.*, 1977).

Both excitatory and inhibitory junction potentials can be

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elicited in the circular muscle of the canine pylorus (Vogalis & Sanders, 1990), and a high density of enkephalinergic nerve fibres exists in the muscularis externa of the dog (Allescher *et al.*, 1988), as well as in man (Ferri *et al.*, 1987) and cat (Edin *et al.*, 1980). Opioids may have a physiological role in pyloric function since opioid agonists have been shown to delay gastric emptying (Sullivan *et al.*, 1981). In the present study we have examined the regulation of junction potentials in the canine pylorus by opioids. Recent studies have suggested that nitric oxide, or a related compound, might serve as an inhibitory transmitter in gastrointestinal muscles (for review, see Sanders & Ward, 1992), but regulation of nitric oxide release by opioids has not yet been addressed. Therefore, we have also attempted to determine whether inhibitory junction potentials, which appear to be mediated by nitric oxide in the canine pylorus (Bayguinov & Sanders, 1992), are regulated by endogenous opioids.

Methods

Mongrel dogs of either sex were anaesthetized with pentobarbitone sodium (100 mg kg^{-1}). The abdomen of each animal was opened by a midline incision and the entire stomach and a short segment of the proximal duodenum was removed. The excised organs were placed in a dish filled with oxygenated, Krebs-Ringer buffer. The pyloric region and adjoining portions of the terminal antrum and duodenum were dissected from the rest of the stomach. The pyloric sphincter was clearly visible, as previously described (Vogalis *et al.*, 1991). The pyloric canal was opened along the lesser curvature. The resulting sheet of tissue was pinned-out flat, and strips of muscle (1–1.5 mm in thickness) were cut parallel to the longitudinal muscle layer. These strips were laid 'on-side' in an electrophysiological chamber in such a manner as to provide a cross-sectional view of the entire muscularis externa. These preparations allowed impalement of smooth muscle cells at any point through the thickness of the circular layer (see Sanders & Vogalis, 1989). For some experiments, muscle strips were dissected to remove the myenteric plexus. These strips were prepared from the cross-sectional muscle strips by cutting away the entire longitudinal muscle layer and a small portion of the circular muscle layer to remove the entire myenteric plexus region.

The muscles strips were continuously perfused with warmed, oxygenated Krebs solution at a rate of 5 ml min^{-1}. The bath temperature was maintained at 37.5 ± 0.5°C, and the muscle strips were allowed to equilibrate for at least 2 h before intracellular recording was started.

Smooth muscle cells of the circular layer were impaled with glass microelectrodes filled with 3 M KCl and having resistances of 20–50 MΩ. Impalements were accepted based on previously described criteria (Sanders & Vogalis, 1989). Transmembrane potential was measured with a standard high-input impedance electrometer (WPI M-707), and the output was displayed on an oscilloscope (Tektronix 5111 A). Data were simultaneously recorded by a chart recorder (Gould 2200) and an FM tape recorder (Hewlett Packard). Data were analyzed from chart recordings or from taped records.

Intrinsic nerves were stimulated by electrical field stimulation (EFS) applied transmurally with platinum wires. Stimulating electrodes were connected via a stimulus isolation unit to a stimulator (Grass S48), and stimulus parameters were chosen for selective nerve stimulation as judged by blockade of responses by tetrodotoxin (pulses 0.5 ms, 30 Hz, 30–100 V).

Solutions and drugs

The standard Krebs-bicarbonate solution (KRB) used in this study contained (in mM): Na^+ 137.4; K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 134, HCO_3^- 15.5, $H_2PO_4^-$ 1.2 and dextrose 11.5. This solution achieved a final pH of 7.3 to 7.4 after equilibration with 97% O_2:3% CO_2. Methionine enkephalin (MetEnk; Sigma) and [D-Ala^2, D-Leu^5] enkephalin (DADLE; Sigma), were used as acetate salts. ICI 174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu; Cambridge Research Biochemicals) and naloxone hydrochloride salt (Sigma) were used to block opioid receptors. N^{ω}-nitro L-arginine methylester (L-NAME), and propranolol (all Sigma) were used as hydrochloride salts, and N^G-monomethyl-L-arginine (L-NMMA) (Calbiochem) was used as the acetate salt. The sulphate salt of atropine (Sigma) and the mesylate salt of phentolamine (Ciba Geigi) were used. Drugs were dissolved in distilled water as stock solutions of 10^{-2} or 10^{-3} M and further serial dilutions were made in KRB as required. Oxyhaemoglobin was prepared as a haemolysate of canine blood as previously described (Ward *et al.*, 1992).

Data were expressed as mean ± s.e.mean, and paired or unpaired Student's *t* tests were used for determination of statistical significance where appropriate; *n* values refer to the number of muscle strips used in each experiment.

Results

Effects of opioids on circular muscle cells near myenteric border

Circular muscle cells near the myenteric border had average resting potentials of − 64 ± 3 mV ($n = 7$). As previously described (Sanders & Vogalis, 1989), spontaneous electrical activity (slow waves) were recorded in this region. MetEnk and DADLE (10^{-10} to 10^{-6} M) did not affect resting potential (− 64 ± 4 mV in the presence of MetEnk and − 65 ± 1 mV in DADLE) or spontaneous slow wave activity ($n = 7$) suggesting that these agonists did not have direct effects on the ionic conductances of smooth muscle cells (Figures 1).

Electrical field stimulation elicited primarily inhibitory junction potentials (i.j.ps) in the myenteric region that

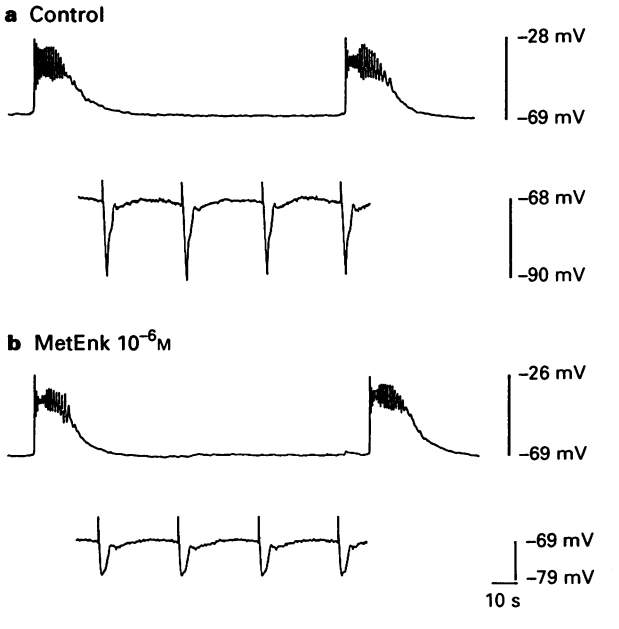


Figure 1 Effects of methionine enkephalin (MetEnk) on spontaneous electrical activity and inhibitory junction potentials (i.j.ps). Top trace in (a) shows spontaneous slow waves recorded from a cell within the myenteric portion of the circular muscle layer. Bottom trace shows i.j.ps elicited by field stimulation (30 Hz, 3 pulses, 60 V). (b) Shows activities after MetEnk (10^{-6} M). MetEnk did not affect slow wave activity or resting potentials, but inhibited i.j.ps. Initial transient upstrokes in records from cells in myenteric region are stimulus artifacts (also in Figures 4, 7 and 8).

averaged 13 ± 6 mV in amplitude ($n = 7$ representative preparations) as previously described (Vogalis & Sanders, 1990). These events persisted after the addition of atropine, phentolamine and propranolol (all at 10^{-6} M), and therefore resulted from the activation of non-adrenergic, non-cholinergic (NANC) enteric inhibitory nerves (see Figure 1a). Addition of MetEnk (10^{-10} to 10^{-6} M) caused a concentration-dependent reduction of the amplitude of i.j.ps ($IC_{50} = 5 \times 10^{-8}$ M). At the maximum effect, MetEnk reduced i.j.ps to an average of 28 ± 2% of the control amplitude. Figure 2b shows the effects of MetEnk on i.j.ps and a concentration-response curve summarizing the effects in 7 muscles is shown in Figure 3.

The δ-selective agonist, DADLE (10^{-11} to 10^{-7} M) had effects similar to MetEnk on i.j.ps (Figure 2e). At the maximum effective concentration, i.j.ps were reduced to an average of 26 ± 3% of the control amplitude. Concentration-response curves showed that DADLE was about 2 orders of magnitude more potent ($IC_{50} = 5 \times 10^{-10}$ M) than MetEnk in reducing i.j.ps (Figure 3).

We have recently shown that L-NAME and L-NMMA, arginine analogues that block nitric oxide synthesis, decrease the amplitude of i.j.ps in the circular muscle of the canine pylorus (Bayguinov & Sanders, 1992). Similar effects were caused by oxyhaemoglobin (HbO). Four muscles were treated with L-NAME (10^{-4} M) and then exposed to MetEnk (10^{-6} M; $n = 3$) and DADLE (10^{-6} M; $n = 3$). After washing out L-NAME, three of the muscles were pretreated with HbO (1%) and then exposed to either MetEnk ($n = 2$) or DADLE ($n = 1$). When i.j.ps were suppressed with drugs to block nitric oxide neurotransmission, MetEnk and DADLE caused no further reduction in i.j.p. amplitude (Figure 4). These data suggest that opioid antagonists act on a population of enteric inhibitory nerves that release nitric oxide.

Naloxone (10^{-6} M) had no effect on resting membrane potential, slow wave activity, or the i.j.ps elicited by EFS. This compound reduced the maximim inhibitory effects of MetEnk (10^{-6} M) and DADLE (10^{-6} M) on i.j.ps restoring the amplitudes of i.j.ps to 90 ± 4% ($n = 4$) and 94 ± 2% ($n = 4$) of control levels, respectively (Figure 2c and f).

The specific δ opioid receptor antagonist, ICI 174,864 (10^{-6} M) had effects similar to naloxone. ICI 174, 864 did not affect resting potential, slow wave activity, or i.j.ps elicited by EFS, but it antagonized the effects of MetEnk and DADLE, restoring the amplitudes of i.j.ps to 91 ± 3% ($n = 3$) and 97 ± 2% ($n = 3$) of control levels, respectively.

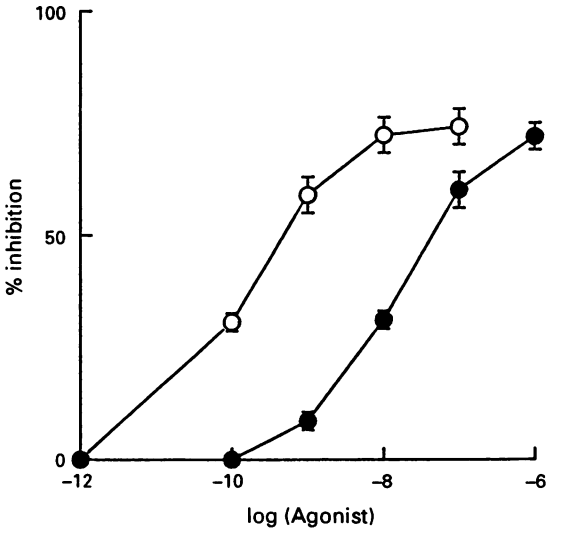


Figure 3 Concentration-response relationships for the effects of methionine enkephalin (MetEnk, ●) and DADLE (○) on inhibitory junction potentials. DADLE was about 2 orders of magnitude more potent than MetEnk in reducing the amplitude of junction potentials. Data are means ± s.e.mean; numbers of experiments given in text.

Effects of opioids on circular muscle cells near the submucosal border

As observed previously, cells in the submucosal region of the circular muscle layer were not spontaneously active. Resting membrane potentials averaged − 70 ± 2 mV, and the application of MetEnk or DADLE did not significantly affect resting potential (i.e. − 71 ± 2 mV and − 69 ± 3 mV in the presence of MetEnk ($n = 4$) and DADLE ($n = 3$), respectively). Field stimulation of this region produced predominantly cholinergically-mediated e.j.ps that averaged 36 ± 2 mV ($n = 7$) as previously described (Vogalis & Sanders, 1990). Both opioids suppressed e.j.ps in a concentration-dependent manner. At the maximum effect, MetEnk (10^{-6} M) decreased the amplitude of e.j.ps by an

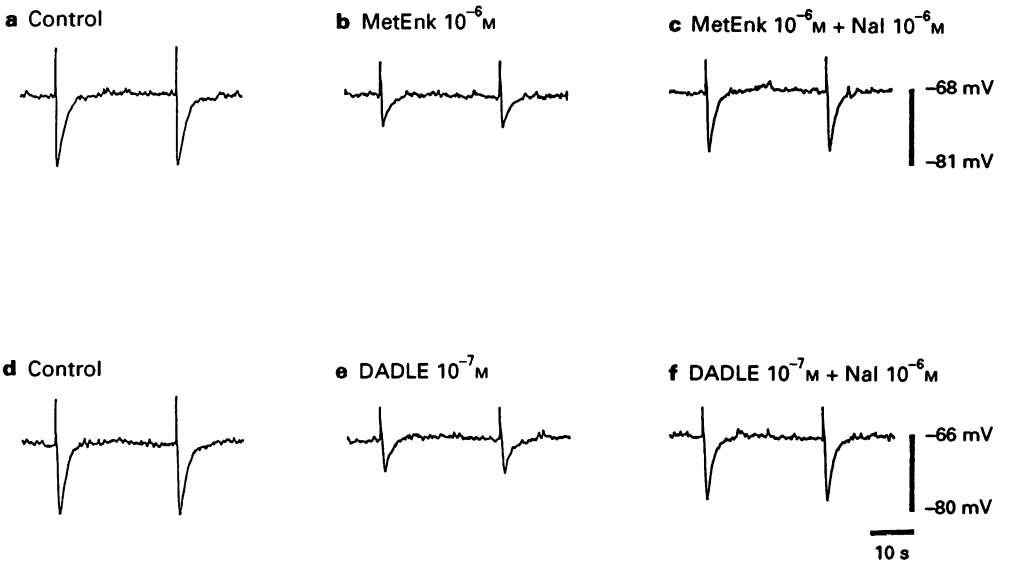


Figure 2 Effects of methionine enkephalin (MetEnk) and DADLE on i.j.ps; (a) shows i.j.ps elicited by EFS (30 Hz, 3 pulses, 60 V); (b) shows reduction in i.j.ps by MetEnk (10^{-6} M). The effect of MetEnk was blocked by naloxone (Nal 10^{-6} M; c), (d) and (e) show that DADLE also reduced i.j.p. amplitude, and these effects were reversed by naloxone (10^{-6} M).

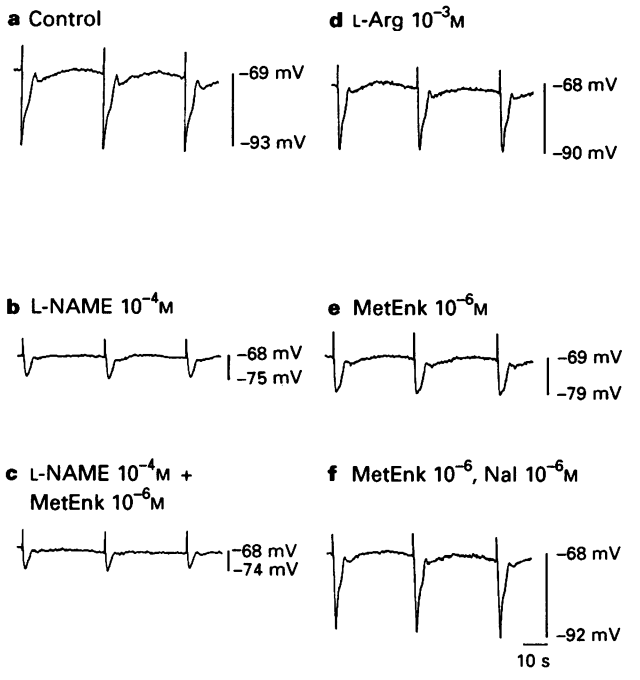


Figure 4 Methionine enkephalin (MetEnk) inhibited nitric oxide-dependent i.j.ps. (a) shows i.j.ps evoked by EFS (30 Hz, 3 pulses, 60 V) in a cell from myenteric region of circular muscle layer; (b) shows reduction in i.j.p. amplitude by treatment (20 min) with N^{ω}-nitro-L-arginine methyl ester (L-NAME). (c) Shows that after L-NAME, MetEnk had little or no effect on remaining i.j.p. The inhibitory effects of MetEnk and L-NAME were reversible. To increase the rate of i.j.p. recovery from the effects of L-NAME, L-arginine (10^{-3} M) was added to the bath solution for 15 min. (d) I.j.ps were inhibited by MetEnk (10^{-6} M) after the wash-out of L-NAME and L-arginine (e) and Panel F (f) shows that naloxone (10^{-6} M; given in continued presence of MetEnk) blocked the inhibitory effects of MetEnk. All recordings were from the same impalement.

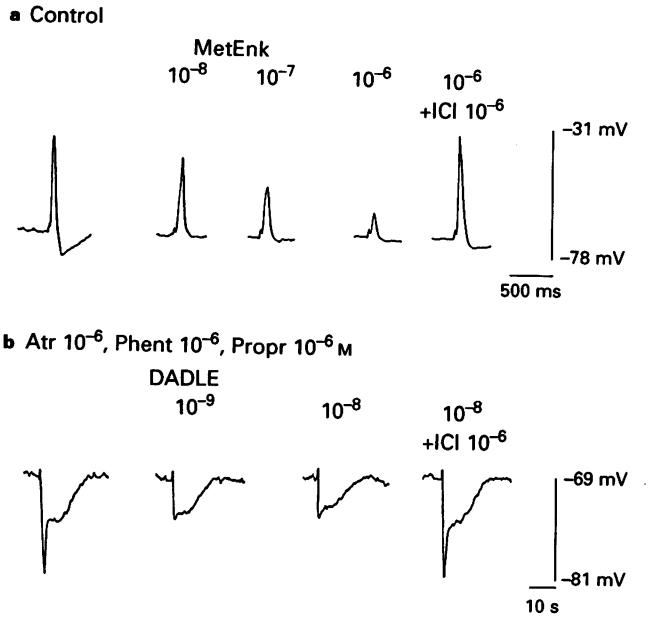


Figure 5 Effects of methionine enkephalin (MetEnk) on e.j.p. and i.j.p. amplitudes recorded from a cell in the submucosal region: (a) shows responses elicited by EFS (30 Hz, 3 pulses, 60 V). The predominant response to EFS in the submucosal region was an e.j.p. MetEnk (10^{-8}–10^{-6} M) decreased e.j.p. amplitude. Representative e.j.ps shown during MetEnk exposure. ICI 174,864 (10^{-6} M) blocked the effects of MetEnk. Representative i.j.ps elicited during the same impalement after treatment with atropine, phentolamine and propranolol (all at 10^{-6} M) are shown in (b). Atropine blocked the e.j.p. response unmasking large non-adrenergic, non-cholinergic i.j.ps. DADLE reduced the amplitude of i.j.ps (similar results were obtained with MetEnk; see text), and these effects were blocked by ICI 174,864.

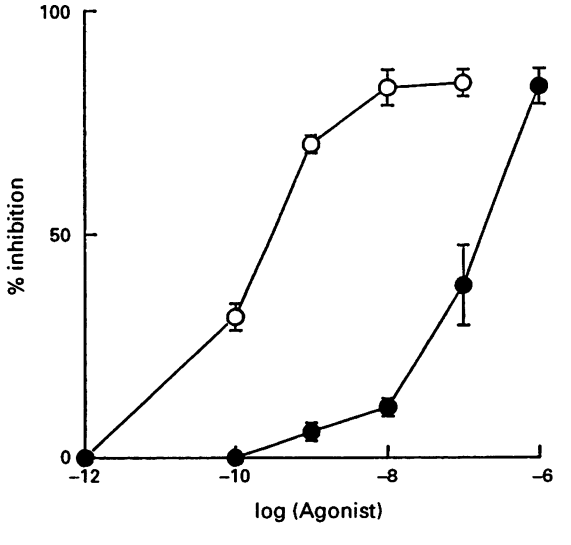


Figure 6 Concentration-response relationships for the effect of methionine enkephalin (MetEnk, ●) and DADLE (○) on excitatory junction potentials elicited in circular muscle cells near the submucosal border. DADLE was about 2 orders of magnitude more potent than MetEnk in reducing the amplitude of excitatory junction potentials. Data are means ± s.e.mean; numbers of experiments given in text.

average of 83 ± 2% (Figure 5a; $n = 4$) and DADLE decreased e.j.ps by 82 ± 4% ($n = 3$; data not shown). Concentration-response curves showed that DADLE was more than 2 orders of magnitude more potent ($IC_{50} = 5 \times 10^{-10}$ M) than MetEnk in reducing e.j.ps (Figure 6).

Application of naloxone (10^{-6} M) had no effect on resting membrane potential or e.j.p. amplitude, but this compound antagonized the inhibitory effects of MetEnk and DADLE, restoring the amplitude of e.j.ps to 96 ± 3% (n = 4) and 92 ± 1% ($n = 3$) of control responses, respectively, in the continued presence of MetEnk and DADLE. ICI 174,864 (10^{-6} M) also had no effect on the resting potential, but it blocked the effects of MetEnk and DADLE (i.e. restoration to 94 ± 1% ($n = 4$) and 93 + 3% ($n = 3$) of control responses, respectively). Figure 5a shows effects of ICI 174,864 on e.j.ps.

Besides an effect on e.j.ps, we noted that MetEnk and DADLE also reduced the amplitude of the small i.j.p. responses that often follow the initial e.j.p. in submucosal muscles. In order to study the effects of opioids on i.j.ps in this region, muscles were exposed to atropine, phentolamine and propranolol (all at 10^{-6} M). Atropine eliminated e.j.ps, leaving the non-adrenergic, non-cholinergic i.j.ps. These events averaged 10 ± 0.5 mV in peak amplitude ($n = 7$). MetEnk and DADLE suppressed i.j.ps (i.e. MetEnk reduced i.j.ps by an average of 56 ± 3%, $n = 4$ and DADLE reduced i.j.ps by 58 ± 3%, $n = 3$) with the same effectiveness as they suppressed the i.j.ps recorded near the myenteric border (Figure 5b). These data suggest that opioid receptors are present on cholinergic and enteric inhibitory nerves in the pylorus. Naloxone and the specific δ opioid antagonist, ICI 174,864, eliminated the effects of MetEnk and DADLE on submucosal i.j.ps. Naloxone restored i.j.p. amplitude to

94 ± 3% of control amplitude in the presence of MetEnk ($n = 4$), and it restored i.j.p. amplitude to 96 ± 4% of control amplitude in the presence of DADLE ($n = 3$). ICI 174,864 restored i.j.p. amplitude to 91 ± 4% of control amplitude in the presence of MetEnk ($n = 4$), and it restored i.j.p. amplitude to 95 ± 1% of control amplitude in the presence of DADLE ($n = 3$; see Figure 5b).

Effects of opioids on responses to EFS in muscles free of the myenteric plexus

Studies on intact muscles suggest that opioids may affect excitatory and inhibitory transmitter release. The site of action of opioids could either be within ganglia or at nerve terminals. We studied this question in preparations from which the myenteric plexus had been removed. Cells in the remaining myenteric region of the circular muscle layer were impaled and i.j.ps were elicited by EFS. Application of MetEnk (10^{-6} M) and DADLE (10^{-6} M) inhibited i.j.p. by an average of 73 ± 3% and 74 ± 5% (both $n = 5$). These data suggest that opioids exert actions directly upon nerve processes within the muscle layer.

Effects of endogenous opioids on responses to field stimulation

The experiments described above do not clarify the role of endogenous opioids that may be released in response to EFS. The observations that naloxone did not affect spontaneous electrical activity or responses to EFS suggest that endogenous opioids do not affect pyloric smooth muscle cells directly nor are they released in sufficient quantity during brief periods of EFS to affect the release of transmitter. Therefore we tested the effects of more sustained EFS.

I.j.p. amplitude was compared before and after a conditioning stimulation period. I.j.ps were elicited with 3 pulses (30 Hz; 60 V). Then a conditioning train of stimuli was applied for 2 min (30 Hz; 30 V). After cessation of the conditioning train and repolarization of the resting potential, i.j.ps were again elicited with 3 pulses (30 Hz; 60 V). I.j.ps elicited after the conditioning train were reduced in amplitude by an average of 17 ± 3% ($n = 5$; $P < 0.01$; Figure 7a). The muscles were then exposed to DADLE (10^{-6} M). This reduced i.j.p amplitude as discussed above, and it also inhibited the reduction in i.j.p. amplitude caused by the conditioning train (Figure 7b). Further addition of naloxone (10^{-6} M in the continued presence of DADLE) antagonized the effects of DADLE on i.j.ps. In the presence of naloxone the conditioning train did not reduce i.j.p. amplitude (i.e. i.j.ps were 97 ± 3% of control amplitude; $P > 0.05$; Figure 7c).

In another series of experiments muscles were stimulated for 20 s (0.5 ms, 60 V, 30 Hz). This caused a sustained hyperpolarization averaging 10 ± 4 mV in amplitude ($n = 4$). Then muscles were treated with naloxone (10^{-6} M) for 5 min and the same stimulus protocol was repeated. Naloxone significantly increased the amplitude of the sustained hyperpolarization by an average of 28 ± 11% ($n = 4$; $P < 0.01$; Figure 8). Taken together these results suggest that effective concentrations of opioids may be released when higher frequency stimuli are applied for a sustained period.

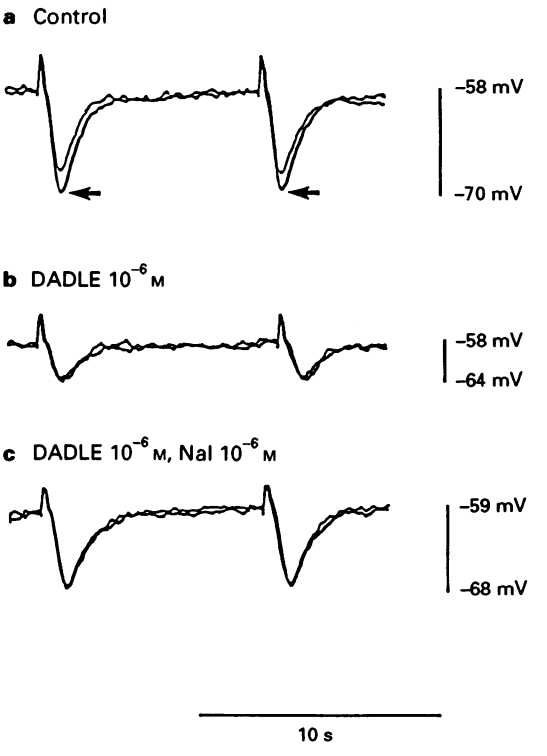


Figure 7 Effects of conditioning stimuli on i.j.p. amplitude: (a) shows 2 superimposed traces of i.j.ps elicited by field stimulation (30 Hz, 3 pulses, 60 V) before (trace denoted by arrows) and after a conditioning period of stimulation (30 Hz, 2 min, 30 V). The amplitude i.j.ps decreased following the conditioning stimuli. (b) Shows superimposed records before and after the conditioning stimulation in the presence of DADLE (10^{-6} M). DADLE reduced the amplitude of i.j.ps and blocked the effect of the conditioning stimulation. The effects of naloxone are shown in (c). Superimposed records were again taken before and after the conditioning stimulation. Naloxone blocked the reduction in i.j.p. amplitude caused by DADLE, and it also blocked the post-stimulation inhibition seen in the control record. All records were taken during a single impalement.

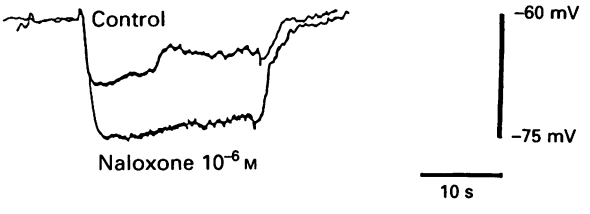


Figure 8 Effects of naloxone on sustained hyperpolarization responses to EFS. Muscle was stimulated by repetitive pulses of EFS (30 Hz, 20 s, 60 V). This stimulation caused a sustained hyperpolarization (control record). Exposure to naloxone increased the amplitude of the hyperpolarization recorded from the same cell, suggesting that release of endogenous opioids during the control record limited the response.

Discussion

A number of studies have shown that enteric neurones contain opioid peptides (Polak *et al.*, 1977; Schultzberg *et al.*, 1980; Furness *et al.*, 1980; 1983), and these compounds can be released by EFS (Glass *et al.*, 1986) and during peristalsis (Donnerer *et al.*, 1984). In the guinea-pig intestine, enkephalins and dynorphin are expressed along with other transmitter substances in excitatory and inhibitory neurones (Brookes *et al.*, 1991), suggesting the possibility that opioids may be co-released with excitatory and inhibitory transmitters. Opioids could have effects either pre- or post-junctionally. The presence of opioid receptors in subcellular fractions containing neural markers, but not in fractions containing smooth muscle markers (Allescher *et al.*, 1989), suggests that the primary site of action of these peptides may be pre-junctional. In support of the concept of pre-junctional modulation, opioids have been shown to decrease the release of enteric transmitter substances (Vizi *et al.*, 1984; Grider & Maklouf, 1987a).

In the present study we characterized the role of opioids in

regulating junction potentials in the circular muscle layer of the canine pyloric sphincter. This region has been shown to contain a very high density of opioid-containing neurones in several species (Edin *et al.*, 1980; Ferri *et al.*, 1987; Allescher *et al.*, 1988). We found that exogenous opioids reduced the amplitude of excitatory and inhibitory junction potentials. Opioids specifically reduced the portion of the i.j.ps that were inhibited by arginine analogues, suggesting that opioids may reduce the synthesis and release of nitric oxide just as they appear to reduce the release of traditional neurotransmitters (Vizi *et al.*, 1984; Grider & Maklouf, 1987a). The fact that opioids affected both excitatory and inhibitory junction potentials suggests that opioid receptors are coupled to a basic signal for transmitter release. A common mechanism may be regulation of Ca^{2+} concentration in varicosities since a rise in $[Ca^{2+}]_i$ appears to be the signal for quantal release of most transmitters and for synthesis and release of nitric oxide (Stark *et al.*, 1991).

Naloxone, a non-specific opioid receptor antagonist, had little or no effect on junction potentials elicited by single or brief trains of stimuli. But naloxone: (i) increased the amplitude of hyperpolarization responses elicited by longer trains of stimulation, and (ii) blocked depression of i.j.ps following periods of high frequency stimulation. These data suggest that repetitive stimuli may be necessary to release enough opioids to affect transmitter release.

Others have suggested a similar role for endogenous opioids in the small intestine. For example, Puig *et al.* (1977) found that higher stimulus frequencies may be necessary for release of opioids in the guinea-pig ileum. These authors found that a conditioning period of stimulation at 10 Hz caused inhibition of electrically evoked twitch responses in the period immediately following the conditioning stimuli. Naloxone protected against the post-stimulus inhibition, suggesting that opioids released during the conditioning stimuli were responsible for the post-stimulus inhibition. Similar results were reported by Fosbraey & Johnson (1980). Others have failed to show effects of naloxone on junction potentials elicited by brief field stimulation. For example, Bauer & Szurszewski (1991) found that naloxone antagonized the reduction in i.j.ps caused by exogenous opioid agonists in the canine duodenum, but opioid receptor antagonists did not significantly affect i.j.ps. Similar results have been reported from experiments on guinea-pig intestine; naloxone had no effect on resting membrane potential or on junction potentials (Ito & Tajima, 1980). These findings also suggest that the concentrations of opioids released by brief stimuli are too low to affect transmitter release. For effective concentrations of opioids to be reached, relatively high frequency stimulation appears to be required.

An important question is whether the enteric nervous system operates at high enough frequencies to utilize endogenous opioids as a means to regulate transmitter release. Bornstein *et al.* (1991) have shown that firing within motor neurones of the enteric nervous system can reach instantaneous frequencies of 100 Hz during reflex activation. Although high instantaneous frequencies are reached, it is unclear whether high frequency firing persists for sufficient periods to activate naloxone-sensitive responses. Physiological evidence, however, tends to suggest that concentrations of opioids may reach effective levels during reflex activity. Opioid peptides and receptor antagonists have been shown to modulate the peristaltic reflex (Kromer *et al.*, 1990), although a recent study suggests that opioids may only be important when the intestine is working against relatively high outflow resistances (Waterman *et al.*, 1992). Grider & Makhlouf (1987b) have attempted to dissect the relative contribution of opioids to the descending relaxation and ascending contraction components of the peristaltic reflex. They found that naloxone caused a concentration-dependent increase in descending relaxation and decreased ascending contraction. The increase in descending relaxation was attributed to an increase in VIP release that coincided with naloxone treatment, and the decrease in ascending contraction may also have been related to an increase in VIP release. Since VIP appears to be expressed in the same inhibitory neurones that synthesize nitric oxide (Costa *et al.*, 1991), it is possible that nitric oxide was also involved in the naloxone-sensitive effects noted by Grider & Makhlouf (1987b). Donnerer *et al.* (1984) measured dynorphin, substance P and somatostatin released from the guinea-pig intestine. They showed that levels of these peptides increased during peristalsis. Release of substance P and dynorphin were blocked by TTX, suggesting these peptides originated from a neural source. Significant levels of substance P were observed only when naloxone was present, suggesting that opioids normally limit the release of substance P released during peristalsis.

Limited evidence suggesting a physiological role for opioids in the pylorus exists. Emptying of solid meals was significantly slowed by the enkephalin analogue DAMME (D-Ala^2, MePhe^4, Met(O-Ol-enkephalin; FK33-824) (Sullivan *et al.*, 1981), but others reported that naloxone did not affect gastric emptying (Feldman *et al.*, 1980). Allescher *et al.* (1988) found that exogenous opioid agonists inhibited motor activity of the canine pylorus *in vivo*. Naloxone antagonized these effects, demonstrating the presence of functional opioid receptors in the canine pylorus. A variety of opioids, directed at μ, δ and κ receptors, however, did not affect basal motor activity in the pyloric region, and blockade of opioid receptors did not affect excitatory responses elicited by duodenal field stimulation or inhibitory responses elicited by antral field stimulation or vagal stimulation. These *in vivo* studies further illustrate the difficulties in demonstrating the role of endogenous opioids. Our results would predict that rather intense or prolonged stimuli would be necessary to elicit naloxone-sensitive responses.

In some species opioids might have pre- and post-junctional effects. For example in the cat, atropine-resistant contractions of the pylorus elicited by vagal stimulation could be blocked by naloxone (Edin *et al.*, 1980). Others have suggested that endogenous opioids in the pyloric region are involved in an excitatory reflex stimulated by duodenal acidification or amino acids (Reynolds *et al.*, 1984; 1985). Taken together it appears that in the cat there may be (i) release of enkephalins from non-cholinergic excitatory nerves, and (ii) direct effects on the smooth muscle. These mechanisms are not apparent in the dog because we found no effect on either resting membrane potential or slow waves in canine pyloric muscles.

In addition to regulation of physiological responses, the receptors and effectors for opioids provide an important means for therapeutic control of motor activity. For example, it has been known for many years that the peristaltic reflex elicited by distension of the bowel is inhibited by morphine (Gyang *et al.*, 1964). In the pylorus it appears that a significant portion of the regulation of junction potentials by opioids occurs via δ receptors since ICI 174,864, a specific δ receptor antagonist, had nearly identical actions to those of the non-specific antagonist, naloxone. Similar results have been reported for the modulation of inhibitory junction potentials by exogenous opioids in the human colon (Hoyle *et al.*, 1990).

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References

ALLESCHER, H.D., AHMAD, S., DANIEL, E.E., DENT, J., KOSTOLANSKA, F. & FOX, J.E.T. (1988). Inhibitory opioid receptors in canine pylorus. *Am. J. Physiol.*, **255**, G352–G360.

ALLESCHER, H.D., AHMAD, S., KOSTKA, P., KWAN, C.Y. & DANIEL, E.E. (1989). Distribution of opioid receptors in canine small intestine: implications for function. *Am. J. Physiol.*, **256**, G966–G974.

BAUER, A.J., SARR, M.G. & SZURSZEWSKI, J.H. (1991). Opioids inhibit neuromuscular transmission in circular muscle of human and baboon jejunum. *Gastroenterology*, **101**, 970–976.

BAUER, A.J. & SZURSZEWSKI, J.H. (1991). Effect of opioid peptides on circular muscle of canine duodenum. *J. Physiol.*, **434**, 409–422.

BAYGUINOV, O. & SANDERS, K.M. (1992). Role of nitric oxide as an inhibitory neurotransmitter in the canine pyloric sphincter. *Gastroenterology*, **102**, A422 (abstract).

BORNSTEIN, J.C., FURNESS, J.B., SMITH, T.K. & TRUSSELL, D.C. (1991). Synaptic responses evoked by mechanical stimulation of the mucosa in morphologically characterized myenteric neurons of the guinea pig ileum. *J. Neuroscience*, **11**, 505–518.

BROOKES, S.J.H., STEELE, P.A. & COSTA, M. (1991). Identification and immunohistochemistry of cholinergic and non-cholinergic circular muscle motor neurons in the guinea-pig small intestine. *Neuroscience*, **42**, 863–878.

CHERUBINI, E. & NORTH, R.A. (1985). Mu and kappa opioids inhibit transmitter release by different mechanisms. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 1860–1863.

COSTA, M., FURNESS, J.B., BROOKES, S.J.H., BREDT, D.S. & SNYDER, S.H. (1991). Presence and chemical coding of neurons with nitric oxide synthase immunoreactivity in the guinea-pig small intestine. *Proc. Aust. Physiol. Pharmacol. Soc.*, **22**, 97P (abstract).

COWIE, A.L., KOSTERLITZ, H.W., ANGELA, A. & WATERFIELD, A. (1978). Factors influencing the release of acetylcholine from the myenteric plexus of the ileum of the guinea-pig and rabbit. *Br. J. Pharmacol.*, **64**, 565–580.

DONNERER, J., HOLZER, P. & LEMBECK, F. (1984). Release of dynorphin, somatostatin and substance P from the vascularly perfused small intestine of the guinea-pig during peristalsis. *Br. J. Pharmacol.*, **83**, 919–925.

DUGGAN, A.W. & NORTH, R.A. (1984). Electrophysiology of opioids. *Pharmacol. Rev.*, **35**, 219–281.

EDIN, R., LUNDBERG, J., TERENIUS, L., DAHLSROM, A., HOKFELT, T., KEWENTER, J. & AHLMAN. (1980). Evidence for vagal enkephalinergic control of the feline pylorus and stomach. *Gastroenterology*, **78**, 492–497.

FELDMAN, M., WALSH, J.H. & TAYLOR, I.L. (1980). Effect of naloxone and morphine on gastric acid secretion and on serum gastrin and pancreatic polypeptide concentrations in humans. *Gastroenterology*, **79**, 294–298.

FERRI, G.L., MORREALE, R.A., SOIMERO, L., BILIOTTI, G. & DOCKRAY, G.J. (1987). Intramural distribution of met^5-enkephalin-arg^6-gly^7-leu^8 in sphincter regions of the human gut. *Neurosci. Lett.*, **74**, 304–308.

FOSBRAEY, P. & JOHNSON, E.S. (1980). Release-modulating acetylcholine receptors on cholinergic neurones of the guinea-pig ileum. *Br. J. Pharmacol.*, **68**, 289–300.

FURNESS, J.B., COSTA, M., FRANCO, R. & LLEWELLYN-SMITH, I.J. (1980). Neuronal peptides in the intestine: distribution and possible functions. *Adv. Biochem. Psychopharmacol.*, **22**, 601–617.

FURNESS, J.B., COSTA, M. & MILLER, R.J. (1983). Distribution and projections of nerves with enkaphalin-like immunoreactivity in the guinea-pig small intestine. *Neuroscience*, **8**, 653–664.

GLASS, J., CHAN, W.C. & GINTZLER, A.R. (1986). Direct analysis of the release of methionine-enkephalin from the guinea-pig myenteric plexus: modulation by endogenous opioids and exogenous morphine. *J. Pharmacol. Exp. Ther.*, **239**, 742–747.

GRIDER, J.R. & MAKHLOUF, G.M. (1987a). Suppression of inhibitory neural input to colonic circular muscle by opioid peptides. *J. Pharmacol. Exp. Ther.*, **243**, 205–210.

GRIDER, J.R. & MAKHLOUF, G.M. (1987b). Role of opioid neurons in the regulation of intestinal peristalsis. *Am. J. Physiol.*, **253**, G226–G231.

GYANG, E.A., KOSTERLITZ, H.W. & LEES, G.M. (1964). The inhibition of autonomic neuroeffector transmission by morphine-like drugs and its use as a screening test for narcotic analgesic drugs. *Arch. Exp. Path. Pharmak.*, **248**, 231–246.

HOYLE, C.H.V., KAMM, M.A., BURNSTOCK G. & LENNARD-JONES, J.E. (1990). Enkephalins modulate inhibitory neuromuscular transmission in circular muscle of human colon via δ-opioid receptors. *J. Physiol.*, **431**, 465–478.

ITO, Y. & TAJIMA, K. (1980). Action of morphine on the neuroeffector transmission in the guinea-pig ileum and in the mouse vas deferens. *J. Physiol.*, **307**, 367–383.

KROMER, W. (1990). Reflex peristalsis in the guinea pig isolated ileum is endogenously controlled by kappa opioid receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 450–454.

MCFADZEAN, I. (1988). The ionic mechanisms underlying opioid action. *Neuropeptides*, **11**, 173–180.

MIHARA, S. & NORTH, R.A. (1986). Opioids increase potassium conductance in submucous neurones of guinea-pig caecum by activating delta-receptors. *Br. J. Pharmacol.*, **88**, 315–322.

MILLER, R.J. & HIRNING, L.D. (1989). Opioid peptides of the gut. In *Handbook of Physiology–The gastrointestinal system II.* ed. Schultz, S.G. & Wood, J.D. pp. 631–660. Bethesda MD: American Physiological Society.

MORITA, K. & NORTH, R.A. (1981). Opiate activation of potassium conductance in myenteric neurons: inhibition by calcium ions. *Brain Res.*, **242**, 145–150.

NORTH, R.A., WILLIAMS, J.T., SUPRENANT, A. & CHRISTIE, M.J. (1987). Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 5487–5491.

POLAK, J.M., BLOOM, S.R., SULLIVAN, S.N., FACER, P. & PEARSE, A.G.E. (1977). Enkephalin-like immunoreactivity in the human gastrointestinal tract. *Lancet*, **i**, 972–974.

PUIG, M.M., GASCON, P., CRAVISO, G.L. & MUSACCHIO, J.M. (1977). Endogenous opiate receptor ligand: electrically induces release in the guinea-pig ileum. *Science*, **195**, 419–420.

REYNOLDS, J.C., OUYANG, A. & COHEN, S. (1984). Evidence for an opiate-mediated pyloric sphincter reflex. *Am. J. Physiol.*, **246**, G130–G136.

REYNOLDS, J.C., OUYANG, A. & COHEN, S. (1985). Opiate nerves mediate feline pyloric response to intraduodenal amino acids. *Am. J. Physiol.*, **248**, G307–G312.

SANDERS, K.M. & VOGALIS, F. (1989). Organization of electrical activity in the canine pyloric canal. *J. Physiol.*, **416**, 49–66.

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.*, **262**, G379–G392.

SCHULTZBERG, M., HOKFELT, T., NILSSON, G., TERENIUS, L., REHFELD, J.F., BROWN, M., ELDE, R., GOLDSTEIN, M. & SAID, S. (1980). Distribution of peptide- and catecholamine-containing neurons in the gastro-intestinal tract of rat and guinea-pig: immunohistochemical studies with antisera to substance P, vasoactive intestinal polypeptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin and dopamine β-hydroxylase. *Neuroscience*, **5**, 689–744.

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.

SULLIVAN, S.N., LAMKI, L. & CORCORAN, P. (1981). Inhibition of gastric emptying by enkephaline analogue. *Lancet*, **ii**, 86–87.

VIZI, E.S., ONO, K., ADAM-VIZI, V., DUNCALF, D. & FOLDES, F.F. (1984). Presynaptic inhibitory effect of met-enkephalin on [^{14}C] acetylcholine release from the myenteric plexus and its interaction with muscarinic negative feedback inhibition. *J. Pharmacol. Exp. Ther.*, **230**, 493–499.

VOGALIS, F. & SANDERS, K.M. (1990). Excitatory and inhibitory neural regulation of canine pyloric smooth muscle. *Am. J. Physiol.*, **259**, G125–G133.

VOGALIS, F., WARD, S.M. & SANDERS, K.M. (1991). Correlation between electrical and morphological properties of canine pyloric circular muscle. *Am. J. Physiol.*, **260**, G390–G398.

WARD, S.M., DALZIEL, H.H., THORNBURY, K.D., WESTFALL, D. & SANDERS, K.M. (1992). Nonadrenergic, noncholinergic inhibition and rebound excitation in canine colon depend on nitric oxide. *Am. J. Physiol.*, **262**, G237–G243.

WATERMAN, S.A., COSTA, M. & TONINI, M. (1992). Modulation of peristalsis in the guinea-pig isolated small intestine by exogenous and endogenous opioids. *Br. J. Pharmacol.*, **106**, 1004–1010.

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Evidence for a nucleotide receptor on adrenal medullary endothelial cells linked to phospholipase C and phospholipase D

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1 We have investigated whether the 'atypical' P_2-purinoceptor previously described on adrenal microvasculature endothelial cells is a nucleotide receptor (responds to pyrimidines and purines) and is linked to phospholipase D as well as phospholipase C.

2 Cultured bovine adrenal medullary endothelial (BAME) cells responded to the pyrimidine UTP, as well as the purines. The total [^{3}H]-inositol phosphate responses were with a rank order of UTP > ATP = adenosine 5'-O-(3-thio-triphosphate) (ATPγS) >> 2MeSATP. The selective P_{2x} agonist β, γ-methylene ATP was inactive.

3 Construction of dose-response curves to ATP, ATPγS and UTP in the presence and absence of additional agonists showed that responses to ATPγS and UTP were not additive, nor were those to UTP and ATP. This suggests that purines and pyrimidines acted via a common nucleotide receptor.

4 ^{32}P-labelled BAME cells, in the presence of butanol, produced [^{32}P]-phosphatidylbutanol (PBut) when stimulated with ATPγS or the protein kinase C activator, tetradecanoyl phorbol acetate (TPA).

5 Cells labelled with [^{3}H]-palmitate and stimulated in the presence of butanol generated [^{3}H]-PBut with the same order of agonist potencies seen for inositol phosphate responses.

6 The protein kinase C inhibitor, Ro 31-8220, abolished TPA and agonist stimulation of [^{3}H]-PBut production.

7 These observations, and our related studies on bovine aortic endothelial cells, provide the first demonstration of a phospholipase C linked nucleotide receptor on vascular endothelial cells. It is concluded that BAME cells express a nucleotide receptor linked to phospholipase C and phospholipase D, but that activation of phospholipase D is probably down-stream of phospholipase C.

Keywords: Nucleotide receptors; phospholipase C; phospholipase D; P_{2y}-purinoceptors; protein kinase C.

Introduction

The role of extracellular adenosine 5'-triphosphate (ATP) as a mediator of a variety of physiological functions was established during the 1970's by Burnstock and co-workers (Burnstock, 1990). Subsequently ATP was found to produce endothelium-dependent vasodilation (De Mey & Vanhoutte, 1981) and stimulate prostacyclin production by endothelial cells (Pearson *et al.*, 1983). Sensitivity of the vascular endothelium to ATP and ADP but not AMP and adenosine identifies these effects as mediated by P_2-purinoceptors (Burnstock, 1978). A subdivision of P_2-purinoceptors was proposed by Burnstock & Kennedy (1985) based, in the absence of specific antagonists, on the rank order of potency of ATP analogues. P_{2x}-receptors are activated with a potency order of α, β-methylene ATP > β, γ-methylene ATP > ATP = ADP < 2-methylthioadenosine 5'-triphosphate (2MeSATP) and P_{2y}-receptors with a potency order of 2MeSATP > ATP = ADP > α,β-methylene ATP > β,γ-methylene ATP. Two other P_2-purinoceptor subtypes have been described, though their location is apparently restricted to platelets (P_{2T}) and mast cells (P_{2z}). Stimulation of endothelium-dependent relaxation (Houston *et al.*, 1987), prostacyclin production (Needham *et al.*, 1987) calcium mobilisation (Carter *et al.*, 1988) and phospholipid breakdown (Allsup & Boarder, 1990; Purkiss & Boarder, 1992) by 2MeSATP therefore indicate the presence of a P_{2y} type receptor on the endothelium of large vessels. These responses are believed to be dependent on the initial activation of phospholipase C and the generation of inositol phosphates and diacylglycerol (DAG). Phospholipase D is also activated in large vessel endothelium by purinoceptor stimulation (Martin & Michaelis, 1989; Pirotton *et al.*, 1990). Endothelial cells from different regions of the vasculature exhibit a high degree of variability in ultrastructural (Majno, 1965) and biochemical (Gerritsen, 1987) characteristics. We have found human brain microvessel endothelium to be insensitive to purinoceptor stimulation (Purkiss *et al.*, 1992) while endothelial cells from the bovine adrenal medulla exhibit an 'atypical' (neither P_{2y} nor P_{2x}) purinoceptor sensitivity (Allsup & Boarder, 1990). Other reports on a range of tissues have found a similarly distinct order of agonist potencies to the adrenal cells, but have also found the pyrimidine UTP to be active (Fine *et al.*, 1989; Davidson *et al.*, 1990; Brown *et al.*, 1991; Keppens *et al.*, 1992; Murrin & Boarder, 1992). A recent review discussed evidence for the existence of a 'nucleotide' receptor defined as one which will respond to both purines (e.g. ATP) and pyrimidines (UTP) (O'Connor *et al.*, 1991).

In the present study we have determined UTP to also be an active agonist on the bovine adrenal medulla cells and provide evidence that pyrimidine and purine nucleotides may act at a single receptor population, stimulation of which activates phospholipase C and phospholipase D.

Methods

Materials

[2-^{3}H]-*myo*-inositol (15 Ci mmol^{-1}) was purchased from Du Pont, New England Nuclear, [^{32}p]-H_3PO_4 (285 Ci mg^{-1} P)

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from ICN Radiochemicals and [^{3}H]-palmitic acid (50 Ci mmol^{-1}) from Amersham (Bucks). ATP, adenosine 5'-O-(3-thio-triphosphate) (ATPγS), β,γ-methylene ATP and UTP were purchased from Sigma (Dorset). 2MeSATP was from Research Biochemicals (St. Albans, Herts). Ro31-8220 was a gift from Dr G. Lawton, Roche Products (Welwyn Garden City, Herts). 1-Stearoyl-2-[1-^{14}C]-arachidonyl phosphatidyl-butanol (PBut) was a gift from Dr R. Bonser, Burroughs Wellcome (Beckenham, Kent). Silica gel G thin layer chromatography (TLC) plates (LK6D) were from Whatman (Bromley, Kent). Cell culture medium, additives and plastics were from Gibco (Paisley, Scotland). Other chemicals were from Sigma or Fisons (Loughborough).

Cell culture

Bovine adrenal medullary cells were dissociated from intact glands by collagenase and protease treatment purified and collected as described previously (Allsup & Boarder, 1990). Liberated cells were cultured for 3 h at 37°C in 175 cm^3 tissue culture flasks at 10^{-6} cells ml^{-1}, 50 ml/flask in medium consisting of Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal calf serum, 2 mM glutamine, 25 units ml^{-1} penicillin, 25 μg ml^{-1} streptomycin, 0.5 mg ml^{-1} gentamycin and 25 μg ml^{-1} fungizone. After 3 h unattached cells (mainly chromaffin cells) were decanted and the adherent cells cultivated in medium until confluent. Once confluent (approximately 3 days) the medium was changed to minimum essential medium with D-valine substituted for L-valine to inhibit fibroblast growth; supplements to the medium were the same as before. These cells exhibited the same morphology and unusual growth patterns as those described by Banerjee *et al.* (1985) which were prepared by the same method and identified as endothelial on the basis of Factor VIII:R immunoreactivity and untrastructural characteristics. Bovine adrenal medulla endothelial cells (BAME) were passaged with 0.25% trypsin into 24 well multidishes and used up to passage 3.

Bovine thoracic aorta endothelial cells (BAE) were prepared essentially as described by Booyse *et al.* (1975). Briefly, an undamaged aorta was clamped at one end, arteries ligated and washed gently inside with balanced salt solution (BSS, composition mM: NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, HEPES 30, NaH_2PO_4 1 mM, $MgSO_4$ 0.8, $CaCl_2$ 1.8, glucose 5.5, pH 7.4). The aorta was filled with collagenase solution (Sigma type 1, 0.5 mg ml^{-1} in BSS) and incubated for 20 min at 37°C, then gently washed and filled with medium (D-valine minimum essential medium as above) and vigorously shaken. The resultant suspension was triturated and seeded into 80 cm^2 flasks. When confluent the cells were passaged with 0.25% trypsin into 24 well multidishes and used up to passage 10.

Cell labelling and [^{3}H]-inositol phosphate assay

Confluent BAME cells on multiwell plates were incubated for 48 h with *myo*-[2-^{3}H]-inositol (1μCi ml^{-1}, 0.5 ml/well) in M199 supplemented with glutamine, penicillin, streptomycin and fungizone as above. After 48 h lipid accumulation of radioactivity was maximal in these cells under these conditions (Allsup & Boarder, 1990) indicating that equilibrium labelling had been achieved. Following the loading period, cells were incubated in BSS with 10 mM LiCl for 30 min in the presence of drugs as indicated. Subsequent extraction of inositol (poly)phosphates onto Dowex-1(Cl$^-$) was as previously described (Plevin & Boarder, 1988).

Cell labelling and [^{32}P]-PBut and [^{32}P]-phosphatidic acid ([^{32}P]-PA) assay

The $^{32}P_i$ loading procedure is fully described and characterized in Purkiss & Boarder (1992). Confluent BAME cells were labelled with $^{32}P_i$ by incubation for 24 h with BSS with NaH_2PO_4 omitted and containing [^{32}P]-H_3PO_4 (6.75 μCi ml^{-1}; 0.4 ml/well). Under these conditions phospholipid incorporation was maximal after 24 h in a preliminary loading time course which extended to 72 h. Labelled cells were stimulated by replacing the loading solution with BSS containing agonist. The 5 min incubations were terminated by removal of stimulating solution and addition of 0.5 ml ice-cold methanol. Cells were scraped and wells washed with 0.4 ml water. Lipids were extracted into 0.5 ml chloroform and extracts dried down under N_2 then redissolved in 80 μl chloroform/methanol (95:5) and applied with non-radioactive standard to oxalate treated silica gel G TLC plates. [^{14}C]-PBut was applied to lanes adjacent to samples. Plates were developed in ethylacetate/acetic acid/2,2,4-trimethylpentane (9:2:5), [^{32}P]-PA, [^{32}P]-PBut and [^{14}C]-PBut standard were visualised by autoradiography. Non-radioactive PA standard was stained with I_2 vapour and found to correspond exactly with a spot on the autoradiographs. [^{32}P]-PA and [^{32}P]-PBut spots were well resolved and free of intervening radioactivity. They were scraped and liquid scintillation counted. Butanol, when added, was present for the last 10 min of the labelling period at a concentration of 50 mM and was included in the stimulating medium at 50 mM.

[^{3}H]-palmitate cell labelling and [^{3}H]-PBut and [^{3}H]-PA assay

Confluent or slightly pre-confluent BAE and BAME cells were labelled with [^{3}H]-palmitate by supplementing medium with 10 μCi ml^{-1} [^{3}H]-palmitic acid for 48 h (0.5 ml/well). After the loading period cells were washed twice and incubated for 1 h then washed again. Butanol, to a concentration of 50 mM, was added to the last wash 10 min before stimulation which was the same as for $^{32}P_i$ labelled cells. Extracted lipids, prepared as before, were applied to TLC plates with non-radioactive PA and PBut standards which were prepared from phosphatidylcholine by the method of Kobayashi & Kanfer (1987) using crude enzyme (acetone precipitated) prepared from Savoy cabbage according to Davidson & Long (1958). Plates were developed, I_2 stained and spots scraped and counted as before. When the protein kinase C (PKC) inhibitor Ro 31-8220 was used it was added to cells 20 min before stimulation to a concentration of 10 μM. The same concentration was present in the stimulating medium.

Statistical analysis

All data are expressed as means ± s.e. The significance of difference between means was determined by Student's *t* test, *P* values <0.05 were considered significant (indicated with asterisk). In figures presented with error bars; wherever no bars are visible, they are smaller than the area of the symbol. EC_{50} values were determined using the 'Graph-Pad' programme.

Results

BAME cell [^{3}H]-inositol phosphate release by purinoceptor agonists and UTP

It has previously been shown in our laboratory that incubation of adrenal cells, loaded as described with [^{3}H]-*myo*-inositol, with 30 μM ATP in the presence of 10 mM LiCl, resulted in a linear accumulation of total [^{3}H]-inositol phosphates over a period of 90 min (Allsup & Boarder, 1990). The concentration-dependent responses to 30 min stimulation with UTP, ATPγS and ATP (Figure 1) were therefore observed over a phase of linear accumulation. EC_{50} values could not be calculated from this series of experiments because maximum responses could not be reliably determined: concentrations of the agonists above 100 μM began to produce a

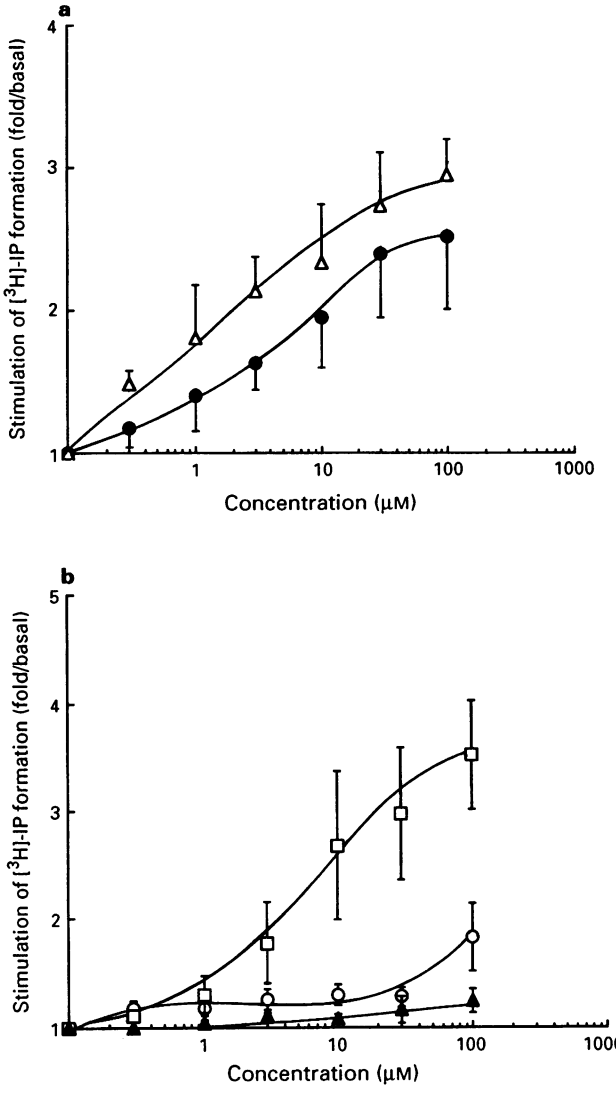


Figure 1 Concentration-effect curves for accumulation of [^{3}H]-inositol phosphates (IP) in BAME cells in the presence of 10 mM LiCl. (a) Responses to increasing concentrations of ATP (●) and UTP (△). (b) Responses to increasing concentrations of ATP analogues, ATPγS (□), 2MeSATP (○) and β,γ-methylene ATP (▲). Stimulation of [^{3}H]-inositol phosphates are expresed as the fold increases over basal. Data are pooled from 3 separate experiments each carried out in triplicate. Typical values were basal, 1835 ± 126 d.p.m.; 100 μM UTP, 6226 ± 165 d.p.m.; 100 μM ATP, 4617 ± 449 d.p.m.; 100 μM ATPγS, 6420 ± 424 d.p.m.; 2MeSATP, 2410 ± 164 d.p.m.; β,γ-methylene ATP, 1822 ± 20 d.p.m. For abbreviations, see text.

non-saturating response which we have previously described (Allsup & Boarder, 1990). However, positions of the curves in Figure 1 indicate rank order of agonist potency of UTP- > ATP = ATPγS > > 2MeSATP. The selective P_{2Y}-purinoceptor agonist, 2MeSATP, had little effect even at 100 μM. The P_{2X}-purinceptor agonist, β,γ-methylene ATP failed to produce a significant increase in [^{3}H]-inositol phosphates at any concentration used.

Interactions of nucleotide agonists

Construction of concentration-effect curves for the stimulation by ATPγS, UTP and ATP of the accumulation of total [^{3}H]-inositol phosphates alone and in the presence of UTP, ATP and ATPγS, are shown in Figure 2a,b and c. The curves of the agonists alone indicate that UTP and ATPγS were at or approaching their maximum effective concentration at 100 μM (Figure 2a and b) whereas ATP appeared to be

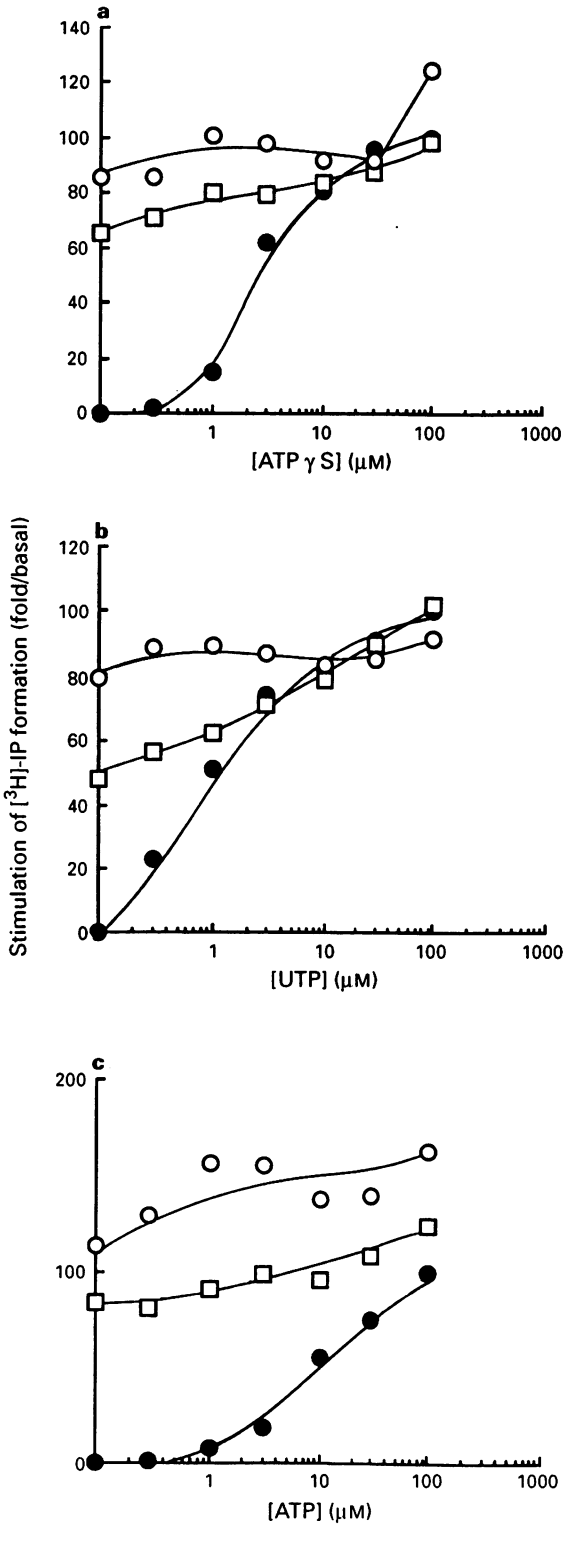


Figure 2 Accumulation of [^{3}H]-inositol phosphates (IP) in BAME cells in the presence of 10 mM LiCl. Curves were constructed for a single agonist (●) and in the presence of 10 μM (□) or 100 μM (○) additional agonist; curves with 30 μM additional agonist were intermediate between 10 μM and 100 μM but have been omitted for clarity. (a) Concentration-effect curves to ATPγS in the absence or presence of UTP. (b) Concentration-effect curves to UTP in the absence or presence of ATP. (c) Concentration-effect curves to ATP in the absence or presence of ATPγS. Error bars have been omitted for clarity but on only two points were they in excess of 15%. Stimulations are expressed relative to the 100 μM agonist alone response, which was taken as 100%. Data are pooled from 3 separate experiments each carried out in triplicate. Typical values were basal 1633 ± 60 d.p.m.; 100 μM UTP, 4634 ± 99 d.p.m.; 100 μM UTP + 100 μM ATP, 4894 ± 45 d.p.m. For abbreviations, see text.

sub-maximal at 100 μM (Figure 2c). Two of the sets of curves appeared to show little or no additivity between the effects of the agonists (Figure 2a and b). The lack of additivity would suggest that the effects of these agonists were mediated through the same receptor. The response to ATP at the highest concentration used was increased by the presence of ATPγS (Figure 2c), although this was much less than additive. This is consistent with action at the same receptor, given that the ATP curve never reached saturation.

Generation of [^{32}P]-PA and [^{32}P]-PBut

In order to determine whether BAME cells exhibit a phospholipase D activity that can be stimulated by nucleotide agonists, we took advantage of the transphosphatidylation reaction unique to phospholipase D. In this reaction a primary alcohol is used by phospholipase D as an acceptor of the phosphatidate moiety of a phospholipid and this process occurs preferentially to hydrolysis. We have previously established in BAE cells that 50 mM butanol, a 10 fold more potent acceptor than ethanol (Bonser *et al.*, 1989), was an optimal concentration for PBut production under resting and stimulated conditions (Purkiss & Boarder, 1992), and that this treatment results in the convertion of all the product of the phospholipase D reaction to PBut. Under these conditions the remaining labelled PA production is formed by the phospholipase C/DAG kinase pathway (Purkiss & Boarder, 1992).

In the absence of butanol, 5 min stimulation of $^{32}P_i$ labelled BAME cells gave rise to the accumulation of [^{32}P]-PA (Table 1). ATPγS was the most active of the compounds used. 2MeSATP was considerably less active than ATPγS and the PKC activator tetradecanoyl phorbol myristate acetate (TPA) at a concentration of 100 nM was not consistently active. In the presence of 50 mM butanol stimulation of [^{32}P]-PA production was enhanced by ATPγS. No stimulation was seen with TPA. Stimulation of [^{32}P]-PBut generation was observed under these conditions demonstrating the presence of a receptor linked phospholipase D. ATPγS was again more effective than 2MeSATP but TPA had a similar effect to ATPγS.

Generation of [^{3}H]-palmitate labelled [^{3}H]-PA and [^{3}H]-PBut in palmitate labelled cells

Phosphatidylcholine has frequently been described as the substrate for phospholipase D in endothelial (Martin & Michaelis, 1988) and other cell types (Cook & Wakelam, 1989; Billah *et al.*, 1989; Martinson *et al.*, 1990; Horowitz, 1991). A high content of palmitic acid has been found in membrane phosphatidylcholine (Lee *et al.*, 1991; Traynor *et al.*, 1982) and cells lablled with [^{3}H]-palmitate have been successfully used in the study of phospholipase D (Horowitz, 1991; Cook *et al.*, 1991). Therefore in order to characterize further the phospholipase D response in BAME cells we labelled them with [^{3}H]-palmitic acid for 48 h and constructed concentration-effect curves for PBut production after 5 min stimulation with ATPγS, UTP, ATP and 2MeSATP (Figure 3). The [^{3}H]-PBut responses to ATPγS and ATP reached a plateau between 100 μM and 300 μM. The UTP response appeared to reach a plateau between 3 μM and 10 μM. The P_{2Y} specific agonist, 2MeSATP, was again virtually inactive and the response to 300 μM 2MeSATP, in terms of fold/basal, for three experiments was 1.47 ± 0.04, compared to maximum response to ATPγS which was nearly twice as large at 2.78 ± 0.46. We found that at 30 μM when UTP, ATPγS and ATP all gave a maximal or close to maximal response; 2MeSATP gave no response at all. EC_{50} values for UTP, ATPγS and ATP were <0.3 μM, 6.19 ± 3.11 and 7.13 ± 2.33 μM respectively. A value could not be determined for 2MeSATP, though clear dose-dependent generation of [^{3}H]-PBut was seen. Stimulation of [^{3}H]-palmitate labelled BAME cells in the presence of 50 mM butanol did not give observable increases in [^{3}H]-PA.

Stimulation of [^{32}P]-PBut generation by TPA suggested a role for PKC in the activation of phospholipase D. In order to investigate this we used the relatively specific PKC inhibitor, Ro 31-8220 (Compound 3 in Davis *et al.*, 1989) at 10 μM, a concentration we have previously shown to inhibit totally purinoceptor agonist activation of phospholipase D in aortic endothelial cells (Purkiss & Boarder, 1992). Stimulation of [^{3}H]-PBut production by 30 μM ATPγS in [^{3}H]-palmitate labelled BAME cells was inhibited by pre-treatment with 10 μM Ro 31-8220 (Figure 4a) to a level below that of basal. For example, in one experiment, control (unstimulated) d.p.m. in [^{3}H]-PBut were 7417 ± 1272, 30 μM ATPγS caused

Table 1 Accumulation of [^{32}P]-phosphatidic acid ([^{32}P]-PA) and [^{32}P]-phosphatidylbutanol ([^{32}P]-PBut) in $^{32}P_i$ labelled BAME cells

	No butanol	*Butanol (50 mM)*	
	[^{32}P]-PA	*[^{32}P]-PA*	*[^{32}P]-PBut*
Control	2405 ± 341	2575 ± 473	1747 ± 256
ATPγS (30 μM)	7705 ± 1285*	8351 ± 776**	3448 ± 352*
2MeSATP (30 μM)	3938 ± 139*	3731 ± 107	2784 ± 97**
TPA (100 nM)	3463 ± 282	2520 ± 290	3350 ± 157**

Control and stimulated counts in PA and PA and PBut. Stimulation was for 5 min, butanol when present was added 10 min prior to and during the stimulation. The data are results of one experiment representative of 2, each value the mean of 3 determinations. For abbreviations, see text. *$P<0.05$, **$P<0.01$, compared to unstimulated controls.

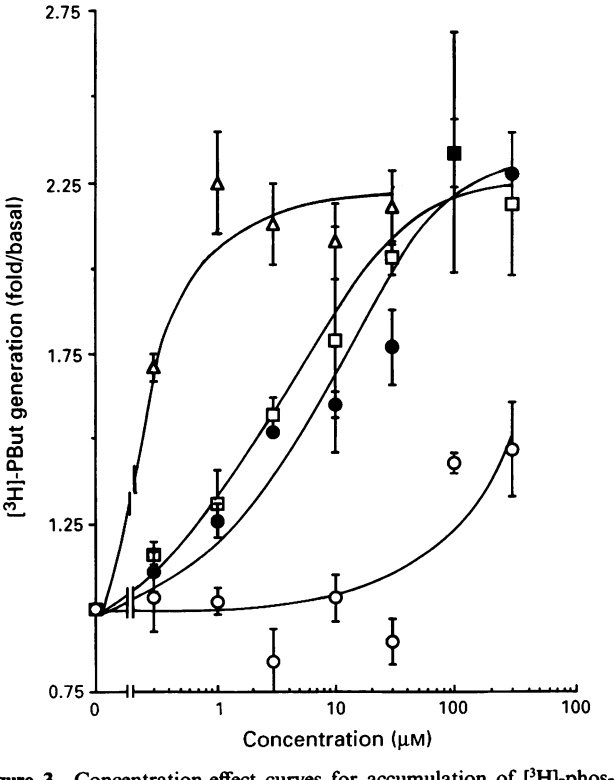


Figure 3 Concentration-effect curves for accumulation of [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) in [^{3}H]-palmitate labelled BAME cells stimulated in the presence of 50 mM butanol. The responses to increasing concentrations of UTP (△), ATPγS (□), ATP (●) and 2MeSATP (○) are expressed as the fold increases over basal. Each point is the mean of triplicate determinations in one representative experiment of three. Basal was 4658 ± 726 d.p.m.

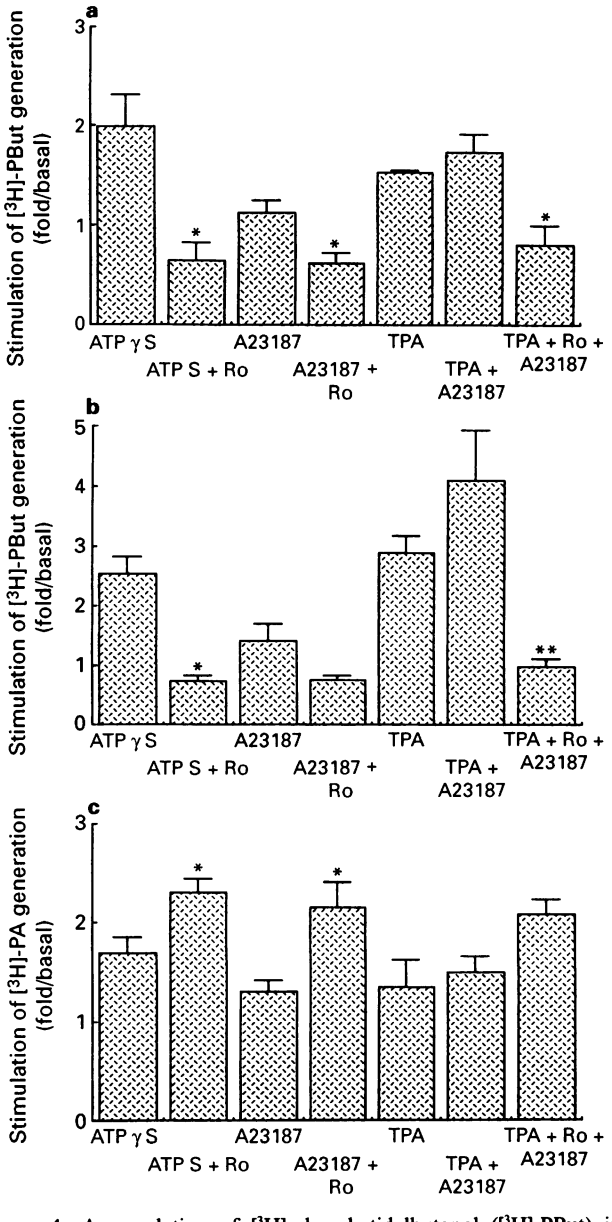


Figure 4 Accumulation of [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) in [^{3}H]-palmitate labelled BAME and BAE cells and of [^{3}H]-phosphatidic acid ([^{3}H]-PA) in BAE cells. Cells were stimulated with 30 μM ATPγS, 10 μM A23187, 100 nM TPA and TPA with A23187 in the absence or presence of 10 μM Ro 31-8220 (Ro). Butanol was present at 50 μM. (a) [^{3}H]-PBut production in BAME cells. (b) [^{3}H]-PBut production in BAE cells. (c) [^{3}H]-PA production in BAE cells. Stimulation of lipid products are expressed as the fold increases over basal. Data are pooled from 3 separate experiments each carried out in triplicate. Significance of the effect of 10 μM Ro 31-8220 on stimulation indicated by $*P<0.05$ or $**P<0.01$. For abbreviations, see text.

stimulation to 13,499 ± 349 and 10 μM Ro 31-8220 treatment prior to and during ATPγS stimulation reduced [^{3}H]-PBut d.p.m. to 2762 ± 314. Ro 31-8220 treatment alone was found to reduce basal d.p.m. in [^{3}H]-PBut by a similar degree (unpublished data). Elevation of intracellular free Ca^{2+} has been reported to activate potently phospholipase D (Reinhold *et al.*, 1990) but treatment with 10 μM of the calcium ionophore A23187 produced a modest stimulation which was significant in only one of the three experiments. The mean stimulation for all three experiments was 112 ± 13% of basal. Combination of PKC inhibitor and ionophore treatments reduced [^{3}H]-PBut production to the same below basal level as found when ATPγS was used in conjunction with Ro 31-8220. In agreement with the findings that $^{32}P_i$-labelled cells, 100 nM TPA stimulated the production of [^{3}H]-PBut to virtually the same extent as 30 μM ATPγS. Addition of 10 μM A23187 with 100 nM TPA marginally, but not significantly, enhanced this response, which was again reduced to below basal by concomitant PKC inhibition.

Figure 4b shows the results for the same range of treatments applied to [^{3}H]-palmitate labelled BAE cells. Again, 30 μM ATPγS stimulation of [^{3}H]-PBut production was reduced below basal by 10 μM Ro 31-8220, for example, in one experiment control (unstimulated) d.p.m. in [^{3}H]-PBut were 5685 ± 761, 30 μM ATPγS stimulated to 12,234 ± 843 and 10 μM Ro 31-8220 treatment reduced [^{3}H]-PBut d.p.m. to 3212 ± 187. A23187 10 μM produced a significant stimulation in only two of the three experiments and the mean stimulation for all three was 142 ± 28% of basal. This was inhibited to below basal by Ro 31-8220. TPA 100 nM stimulated [^{3}H]-PBut production to a level statistically indistinguishable from ATPγS and this stimulation was enhanced by A23187, but not to a significant degree. Ro 31-8220 (10 μM) treatment reduced stimulation by phorbol ester with ionophore back to basal, not below.

In contrast, to the lack of [^{3}H]-PA stimulation found in [^{3}H]-palmitate labelled BAME cells, BAE cells gave small but detectable responses (Figure 4c). [^{3}H]-PA production was stimulated by ATPγS significantly in two of the three experiments. In contrast to [^{3}H]-PBut production, this stimulation was significantly enhanced by 10 μM Ro 31-8220. In one typical experiment basal ^{3}H d.p.m. were 3731 ± 531, stimulation with 30 μM ATPγS gave 7504 ± 63 and the response was increased to 9592 ± 1117 by Ro 31-8220. Ionophore treatment marginally stimulated [^{3}H]-PA production but PKC inhibition again enhanced the response to a significant level in all three experiments. TPA (100 nM) alone or with A23187 (10 μM) stimulated [^{3}H]-PA production poorly but Ro 31-8220 again elevated the response though not significantly.

Discussion

The observation was made in this laboratory (Allsup & Boarder, 1990) that the production of inositol phosphates in BAME cells was stimulated by ATP and its analogues with a rank order of agonist potencies that was 'atypical' for widely accepted P_2-purinoceptors. Concurrently the notion of 'nucleotide' receptors was proposed and subsequently developed (Davidson *et al.*, 1990; O'Connor *et al.*, 1991; Brown *et al.*, 1991; Murrin & Boarder, 1992). The distinctive pharmacological property of the nucleotide receptor was sensitivity to both purines and pyrimidines. Some reports (e.g. Brown *et al.*, 1991; Murrin & Boarder, 1992) further characterize the nucleotide receptor as responding to UTP, ATP and ATPγS but not the P_{2Y}-specific agonist, 2MeSATP and P_{2X}-specific agonist, β,γ-methylene ATP. The sensitivity of BAME cells therefore corresponded to that of a nucleotide receptor, prompting investigation into whether these cells respond to the pyrimidine UTP. In this study we show that extracellular UTP did indeed stimulate inositol lipid hydrolysis in BAME cells and did so with a higher potency than ATP and ATPγS, indicating that this response may be mediated by a nucleotide receptor. It has however been proposed that in the HL-60 leukaemic cell, activation of NADPH oxidase by UTP and ATP is via two distinct receptors (Seifert *et al.*, 1989) so that responses to UTP and ATP by the same cell need not necessarily be mediated by a single type of receptor.

To investigate further whether UTP, ATP and ATPγS exerted their inositol phosphate mobilizing effects on BAME cells via the same or separate receptors, we constructed concentration-effect curves for these agonists in the presence of 0, 10, 30 and 100 μM of one of the other agonists. The presence of 10, 30 or 100 μM interacting agonist elevated basal inositol phosphate d.p.m. in a stepwise fashion in every

case. However, response curves to UTP and ATPγS were not then elevated by the same amount as the rise in basal. Indeed, there was no difference in the response to 100 μM UTP in the absence or presence of 100 μM ATP (2.99 ± 0.08 fold/basal vs 2.89 ± 0.50 fold over basal) or to 100 μM ATPγS in the absence or presence of 100 μM UTP (3.70 ± 0.92 fold/basal vs 3.62 ± 1.10 fold/basal). This lack of additivity of responses was therefore supportive of the proposal of a single nucleotide receptor. It could be argued that the effector enzyme, phosphoinositidase C, was at its maximum capacity to respond. However, increased inositol phosphate generation found in the non-saturating response to concentrations of nucleotide agonists higher than 100 μM showed that the maximum capacity for response had not been reached. This does not preclude the maximum activation of relevant G-proteins occurring. Our attempts to find another agonist capable of producing a major response, with which to test this in additivity experiments with nucleotide agonists was unsuccessful, with bradykinin and histamine giving small and unreliable responses. Having established that the effects of ATPγS and UTP (Figure 2a) and, UTP and ATP (Figure 2b) were not additive, the partial additivity seen between ATP and ATPγS (Figure 2c) was, on first sight, surprising. However, the ATP dose-response curve failed to plateau, i.e. it was submaximal at 100 μM. This would allow further receptor-mediated inositol phosphate response on addition of ATPγS, even when the two agonists act at the same receptor.

These studies on the generation of total [^{3}H]-inositol (poly)phosphates in the presence of lithium provide the two pieces of evidence required to designate the response as nucleotide receptor-mediated. Firstly, the agonist profiles show response to the pyrimidine UTP and the purines ATP and ATPγS, but not to 2MeSATP and β,γ-methylene ATP. Secondly, the construction of dose-response curves in the presence of an interacting agonist indicates the purine and pyrimidine responses are on the same receptor population.

Activation of phospholipase C has been clearly demonstrated for nucleotide receptors in BAME cells (this paper), PC12 cells (Murrin & Boarder, 1992), hepatocytes (Keppens *et al.*, 1992), airway epithelial cells (Brown *et al.*, 1991) and sheep anterior pituitary cells (Davidson *et al.*, 1990). Phospholipase D activation has been reported by ourselves (Purkiss & Boarder, 1992) and others (Martin & Michaelis, 1988; Pirotton *et al.*, 1990) to be a consequence of purinoceptor stimulation of large vessel endothelium. We have recently shown that these cells contain a mixture of both P_{2Y} and nucleotide receptors (Wilkinson & Boarder, 1992), and so the phospholipase D response may in part be a nucleotide receptor response. More specifically we have recently shown that PC12 cells contain nucleotide receptors linked to phospholipase D (Murrin & Boarder, 1992).

To investigate further the nature of the regulation by purines and pyrimidines acting together on these BAME cell nucleotide receptors, we have studied the relationship between phospholipase C (of which phosphoinositide C is an example) and phospholipase D. Phospholipase C will generate DAG as its immediate lipid product, which may then be converted to PA by DAG kinase. Phospholipase D generates PA directly, although this may be converted to DAG by phosphatidase phosphohydrolase. The two activities may be distinguished by the addition of butanol, when phospholipase D only will generate the transphosphatidylation product PBut. We have used two labelling procedures: labelling with $^{32}P_i$ will enable estimation of phospholipase D regardless of the phospholipid head group and acyl substitutions, while specific labelling of the palmitate group will probably preferentially label phosphatidylcholine. We show here that labelled PBut accumulates in response to both UTP and purine agonists. The concentration-effect curves in the palmitate loading experiments form plateau at higher concentrations, enabling the computation of EC_{50} values which confirm the rank order of agonist potency suggested by the [^{3}H]-inositol experiments. These results indicate that the nucleotide receptor on these cells is linked to phospholipase D as well as phospholipase C.

The PKC stimulating phorbol ester, TPA activated phospholipase D, giving a similar increase in [^{32}P]-PBut production to 30 μM ATPγS, but no [^{32}P]-PA generation. This indicated a role for PKC in the elevation of phospholipase D in these cells. Sensitivity of [^{3}H]-PBut production, stimulated by ATPγS or TPA, to the specific PKC inhibitor Ro 31-8220 confirmed that both agonist and phorbol ester exerted their phospholipase D stimulating effects via PKC. Maximal stimulation of PKC would be expected with 100 nM TPA, the similarity in size of response to agonist and phorbol ester (Figure 4) would therefore suggest that PLD activation by agonist was full and so explains the plateau in dose-response curves for [^{3}H]-PBut generation, though they did not occur when inositol phosphate accumulation was studied. Extracellular Ca^{2+} influx, as shown by experiments with A23187, appeared to play a minor role in this process (Figure 4a). Comparison of the activation of phospholipase D in BAME with BAE cells revealed an identical sensitivity to PKC inhibition. In contrast to BAME cells the BAE cells when labelled with [^{3}H]-palmitate gave, in the presence of 50 mM butanol, detectable [^{3}H]-PA stimulations, indicating either different lipid incorporation of palmitate or different lipid specificity by a phospholipase C. These PA stimulations were significantly enhanced by Ro 31-8220, indicating negative feedback control of phospholipase C by PKC.

The results in this paper indicate that microvascular endothelial cells from the bovine adrenal medulla respond to both purines and pyrimidines by action at a single nucleotide receptor (and not a P_{2Y}-receptor as previously reported by Forsberg *et al.*, 1987). This, and our related studies on BAE cells (Wilkinson & Boarder, 1992) provide the first demonstration of a phospholipase C-linked nucleotide receptor on vascular endothelial cells. The observation that such a receptor is found on endothelial cells of such diverse origin as aortic and adrenal microvasculature, suggests a widespread role for the nucleotide receptors in regulation of the vascular system. The nucleotide receptor is linked to phospholipase D as well as phospholipase C. The phospholipase D response is dependent on PKC and may therefore be downstream of phospholipase C by generation of the PKC activating lipid DAG. These findings further extend the significance of nucleotide receptors in the regulation of cellular functions.

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References

ALLSUP, D.J. & BOARDER, M.R. (1990). Comparison of P_2 purinergic receptors of aortic endothelial cells with those of adrenal medulla: evidence for heterogeneity of receptor subtype of inositol phosphate response. *Mol. Pharmacol.*, **38**, 84–91.

BANERJEE, D.K., ORNBERG, R.L., YOUDIN, M.B.H., HELDMAN, E. & POLLARD, H.B. (1985). Endothelial cells from bovine adrenal medulla develop capillary-like growth patterns in culture. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 4702–4706.

BILLAH, M.M., ECKEL, S., MULLMAN, T.J., EGAN, R.W. & SIEGEL, M.I. (1989). Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidic acid and diacylglyceride levels in chemotactic peptide-stimulated human neutrophils. *J. Biol. Chem.*, **264**, 17069–17077.

BONSER, R.W., THOMPSON, N.T., RANDALL, R.W. & GARLAND, L.G. (1989). Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem. J.*, **264**, 617–620.

BOOYSE, F.M., SEDLAK, B.J. & RAFELSON, M.E. (1975). Culture of arterial endothelial cells: characterisation and growth of bovine aortic cells. *Thromb. Diath. Haemorrh.*, **34**, 825–839.

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. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*. ed. Bohs, L. & Straub, R.W. pp. 107–118. New York: Raven Press.

BURNSTOCK, G. (1990). Overview: purinergic mechanisms. In *Biological Actions of Extracellular ATP*, ed. Dubyak, G.R. & Fedan, J.S. *Ann. N.Y. Acad. Sci.*, **603**, 1–17.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CARTER, T.D., HALLAM, T.J., CUSACK, N.J. & PEARSON, J.D. (1988). Regulation of P_2-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br. J. Pharmacol.*, **95**, 1181–1190.

COOK, S.J., BRISCOE, C.P. & WAKELAM, M.J.O. (1991). The regulation of phospholipase D activity and its role in sn-1,2-diacylglycerol formation in bombesin- and phorbol-12-myristate 13-acetate-stimulated Swiss 3T3 cells. *Biochem. J.*, **280**, 431–438.

COOK, S.J. & WAKELAM, M.J.O. (1989). Analysis of the water soluble products of phosphatidylcholine breakdown by ion exchange chromatography: bombesin and TPA stimulate choline generation in Swiss 3T3 cells by a common mechanism. *Biochem. J.*, **263**, 581–587.

DAVIDSON, F.M. & LONG, C. (1958). The structure of the naturally occurring phosphoglycerides 4. Action of cabbage-leaf phospholipase D on ovolecithin and related substances. *Biochem. J.*, **69**, 458–467.

DAVIDSON, J.S., WAKEFIELD, I.K., SOHNIUS, U., VAN DER MERWE, P.A. & MILLAR, R.P. (1990). A novel extracellular nucleotide receptor coupled to phosphoinositide C in pituitary cells. *Endocrinology*, **126**, 80–87.

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*, **259**, 61–63.

DE MEY, J.G. & VANHOUTTE, P.M. (1981). Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. *J. Physiol.*, **316**, 347–355.

FINE, J., COLE, P. & DAVIDSON, J.S. (1989). Extracellular nucleotides stimulate receptor mediated calcium mobilization and inositol phosphate production in human fibroblasts. *Biochem. J.*, **263**, 371–376.

FORSBERG, E.J., FEURESTEIN, G., SHOHAMI, E. & POLLARD, H.B. (1987). Adenosine triphosphate stimulates inositol phospholipid metabolism and prostacyclin formation in adrenal medullary endothelial cells by means of P_2-purinergic receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 5630–5634.

GERRITSEN, M.E. (1987). Functional heterogeneity of vascular endothelial cells. *Biochem. Pharmacol.*, **36**, 2701–1711.

HOROWITZ, J. (1991). Bradykinin activates a phospholipase D that hydrolyses phosphatidylcholine in PC12 cells. *J. Neurochem.*, **56**, 509–517.

HOUSTON, D.A., BURNSTOCK, G. & VANHOUTTE, P.M. (1987). Different P_2-purinergic receptor subtypes of endothelium and smooth muscle in canine blood vessels. *J. Pharmacol. Exp. Ther.*, **241**, 501–509.

KEPPENS, S., VANDEHERCKHOVE, A. & DE WOLF, H. (1992). Extracellular ATP and UTP exert similar effects on rat isolated hepatocytes. *Br. J. Pharmacol.*, **105**, 475–479.

KOBAYASHI, M. & KANFER, J.N. (1987). Phosphatidylethanol formation via transphosphatidylation by rat brain synatosomal phospholipase D. *J. Neurochem.*, **48**, 1597–1603.

LEE, C., FISHER, S.K., AGRANOFF, B.W. & HAJRA, A.K. (1991). Qualitative analysis of molecular species of diacylglycerol and phosphatidate formed upon muscarinic receptor activation of human SK-N-SH neuroblastoma cells. *J. Biol. Chem.*, **266**, 22837–22846.

MAJNO, G. (1965). Ultrastructure of the vascular membrane. In *The Handbook of Physiology* (Section 2, Circulation, Vol III), ed. W.F. Hamilton & P. Dow pp. 2293–2375. Washington D.C.: Am. Physiological Society.

MARTIN, T.W. & MICHAELIS, K.C. (1988). Bradykinin stimulates phosphodiesteratic cleavage of phosphatidylcholine in cultured endothelial cells. *Biochem. Biophys. Res. Commun.*, **157**, 1271–1279.

MARTIN, T.W. & MICHAELIS, K.C. (1989). P_2-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells. *J. Biol. Chem.*, **264**, 8847–8856.

MARTINSON, E.A., TRILIVAS, I. & BROWN, J.H. (1990). Rapid protein kinase C-dependent activation of phospholipase D leads to delayed 1,2-diglyceride accumulation. *J. Biol. Chem.*, **265**, 22282–22287.

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.

NEEDHAM, L., CUSACK, N.J., PEARSON, J.D. & GORDON, J.L. (1987). Characteristics of the P_2-purinoceptor that mediates prosacyclin production by pig aorta endothelial cells. *Eur. J. Pharmacol.*, **134**, 199–209.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

PEARSON, J.D., SLAKEY, L.L. & GORDON, J.L. (1983). Stimulation of prostaglandin production through purinoceptors on cultured porcine endothelial cells. *Biochem. J.*, **214**, 273–276.

PIROTTON, S., RABAYE, B., LAGNEAU, C. & BOEYNAEMS, J.-M. (1990). Adenine nucleotides modulate phosphatidylcholine metabolism in aortic endothelial cells. *J. Cell Physiol.*, **142**, 449–457.

PLEVIN, R. & BOARDER, M.R. (1988). Stimulation of formation of inositol phosphates in primary cultures of bovine adrenal chromaffin cells by angiotensin II, histamine, bradykinin and carbachol. *J. Neurochem.*, **51**, 634–641.

PURKISS, J.R. '& BOARDER, M.R. (1992). Stimulation of phosphatidate synthesis in endothelial cells in response to P_2-receptor activation: evidence for phospholipase C and phospholipase D involvement, phosphatidate and diacylglycerol interconversion and the role of protein kinase C. *Biochem. J.*, **287**, 31–36.

PURKISS, J.R., WILKINSON, G.F., WEST, D.C. & BOARDER, M.R. (1992). Human brain capillary endothelial cells respond differently to those of the bovine aorta to P_2-purinergic stimulation. *Br. J. Pharmacol.*, **103**, 413P.

REINHOLD, S.L., PRESCOTT, S.M., ZIMMERMAN, G.A. & MCINTYRE, T.M. (1990). Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB J.*, **4**, 208–214.

SEIFERT, R., BURDE, R. & SCHULTZ, G. (1989). Activation of NADPH oxidase by purine and pyrimidine nucleotides involves G-proteins and is potentiated by chemotactic peptides. *Biochem. J.*, **259**, 813–819.

TRAYNOR, A.E., SCHUBERT, D. & ALLEN, W.R. (1982). Alterations of lipid metabolism in response to nerve growth factor. *J. Neurochem.*, **39**, 1677–1683.

WILKINSON, G.F. & BOARDER, M.R. (1992). Evidence for two distinct receptors for ATP on bovine aortic endothelial cells, both linked to phospholipase C activation. *Br. J. Pharmacol.*, **107**, 107P.

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Inhibition of nerve stimulation-induced vasodilatation in corpora cavernosa of the pithed rat by blockade of nitric oxide synthase

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1 The effect of inhibition of nitric oxide synthase by N^G-nitro-L-arginine methyl ester (L-NAME) on nerve stimulation-induced vasodilatation in corpora cavernosa was studied in the pithed rat. Corporal vasodilatation was estimated by the increase in ratio (corpora cavernosal pressure/systemic blood pressure; CP/BP) following electrical stimulation of the sacral part of the spinal cord.

2 L-NAME (2, 5, 10 and 25 mg kg^{-1}) caused an increase in BP and a dose-dependent inhibition of the rise in the CP/BP ratio following stimulation.

3 The inhibitory effect of L-NAME (25 mg kg^{-1}) on the corporal response to spinal cord stimulation, as well as the pressor response, was partially prevented by prior administration of L- but not D-arginine (400 mg kg^{-1}, i.v.).

4 L-NAME (20 mg kg^{-1}, i.v.) did not inhibit the rise in corporal pressure resulting from direct intracavernosal administration of papaverine (400 μg over 2 min). However, this response was inhibited by 5-hydroxytryptamine (20 μg kg^{-1}, i.v.).

5 The results are indicative of a role of nitric oxide (NO) in the corporal vasodilator response to erectile stimulation.

Keywords: Penile erection; nitric oxide; papaverine; 5-hydroxytryptamine; corpora cavernosa; N^G-nitro-L-arginine; pithed rat

Introduction

Penile erection is initiated by neuronal impulses in parasympathetic pelvic nerves which cause arteriolar vasodilatation and relaxation of smooth muscle elements in the corpora cavernosa, the erectile tissue of the penis. Inflow of blood in the corporal lacunae, together with relaxation of the smooth muscle surrounding the lacunae, and venous outflow obstruction (Lue & Tanagho, 1987; Aoki *et al.*, 1989), leads to turgor of the erectile tissue. Although the corpora are extensively innervated with cholinergic nerves (Dail & Hamill, 1989), acetylcholine is not thought to play a major role as a vasodilator neurotransmitter, because atropine-like drugs (which effectively antagonize other vasodilator effects of acetylcholine), have little effect on the erectile response (Brindley, 1986; Stief *et al.*, 1989). Peptide neurotransmitters, especially vasoactive intestinal peptide (VIP; Willis *et al.*, 1981) have also been proposed to play a role in the arteriolar vasodilatation but there is marked tachyphylaxis to the vasodilator effect of VIP and the peptide causes a relatively poor vasodilatation even after direct administration to the corpora (Roy *et al.*, 1990).

Nitric oxide (NO) is a vasodilator and smooth-muscle relaxant substance which is produced in a number of vascular and non-vascular tissues by the enzyme nitric oxide synthase (see Moncada *et al.*, 1991, for review). It has been proposed that release of NO mediates the smooth muscle relaxant effect of non-adrenergic, non-cholinergic (NANC) nerve stimulation in tissues such as human corpora cavernosa (Ignarro *et al.*, 1990; Kim *et al.*, 1991; Pickard *et al.*, 1991), rat anococcygeus muscle, bovine penile artery and bovine retractor penis (Liu *et al.*, 1991). Although evidence exists for a role of NO in mediating the NANC relaxation of isolated muscle strips of corporal tissue, it is still not clear whether NO is responsible for the vasodilator phase of penile erection.

We have studied the effect of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; Moore *et al.*, 1990) on erectile stimulation in our *in vivo* model of erectile function in the pithed rat (Vardi *et al.*, 1989). In this preparation, electrical stimulation of the sacral part of the spinal cord causes a rise in corporal pressure, which is the result of active vasodilatation in the corporal arterioles. The response is blocked by chlorisondamine but not by atropine. In this preparation, our preliminary data showed that L-NAME antagonized the corporal response to spinal stimulation (Finberg & Vardi, 1991). Holmquist *et al.* (1991) reported that intra-corporal L-NAME antagonized the penile erection and increase in corporal pressure caused by direct stimulation of the cavernous nerve in the anaesthetized rabbit. Recently, Burnett *et al.* (1992) reported inhibition of penile erectile response to direct stimulation of the cavernous nerve by L-NAME in the anaesthetized rat, as well as describing the location of NO synthase in penile tissue. Since L-NAME causes powerful peripheral vasoconstriction, we have examined the possibility that inhibition of erectile stimulation by L-NAME results from generalized vasoconstriction, as opposed to a specific antagonism of neuronally released NO.

Methods

Pithed rat preparation

Rats (Sprague-Dawley derived, 250–280 g body weight) were pithed under halothane anaesthesia, respired with oxygen-enriched room air at a rate of 1 ml kg^{-1}, 60 strokes min^{-1}, and prepared for preganglionic stimulation of the spinal outflow to the pelvic nerves as described previously (Vardi *et al.*, 1989). Rectal temperature was maintained at 37°C with a small electric pad underneath the animal, and (+)-tubocurarine (1 mg kg^{-1}) was administered i.v. to prevent contraction of skeletal muscle during stimulation. The

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stimulating electrode (diameter 1.5 mm) was positioned in the sacral part of the spinal cord, and its optimal location determined by monitoring corporal response to test stimuli. The indifferent electrode was inserted underneath the dorsal skin. Corporal pressure was measured from a 26 gauge needle inserted into one corpus cavernosum, after removing the penile skin. Drugs were administered by intravenous injection via the jugular vein, and systemic arterial pressure was recorded from a carotid artery. Pressures were measured with Statham P23dB transducers, and monitored on a Graphtec direct-writing recorder. Since the hydrostatic pressure measured in the corpora (CP) during a vasodilator response is influenced by the level of the systemic arterial blood pressure (BP), corporal pressure was expressed as a function of mean blood pressure (CP/BP ratio).

Experimental protocols

A 45 min equilibration period was allowed after pithing, and then spinal stimulation was applied for 60 s periods at 2, 5, 10 and 20 Hz, with square-wave uniphasic pulses (1 ms) at maximal voltage. Four min rest periods were allowed between individual stimuli. Following establishment of at least two control responses to stimulation, L-NAME was administered at one of the indicated dose levels. Fifteen min later, application of spinal stimulation was recommenced, and frequency-response curves were repeated, with intervals of 15 min between each set of stimuli. When L- or D-arginine was administered, the amino acid was given slowly by intravenous injection in two divided doses, with 2 min between each dose, 10 min before the second set of control electrical stimuli. Either L-NAME or an equivalent volume of diluent was administered i.v., and determination of frequency-response curves recommenced 15 min later.

In another series of experiments, vasodilator responses to papaverine were obtained by infusing this drug into one corpus cavernosum (20 μl min^{-1} of a 10 mg ml^{-1} solution for 2 min) and measuring pressure from the contralateral corpus. Control CP values were measured 2 min after the papaverine infusion, and again 60 min later. L-NAME or physiological saline was then administered as above, and a second intracorporal papaverine bolus was infused 30 min later. Corporal pressure was again recorded 2 min after the papaverine bolus. Spinal stimulation was carried out before the first papaverine bolus, and again after administration of L-NAME or saline. In an additional series of experiments, the effect of 5-HT was examined on papaverine-induced corporal vasodilatation. In these experiments, 5-HT was injected i.v. 5 min after the papaverine bolus; at this time, in control experiments, CP was not significantly different from the value measured 2 min after the bolus.

Values of CP/BP ratio following administration of L-NAME or arginine are presented as a percentage of control values recorded at the start of the experiment, or, in the papaverine experiments, as a ratio. Corporal pressures used for calculation of CP/BP ratio during electrical stimulation were the average values determined between 30 and 60 s after the onset of electrical stimulation.

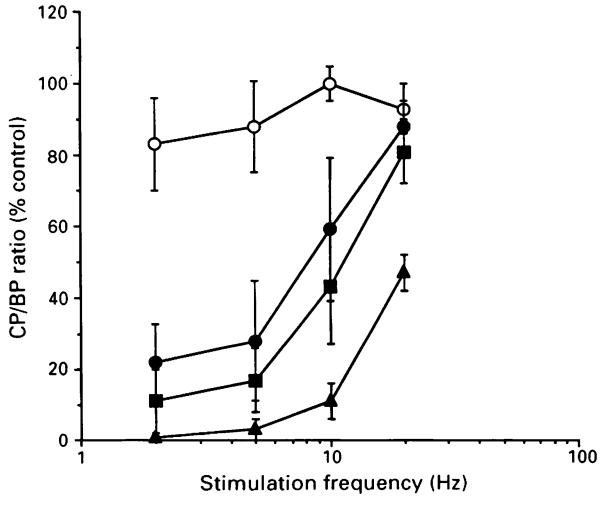


Figure 1 Inhibition of spinal stimulation-induced increase in corporal pressure following i.v. injection of N^G-nitro-L-arginine methyl ester (L-NAME) in the pithed rat. Corporal pressure (CP) during stimulation at frequencies shown was divided by simultaneous mean arterial pressure (BP) and expressed as percentage of control response measured before drug injection. Doses of L-NAME administered were 2 (○), 5 (●), 10 (■) and 25 (▲) mg kg^{-1}, i.v. Responses are shown as mean ± s.e.mean for $n = 4$ to 5 animals in each treatment group.

Statistical method

Significance of difference between group means was estimated by Student's t test. A probability of less than 0.05 was considered significant.

Drugs

N^G-nitro-L-arginine methyl ester (L-NAME), L-arginine, D-arginine and 5-HT creatinine sulphate were obtained from Sigma (St Louis, MO, U.S.A.). L-NAME, L-arginine and D-arginine were dissolved in saline. 5-HT was dissolved in 0.01 M HCl, and diluted in saline immediately before use. Papaverine HCl was obtained from Teva Inc. (Petah Tikva, Israel). All other reagents were Analar grade.

Results

Intravenous injection of L-NAME caused an increase in BP, together with a suppression of the CP response to spinal stimulation (i.e. a decrease in CP/BP ratio, see Figures 1 and 2). The increase in BP reached maximum after about 5 to 10 min, while maximal suppression of CP/BP response to stimulation was evident about 30 min after L-NAME injection. Corporal responses were suppressed for the duration of the experiment (up to 3 h), and did not show any indication of a return over this time period. Responses to the lower frequency stimulation used (2, 5 and 10 Hz) were suppressed to a greater degree than those to the highest frequency (20 Hz). Previous work with this model has shown that there is no significant decline of responses to spinal stimulation over a 1 to 2 h period in control animals (Finberg & Vardi, 1990). Prior injection of L-arginine (400 mg kg^{-1}), which itself had no significant effect on corporal pressure response to spinal stimulation, partially prevented the suppression of the corporal response and the increase in BP caused by L-NAME (Figures 3 and 4). The same dose of D-arginine, however, had no effect on the L-NAME response (see Figure 3).

In order to determine whether inhibition of the corporal response to stimulation was a result of vasoconstriction in the arterial vessels supplying the corpora cavernosa, another vasodilator, papaverine, was infused into the corpus cavernosum of the rat during treatment with L-NAME. Intracorporal infusion of a pulse of papaverine (20 μl min^{-1} of a 10 mg ml^{-1} solution for 2 min) produced an increase in corporal pressure with no change in arterial pressure. The corporal pressure remained at a stable level for about 15 min after stopping papaverine infusion, before dropping gradually towards control levels over a period of about 20 to 40 min. Following administration of L-NAME (20 mg kg^{-1}, i.v.), intra-corporal infusion of papaverine now produced a greater rise in corporal pressure as a result of the increase in BP, but the ratio CP/BP was equivalent before and after administration of L-NAME (Table 1). In addition, there was no significant difference in the mean duration of papaverine-

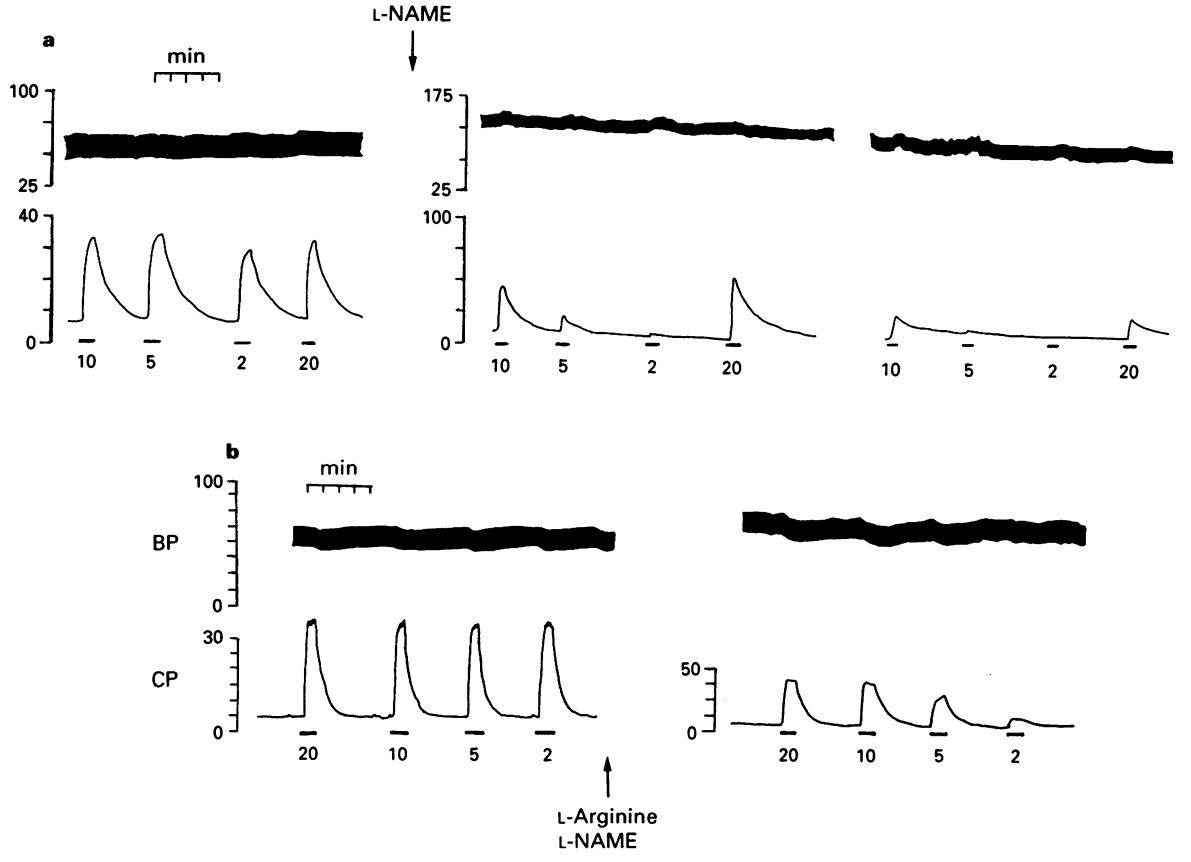


Figure 2 Experimental records of arterial blood pressure (BP) and corporal pressure (CP) in two separate pithed rat experiments. At horizontal bars, electrical stimulation was applied to sacral spinal cord at frequency shown in Hz. Pressure scales are given as mmHg. (a) Right hand portion of record 15 and 30 min after administration of N^G-nitro-L-arginine methyl ester (L-NAME; 25 mg kg^{-1}, i.v.). (b) After left hand part of record, L-arginine (400 mg kg^{-1}, i.v.) was administered, followed, 10 min later, by L-NAME (25 mg kg^{-1}, i.v.). Right hand portion of record was made 15 min after L-NAME.

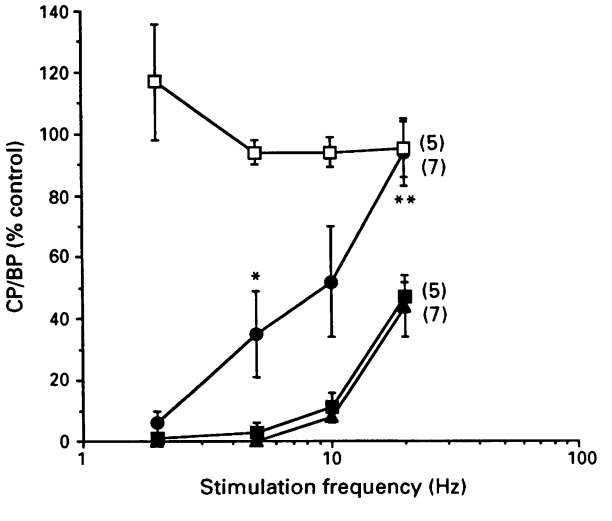


Figure 3 Prevention of N^G-nitro-L-arginine methyl ester (L-NAME)-induced suppression of corporal response to spinal stimulation in the pithed rat by L-arginine. Graph shows inhibition of stimulation-induced increase in CP/BP ratio by L-NAME (25 mg kg^{-1}) alone (■), after prior injection of L-arginine (400 mg kg^{-1}, i.v.; ●) or after prior injection of D-arginine (400 mg kg^{-1}, i.v.; ▲). Also shown is effect of L-arginine alone on corporal response to spinal stimulation (□). Responses are shown as mean ± s.e.mean for numbers of animals shown in parentheses. $*P<0.05$, $**P<0.01$ for significance of difference between L-arginine + L-NAME and L-NAME alone. Numbers of animals per group shown in parentheses.

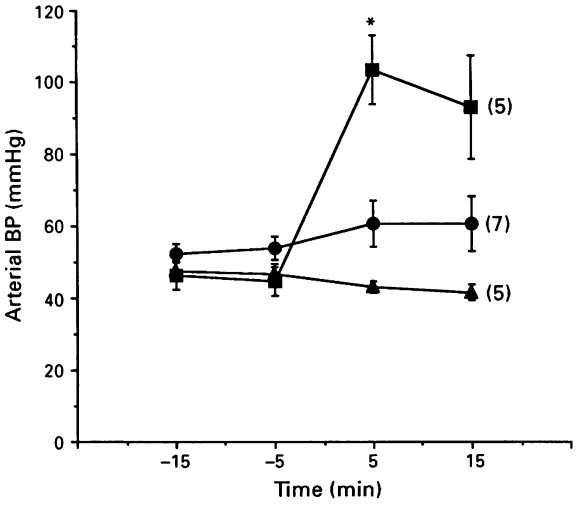


Figure 4 Mean arterial blood pressure (BP) response to N^G-nitro-L-arginine methyl ester (L-NAME) in pithed rat. Mean BP was measured at indicated times before and after injection of either L-NAME 25 mg kg^{-1} (■—■), L-NAME 25 mg kg^{-1} after prior injection of L-arginine (400 mg kg^{-1} at − 30 min; ●—●), or saline after prior injection of L-arginine (▲—▲). $*P<0.05$ for significance of difference between L-NAME alone and L-NAME + L-arginine. Responses are shown as mean ± s.e.mean for numbers of animals shown in parentheses.

induced increase in CP following L-NAME (18 ± 3.5 min in control to 24.8 ± 4.4 min following L-NAME; mean ± s.e.mean for 6 experiments). Prior administration of papaverine did not alter the corporal response to spinal stimulation, given after return of CP to control levels, and prior administration of papaverine did not affect the inhibitory action of L-NAME on the increase in CP/BP induced by nerve stimulation (Table 1). The papaverine pulse produced a sub-maximal degree of vasodilatation, since an increased duration of the intra-corporal infusion of papaverine produced a greater increase in the ratio CP/BP.

In order to compare the effect of another vasoconstrictor substance with that of L-NAME, 5-HT was injected intravenously in pithed rats which had just received an intracorporal infusion of papaverine. In these conditions, 5-HT (20 $\mu g\ kg^{-1}$, i.v.) caused a marked suppression of the CP/BP ratio (Figure 5).

Table 1 Corporal pressure responses to spinal stimulation and intra-corporal papaverine following systemic administration of N^G-nitro-L-arginine methyl ester (L-NAME) in the pithed rat

	Stimulation frequency (Hz)				*Papaverine*
	2	*5*	*10*	*20*	(400 μg)
(1) *Control series* ($n = 5$)					
Control	0.57	0.73	0.72	0.73	0.64
s.e.mean	0.09	0.06	0.06	0.06	0.07
Saline	0.49	0.71	0.76	0.82	0.57
s.e.mean	0.13	0.04	0.04	0.04	0.01
(2) *L-NAME series* ($n = 6$)					
Control	0.40	0.53	0.56	0.59	0.55
s.e.mean	0.07	0.06	0.05	0.04	0.07
L-NAME	0.00*	0.10*	0.22*	0.30*	0.61
s.e.mean	0.00	0.09	0.09	0.08	0.08

CP/BP (cavernosal:arterial blood pressure) ratios are shown during spinal stimulation at 2, 5, 10 and 20 Hz, and 2 min after start of the infusion of papaverine intra-corporally (200 $\mu g\ min^{-1}$ for 2 min). An initial set of stimuli was followed by papaverine infusion. Either saline or L-NAME (20 $mg\ kg^{-1}$) was then administered i.v., and a second set of stimuli carried out 30 min later. The second papaverine infusion was administered following completion of the second set of stimuli.

*$P < 0.01$ for difference between first and second set of stimuli (t test).

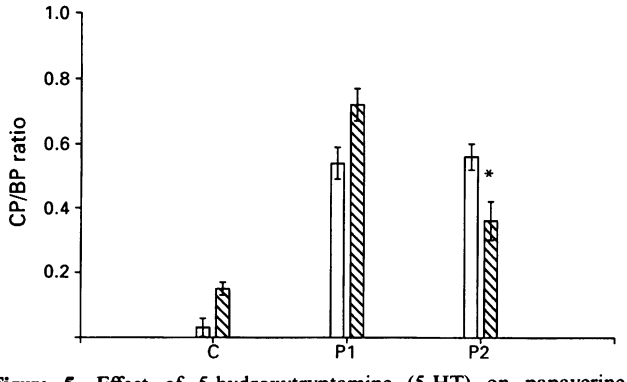


Figure 5 Effect of 5-hydroxytryptamine (5-HT) on papaverine-induced elevation in CP/BP ratio. Values of CP/BP ratio shown in control period (C), 2 min after intracorporal papaverine (200 $\mu g\ min^{-1}$ for 2 min; P1), and 5 min after 5-HT (20 $\mu g\ kg^{-1}$, i.v.) or normal saline injected immediately after completion of papaverine infusion (P2). Control animals (open columns); 5-HT (hatched columns). * indicates significant difference ($P < 0.05$) between P1 and P2; $n = 6$ animals per treatment group.

Discussion

The results of these experiments are consistent with the hypothesis that stimulation of the pelvic nerve induces the release of NO, which causes corporal vasodilatation. The effect of L-NAME in inhibiting neuronally-mediated corporal vasodilatation is presumably the result of inhibition of NO production. The irreversible nature of the L-NAME-induced blockade of corporal vasodilatation is compatible with the characteristics of blockade of NO-synthase observed by other workers (Mulsch & Busse, 1990). The inhibition of CP/BP response to pelvic nerve stimulation by L-NAME could be prevented by pretreatment with a large dose of L-arginine. In preliminary experiments, we found that the 400 $mg\ kg^{-1}$ dose of L-arginine was required in order to prevent effectively the inhibitory effect of L-NAME. The stereo-selective reversal of the effect of L-NAME by L-arginine is strongly indicative of a selective action of L-NAME inhibiting NO generation. Reversal of the inhibitory action of L-NAME on corporal vasodilator response to erectile stimulation by L-arginine but not by D-arginine was also demonstrated by Burnett *et al.* (1992) but not by Holmquist *et al.* (1991). In the latter study, L-NAME was administered intra-corporally, and the ability to demonstrate reversal by L-arginine could have resulted from insufficient concentration of L-arginine, or unfavourable timing of the L-arginine administration.

The inhibitory effect of L-NAME on cavernosal vasodilatation could be the result of inhibition of NO released as a NANC neurotransmitter from pelvic nerve endings, or a result of a direct vasoconstrictor action of L-NAME in the cavernosal arterioles. Administration of L-NAME caused extensive systemic vasoconstriction, as shown by the large pressor response. The pressor effect of L-NAME has been well described in conscious rats (Gardiner *et al.*, 1990); however, it is interesting that even in the pithed rat preparation, which is devoid of neural tone, we still find a marked pressor response. This effect is evidence of the degree of basal NO release. Such a profound vasoconstrictor effect of L-NAME in cavernosal vessels, therefore, would antagonize the effect of any vasodilator substance released from the parasympathetic nerves. In an attempt to distinguish between such a physiological antagonism at the post-synaptic level, and blockade of neuronally-released NO, we also examined the ability of L-NAME to antagonize the effect of a direct vasodilator, papaverine. It is interesting that L-NAME did not antagonize the papaverine-induced vasodilatation, while 5-HT did. The lack of antagonism of papaverine-induced vasodilatation by L-NAME provides evidence that the inhibition of stimulation-induced vasodilatation by L-NAME did not result from generalized vasoconstriction at a site central to the origin of the corporal blood vessels.

This finding adds credence to the hypothesis that production of NO is important for neuronally induced corporal vasodilatation. Further evidence that NO plays a neurotransmitter role in corporal vasodilatation is provided by the demonstration by Burnett *et al.* (1992) that nitric oxide synthase is localized to neuronal cells in the corpora cavernosa. It is, however, impossible to rule out completely the possibility that physiological antagonism between vasoconstrictor and vasodilator influences plays a role in the inhibitory effect of L-NAME seen in the present study. The NO generated by pelvic nerve stimulation could be released from the nerve endings themselves, and, in addition, could be released from the corporal endothelial cells in response to a neuromodulator released from the nerves. Localization of nitric oxide synthase to endothelial layer of cavernosal vessels was also demonstrated by Burnett *et al.* (1992). The identity of such a neuromediator is at present unknown, but it is unlikely to be acetylcholine, since atropine is largely ineffective in blocking penile erection in this (Vardi *et al.*, 1989) and other models of penile erection (Brindley, 1986; Stief *et al.*, 1989).

It is interesting to compare the effect of L-NAME with

that of 5-HT. The latter has previously been shown by us to inhibit the effect of spinal stimulation on CP/BP elevation in the pithed rat preparation (Finberg & Vardi, 1990). Again, this inhibitory effect of 5-HT could be the result of a direct post-synaptic vasoconstriction of the cavernosal arterioles, or pre-synaptic inhibition of released NANC neurotransmitter. The fact that 5-HT antagonized papaverine-induced vasodilatation, is evidence in favour of the former possibility.

The pithed rat model used in these experiments is devoid of CNS mediated peripheral nervous tone. It is interesting, therefore, that corporal arterioles are maintained in a state of vasoconstriction. This vasoconstriction may be the result of local production of vasoactive substances such as endothelin in the endothelial cells of the corporal vasculature. 5-HT apparently does not play a role in this tonic vasoconstriction, since 5-HT antagonists did not produce an increase in the CP/BP ratio (Finberg & Vardi, 1990).

The relatively greater effectiveness of L-NAME in antagonizing low frequency stimulation may indicate the presence of an additional vasodilator neurotransmitter, which is released to a greater extent at the higher frequency. Alternatively, since we used a fixed duration of stimulation, the apparent resistance of 20 Hz stimulation to L-NAME may simply be the result of release of a greater amount of neurotransmitter, by a greater number of pulses. Candidate substances for alternative neurotransmitters have been mentioned above. Cholinergic and peptidergic (VIP) nerves have been localized in the corpora by immunohistochemical techniques (Dail & Hamill, 1989; Willis *et al.*, 1981), but the role of these neurotransmitters in control of trabecular, as opposed to vascular, responsiveness in the corpora is at present unknown.

Our findings are therefore indicative of a physiological role for NO as a NANC neurotransmitter in erectile function.

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References

AOKI, H., TAKAGANE, H., MATSUZAKA, J., BANYA, Y., FUJIOKA, T., NAKAYA, S., KUBO, T. & OHORI, T. (1989). Quantitative analysis of outflow pathway of the corpora cavernosa by pressure flow technique. *J. Urol.*, **141**, 645–650.

BRINDLEY, G.S. (1986). Pilot experiments on the actions of drugs injected into the human corpus cavernosum penis. *Br. J. Pharmacol.*, **87**, 495–500.

BURNETT, A.L., LOWENSTEIN, C.J., BREDT, D.S., CHANG, T.S.K. & SNYDER, S.H. (1992). Nitric oxide: a physiologic mediator of penile erection. *Science*, **257**, 401–403.

DAIL, W.G. & HAMILL, R.W. (1989). Parasympathetic nerves in penile erectile tissue of the rat contain choline acetyltransferase. *Brain Res.*, **487**, 165–170.

FINBERG, J.P.M. & VARDI, Y. (1990). Inhibitory effect of 5-hydroxytryptamine on penile erectile function in the rat. *Br. J. Pharmacol.*, **101**, 698–702.

FINBERG, J.P.M. & VARDI, Y. (1991). Evidence for a role of EDRF in penile erection. *Br. J. Pharmacol.*, **102**, 82P.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990). Regional and cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats. *Br. J. Pharmacol.*, **101**, 625–631.

HOLMQUIST, F., STIEF, C.G., JONAS, U. & ANDERSSON, K.-E. (1991). Effects of the nitric oxide synthase inhibitor N^G-nitro-L-arginine on the erectile response to cavernous nerve stimulation in the rabbit. *Acta Physiol. Scand.*, **143**, 299–304.

IGNARRO, L.J., BUSH, P.J., BUGA, G.M., WOOD, K.S., FUKUTO, J.M. & RAJFER, J. (1990). NO and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Commun.*, **170**, 843–850.

KIM, N., AZADZOI, K.M., GOLDSTEIN, I. & DE TEJADA, I.S. (1991). A nitric oxide-like factor mediates nonadrenergic-noncholingeric neurogenic relaxation of penile corpus cavernosum smooth muscle. *J. Clin. Invest.*, **88**, 112–118.

LIU, X., GILLESPIE, J.S., GIBSON, I.F. & MARTIN, W. (1991). Effects of N^G-substituted analogues of L-arginine on NANC relaxation of the rat anococcygeus and bovine retractor penis muscles and the bovine penile artery. *Br. J. Pharmacol.*, **104**, 53–58.

LUE, T.F. & TANAGHO, E.A. (1987). Physiology of erection and pharmacological management of impotence. *J. Urol.*, **137**, 829–836.

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

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-N^G-nitro arginine (L-NAME), a novel, L-arginine-derived reversible inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

MULSCH, A. & BUSSE, R. (1990). N^G-nitro-L-arginine (N^5-[imino-(nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 143–147.

PICKARD, R.S., POWELL, P.H. & ZAR, M.A. (1991). The effect of inhibitors of nitric oxide biosynthesis and cyclic GMP formation on nerve-evoked relaxation of human cavernosal smooth muscle. *Br. J. Pharmacol.*, **104**, 755–759.

ROY, J.B., PETRONE, R.L. & SAID, S.I. (1990). A clinical trial of intracavernous vasoactive intestinal peptide to induce penile erection. *J. Urol.*, **143**, 302–304.

STIEF, C., BENARD, F., BOSCH, R., ABOSIEF, S., NUNES, L., LUE, T.F. & TANAGHO, E.A. (1989). Acetylcholine as a possible neurotransmitter in penile erection. *J. Urol.*, **141**, 1444–1448.

VARDI, Y., YOUDIM, M.B.H., LEVIN, R.D. & FINBERG, J.P.M. (1989). A model for the study of penile erection in the rat. *Int. J. Impotence Res.*, **1**, 11–17.

WILLIS, E., OTTESEN, B., WAGNER, F., SUNDLER, F. & FAHRENKRUG, J. (1981). Vasoactive intestinal polypeptide (VIP) as a possible neurotransmitter involved in penile erection. *Acta Physiol. Scand.*, **113**, 545–547.

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Digoxin activates sarcoplasmic reticulum Ca^{2+}-release channels: a possible role in cardiac inotropy

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1 The effect of digoxin on rapid $^{45}Ca^{2+}$ efflux from cardiac and skeletal sarcoplasmic reticulum (SR) vesicles was investigated. Additionally the interaction of digoxin with single cardiac and skeletal muscle SR Ca^{2+}-release channels incorporated into planar phospholipid bilayers and held under voltage clamp was determined.

2 Digoxin (1 nM) increased the initial rate and amount of Ca^{2+}-induced release of $^{45}Ca^{2+}$ from cardiac SR vesicles, passively loaded with $^{45}CaCl_2$, at an extravesicular [Ca^{2+}] of 0.1 μM. The efflux in the presence and absence of digoxin was inhibited at pM extravesicular Ca^{2+} and blocked by 5 mM Mg^{2+}.

3 To elucidate the mechanism of action of digoxin, single-channel recording was used. Digoxin (1–20 nM) increased single-channel open probability (P_o) when added to the cytosolic but not the luminal face of the cardiac channel in the presence of sub-maximally activating Ca^{2+} (0.1 μM–10 μM) with an EC_{50} of 0.91 nM at 10 μM Ca^{2+}. The mechanisms underlying the action of digoxin appear to be concentration-dependent. The activation observed at 1 nM digoxin appears to be consistent with the sensitization of the channel to the effects of Ca^{2+}. At higher concentrations the drug appears to interact synergistically with Ca^{2+} to produce values of P_o considerably greater than those seen with Ca^{2+} as the sole activating ligand.

4 Digoxin had no effect on single-channel conductance or the Ca^{2+}/Tris permeability ratio. In channels activated by digoxin the P_o was decreased by Mg^{2+}. Single-channels were characteristically modified to a long lasting open, but reduced, conductance state when 100 nM ryanodine was added to the cytosolic side of the channel.

5 Activation of the cardiac SR Ca^{2+}-release channel was observed with similar concentrations of digitoxin, however, higher concentrations of ouabain were required to increase P_o. In contrast, a steroid which is not positively inotropic, chlormadinone acetate, had no effect on either cardiac or skeletal SR Ca^{2+}-release channel activity.

6 At concentrations up to 1 μM, digoxin had no effect on Ca^{2+}-induced $^{45}Ca^{2+}$ efflux from skeletal muscle SR vesicles nor did it affect skeletal SR Ca^{2+}-release channel P_o, reflecting a difference between the cardiac and skeletal isoforms of the Ca^{2+}-release channel.

7 Since activation of the cardiac SR Ca^{2+}-release channel occurs within the range of concentrations of digoxin encountered therapeutically, it is possible that activation of this channel contributes to the positive inotropic effect observed with this drug. Further, activation of the channel by higher concentrations of digoxin may contribute to the toxic effects seen clinically.

Keywords: Sarcoplasmic reticulum; cardiac Ca^{2+}-release channel; digoxin; cardiac glycosides

Introduction

Cardiac glycosides increase the force of contraction of cardiac muscle and are used clinically in the treatment of heart failure, yet how these drugs achieve their positive inotropic effect remains controversial. Digoxin and other cardiotonic steroids cause an increase in intracellular free calcium when applied to cardiac muscle (Wier & Hess, 1984; Allen *et al.*, 1984; 1985). The mechanism of this increase is thought to be due primarily to Na^+/K^+ ATPase inhibition by the glycoside, which raises intracellular sodium thereby modulating Na^+/Ca^{2+} exchange and increasing the uptake and subsequent release of calcium by the sarcoplasmic reticulum (SR) (Eisner & Lederer, 1979; Wier & Hess, 1984; Morgan, 1985; Bers & Bridge, 1988; Eisner, 1990). An enhancement of the transient inward current by glycosides has also been reported and may contribute to the increase in intracellular calcium (Weingart *et al.*, 1978; Marban & Tsien, 1982). However, in some experiments glycosides stimulate, rather than inhibit, the Na^+/K^+ ATPase at positively inotropic concentrations (Cohen *et al.*, 1976; Godfraind & Ghysel-Burton, 1977; Ghysel-Burton & Godfraind, 1979); in others, compounds which inhibit the Na^+/K^+ ATPase do not cause an increased contraction even though they displace [^{3}H]-ouabain from binding sites (LaBella *et al.*, 1979). Further, it has been reported that strophanthidin may cause an increase in developed tension prior to an increase in intracellular sodium (Boyett *et al.*, 1986), suggesting that inhibition of the Na^+/K^+ ATPase is not the initial mechanism of inducing positive inotropy. Given these, at times, contradictory reports of the mechanisms of action of cardiotonic steroids, there may be an additional action of these drugs to raise intracellular calcium and increase tension in cardiac muscle (Hart *et al.*, 1983). Since glycosides are able to cross the sarcolemma (Dutta *et al.*, 1968; Fricke & Klaus, 1978), and it has been shown that a range of positively inotropic agents directly activate the cardiac SR Ca^{2+}-release channel (Williams, 1992), the possibility exists that glycosides induce positive inotropy by acting at this intracellular site. In view of this possibility we have investigated the effects of therapeutic and toxic concentrations of cardiac glycosides on the release of calcium from SR vesicles and on the open probability of the SR Ca^{2+}-release channel.

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Methods

Isolation of SR membrane vesicles

The methods for the isolation of SR membrane vesicles were as previously described (Sitsapesan & Williams, 1990). Briefly, sheep hearts or skeletal muscles were obtained from a local abattoir and transported to the laboratory in cold modified cardioplegic solution (Tomlins *et al.*, 1986), which contained (mM): NaCl 102, KCl 21.4, $MgCl_2$ 16, sodium lactate 28.7, $CaCl_2$ 2.4 and procaine 1.0. Tissue was stripped of fat and minced prior to homogenization in a solution containing phenylmethyl-sulphonide chloride (PMSF) 1 mM, sucrose 300 mM and piperazine-N'N'-*bis*-2-ethanesulphonic acid (PIPES) 20 mM, pH 7.4. The homogenate was centrifuged at 8500 g_{av} in a Sorvall GSA rotor for 20 min and the pellet discarded. The supernatant was then centrifuged at 100000 g_{av} for 40 min in a Sorvall A641 or T647.5 rotor. The mixed membrane population sedimented by this step was resuspended in a solution containing mM: KCl 400, $MgCl_2$ 0.5, $CaCl_2$ 0.5, 1,2 di(2-aminoethyl)ethane-N,N,N',N'-tetra-acetic acid (EGTA) 0.5 and PIPES 25, pH 7.0 plus 10% sucrose w/v and fractionated on discontinuous sucrose-density gradients. The membrane suspension was layered over identical salt solutions containing 20, 30 and 40% sucrose w/v and sedimented at 100000 g_{av} for 120 min in a Sorvall AH629 rotor. Heavy sarcoplasmic reticulum (HSR) membrane vesicles collecting at the 30–40% interface were diluted in KCl 400 mM and pelleted by centrifugation at 100000 g_{av} for 40 min in a Sorvall T647.5 rotor before resuspension in a solution containing sucrose 400 mM and N'-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 5 mM titrated to pH 7.4 with tris(hydroxymethyl)-methylamine (Tris). Membrane vesicles were snap frozen in liquid nitrogen and stored at − 80°C.

Rapid $^{45}Ca^{2+}$ efflux from SR vesicles

The kinetics of $^{45}Ca^{2+}$ efflux from SR vesicles passively loaded with $^{45}Ca^{2+}$ were investigated by use of the Bio-Logic rapid filtration apparatus (Intracel, Cambridge, U.K.), which allows resolution of ionic fluxes in the millisecond time range, utilizing a modification of the methods of DuPont (DuPont, 1984; Moutin & DuPont, 1988). HSR vesicles were thawed, resuspended in KCl 100 mM and HEPES 20 mM, pH 7.4 and pelleted by centrifugation in a Sorvall T647.5 rotor at 100000 g_{av} to remove residual sucrose prior to resuspension and incubation at a protein concentration of 4 mg ml^{-1} in an uptake solution containing KCl 100 mM, HEPES 20 mM, $CaCl_2$ 4.95 mM and $^{45}CaCl_2$ 50 μM for 120 min at 22°C. $^{45}Ca^{2+}$-loaded vesicles were used within 1 h following loading, during which time uptake remained constant. Under the conditions described, uptake of 25–35 nmol of $^{45}Ca^{2+}$ into the vesicles mg^{-1} protein for cardiac and 45–55 nmol of $^{45}Ca^{2+}$ mg^{-1} protein for skeletal vesicles was consistently achieved, with 50% of maximal loading occurring after approximately 1 min. Maximum uptake occurred after 1 h and remained constant thereafter. This loading is higher than has been achieved by passive loading of canine cardiac SR vesicles under similar conditions; however, the loading for skeletal vesicles is similar to that obtained by others (Meissner & Henderson, 1987). Aliquots of vesicles containing 200 μg protein were placed on 0.45 μm pore diameter Millipore filters, pre-soaked in non-release buffer, which contained KCl 100 mM, HEPES 20 mM, EGTA 1 mM, $MgCl_2$ 5 mM and $CaCl_2$ 5 mM. Vesicles and filter were washed with 5 ml of non-release buffer under vacuum to remove extravesicular $^{45}Ca^{2+}$. The filter was then placed in the efflux apparatus and washed for controlled times (10–1000 ms) with a solution consisting of KCl 100 mM, HEPES 20 mM, EGTA 1 mM and $CaCl_2$ 695 μM (free $[Ca^{2+}] = 0.1$ μM). Digoxin was added to the injection syringe from a stock solution of 100 nM. After the efflux was completed, filters were removed and dissolved in 10 ml Packard scintillant and the remaining radioactivity on the filters counted in a liquid scintillation counter. Initial efflux rates were calculated from the derivative of the single exponential decay curve fitted to each set of data.

Planar lipid bilayer methods

Experiments were performed essentially as previously described by Sitsapesan & Williams (1990). Bilayers, composed of phosphatidylethanolamine dispersed in decane (30 mg ml^{-1}) were painted across a 200 μm diameter hole in the partition between two fluid-filled styrene co-polymer chambers designated *cis* and *trans*. The *trans* chamber was held at ground and the *cis* chamber clamped at holding potentials relative to this. Current fluctuations were monitored with an operational amplifier as a current-voltage converter (Miller, 1982). Initially, both chambers contained choline chloride 50 mM, HEPES 10 mM and $CaCl_2$ 5 mM, with the pH adjusted to 7.4 with Tris. HSR vesicles were added to the *cis* chamber and the choline chloride concentration increased to give a 7:1 gradient *cis-trans* to promote vesicle fusion with the bilayer. Fusion was marked by the appearance of Cl^--selective channels (Smith *et al.*, 1985). SR vesicles incorporated into the planar bilayer in a fixed orientation such that the cytosolic face of the Ca^{2+}-release channel was directed towards the *cis* chamber and the luminal face to the *trans* chamber. Following vesicle fusion, the *cis* and *trans* chambers were perfused with solutions allowing resolution of Ca^{2+}-release channel currents. The *cis* chamber was perfused with HEPES 250 mM, Tris 125 mM, pH 7.4; and the *trans* with glutamic acid 250 mM, HEPES 10 mM with the pH adjusted to 7.4 with $Ca(OH)_2$, giving a $[Ca^{2+}]$ on the *trans* side of 67 mM. The concentration of Ca^{2+} in the *cis* chamber was buffered to the desired level by the addition of $CaCl_2$ and EGTA and calculated using EQCAL (BioSoft, Cambridge). Experiments were performed at room temperature (22 ± 1°C).

Data acquisition and analysis

Channel data were displayed on an oscilloscope and recorded on video tape. Current recordings were replayed, low pass filtered at 1 kHz using an eight pole Bessel filter and digitized at 2 kHz with either an AT-based system (Intracel, Cambridge) or a PDP 11/73 based system (Indec, Sunnyvale, U.S.A.). Channel open probability (P_o) and the lifetimes of open and closed events were monitored by 50% threshold analysis at a holding potential of 0 mV from 3 min of continuous recordings. P_o was determined by the equation $P_o = T_{open}/(T_{open} + T_{closed})$, where T_{open} and T_{closed} are the mean open and closed lifetime durations. Lifetimes, accumulated from 3 min steady-state recordings were stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to a probability density function by the method of maximum likelihood (Colquhoun & Sigworth, 1983). Lifetimes lasting less than 1 ms were not fully resolved under these conditions of data acquisition and were therefore excluded from the fitting procedure. A missed events correction was applied (Colquhoun & Sigworth, 1983) and a likelihood ratio test was used to compare fits to double and triple exponentials (Blatz & Magleby, 1986).

Single-channel conductance was obtained from the slope of linear regression lines drawn through single-channel current amplitude data monitored at holding potentials within the range (+40 to −40 mV). Reversal potentials (E_{rev}) were obtained from extrapolations of these lines. The $Ca^{2+}/Tris^+$ permeability ratio was calculated from the equation given by Fatt & Ginsborg (1958):

$$pX^{2+}/pY^+ = [Y^+]/4[X^{2+}].\exp(E_{rev}F/RT).[\exp(E_{rev}F/RT) + 1]$$

Drugs used

Drugs used in the study were: digoxin, digitoxin, ouabain octahydrate, chlormadinone acetate and spironolactone (Sigma). Stock solutions of digoxin, digitoxin, chlormadinone and spironolactone were prepared in ethanol. The final concentration of ethanol did not exceed 0.03% in either the rapid efflux experiments or the single-channel experiments. In both cases, this concentration of ethanol was without effect. Ouabain was prepared in distilled water. Phosphatidylethanolamine was from Avanti Polar Lipids, Birmingham, U.S.A.

Results

Enhancement of Ca^{2+}-induced $^{45}Ca^{2+}$ efflux from cardiac SR vesicles by digoxin

Isolated SR vesicles are of a fixed orientation with the outside of the vesicle equivalent to the cytosolic face of the membrane. Efflux from cardiac (and skeletal) SR vesicles was dependent upon the extravesicular Ca^{2+} concentration. There was no measurable efflux of $^{45}Ca^{2+}$ at pM Ca^{2+} in the presence or absence of digoxin. However, the initial rate and total amount of $^{45}Ca^{2+}$ release increased as extravesicular [Ca^{2+}] was raised. In all cases efflux could be described by a single exponential. Release was inhibited by 5 mM Mg^{2+} (data not shown). In the presence of digoxin, the Ca^{2+}-induced release of $^{45}Ca^{2+}$ was enhanced (Figure 1). At 0.1 μM Ca^{2+} the initial rate of efflux increased from 144 ± 20 to 258 ± 24 nmol $^{45}Ca^{2+}$ mg^{-1} protein s^{-1} (± s.e.mean, $n = 5$) when 1 nM digoxin was present. The digoxin-enhanced efflux was also blocked by 5 mM Mg^{2+} (not shown).

Lack of effect of digoxin on Ca^{2+}-induced $^{45}Ca^{2+}$ efflux from skeletal SR vesicles

Digoxin failed to increase the initial rate or total amount of $^{45}Ca^{2+}$ released from skeletal SR vesicles in the same conditions. Initial efflux rates at an extravesicular [Ca^{2+}] of 0.1 μM were 502 ± 32 nmol $^{45}Ca^{2+}$ mg^{-1} protein s^{-1} (control) and 453 ± 59 nmol $^{45}Ca^{2+}$ mg^{-1} protein s^{-1} in the presence of 1 nM digoxin (± s.e.mean, $n = 4$).

Whilst these experiments establish that therapeutic concentrations of digoxin increase the rapid efflux of $^{45}Ca^{2+}$ from cardiac, but not skeletal SR vesicles this method does not elucidate the exact route and mechanism of this enhanced $^{45}Ca^{2+}$ efflux in the presence of digoxin.

Single-channel recording

To study the mechanism of action of digoxin, HSR vesicles from at least six different preparations were incorporated into planar phospholipid bilayers and Ca^{2+}-release channel conduction and gating properties studied under voltage clamp conditions.

Cardiac SR Ca^{2+}-release channels

Single Ca^{2+}-release channels were activated by adding Ca^{2+} 0.1 μM–10 μM to the cytosolic side of the channel (Figure 2). Digoxin (1 nM) further increased single-channel P_o when added to the cytosolic, but not the luminal face of the channel. At an activating [Ca^{2+}] of 0.1 μM, 1 nM digoxin increased P_o from 0.012 ± 0.003 to 0.110 ± 0.029 (± s.e.mean, $n = 8$). At 10 μM Ca^{2+}, 1 nM digoxin increased P_o from 0.048 ± 0.012 to 0.281 ± 0.065 ($n = 4$). As digoxin caused an increase in P_o when added from only one side of the bilayer, it is likely that it is acting at a site on the cytosolic face of the Ca^{2+}-release channel rather than indirectly affecting the channel by partitioning into the lipid bilayer.

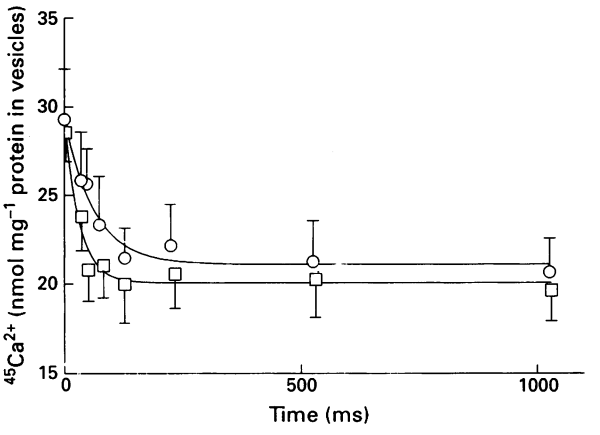


Figure 1 Rapid Ca^{2+}-induced $^{45}Ca^{2+}$ efflux at 0.1 μM Ca^{2+} (○) and at 0.1 μM Ca^{2+} + 1 nM digoxin (□). Digoxin increased both the initial rate of efflux and total amount of $^{45}Ca^{2+}$ released at an extravesicular [Ca^{2+}] of 0.1 μM. Each set of efflux data was best fitted by a single exponential curve, using non-linear regression and initial efflux rates were calculated from the differential of the single exponential decay equation. Fits to single and double exponentials were compared by an F test. Each point is the mean of five experiments conducted in duplicate and results are displayed as means ± s.e.mean.

Lifetime analysis of the channel open and closed states in the presence and absence of digoxin (Figure 3) indicated that the increase in P_o caused by 1 nM digoxin at 0.1 μM and 10 μM activating Ca^{2+} appeared to be similar to that observed as the concentration of activating cytosolic Ca^{2+} is increased. That is, P_o was increased as a result of increased frequency of channel opening with no significant increase in channel open lifetimes.

The interpretation that 1 nM digoxin increased P_o by increasing the frequency of channel opening and hence sensitized the channel protein to Ca^{2+}, is supported by the observation that digoxin failed to activate the channel at sub-activating (pM) Ca^{2+}. Similarly at a [Ca^{2+}] of 100 μM, digoxin induced no further increase in P_o. At 100 μM Ca^{2+}, P_o was 0.160 ± 0.043 in the absence and 0.143 ± 0.038 (± s.e.mean, $n = 3$) in the presence of 1 nM digoxin. In addition Mg^{2+}, which is thought to compete with Ca^{2+} for the activation site on the Ca^{2+}-release channel (Ashley & Williams, 1990), reduced P_o following activation by digoxin (not shown).

Digoxin had no effect on single-channel conductance (86 ± 3 pS, control vs. 88 ± 2 pS + 1 nM digoxin, ± s.e.mean, $n = 5$) or the $Ca^{2+}/Tris^{+}$ permeability ratio (12.7 ± 0.3, control vs. 13.0 ± 0.3 + digoxin, $n = 5$). Channels activated by Ca^{2+} alone or by Ca^{2+} and digoxin together were characteristically modified to a long lasting open, but reduced conductance state when 100 nM ryanodine was added to the cytosolic side of the channel (Figure 4).

Table 1 summarizes the effects of increasing cytosolic Ca^{2+} and digoxin on single SR Ca^{2+}-release channel P_o and mean open and closed lifetimes.

Higher concentrations of digoxin (10 nM and 20 nM) increased P_o at 10 μM Ca^{2+} to 0.480 ± 0.049 (± s.e.mean, $n = 5$) and 0.531 ± 0.032 ($n = 4$) from control values. Given these results, an approximate apparent EC_{50} of 0.91 nM is obtained for digoxin from this data. At these higher concentrations of digoxin, there was an increase in channel open lifetime duration as well as an increase in the frequency of opening (not shown). Single-channel open and closed lifetimes could be best fitted by triple exponential curves, indicating that digoxin interacts with at least one of the open states as well as at least one of the closed states of the Ca^{2+}-release channel. However, the interaction with the open state, resulting in an increase in the channel open lifetime

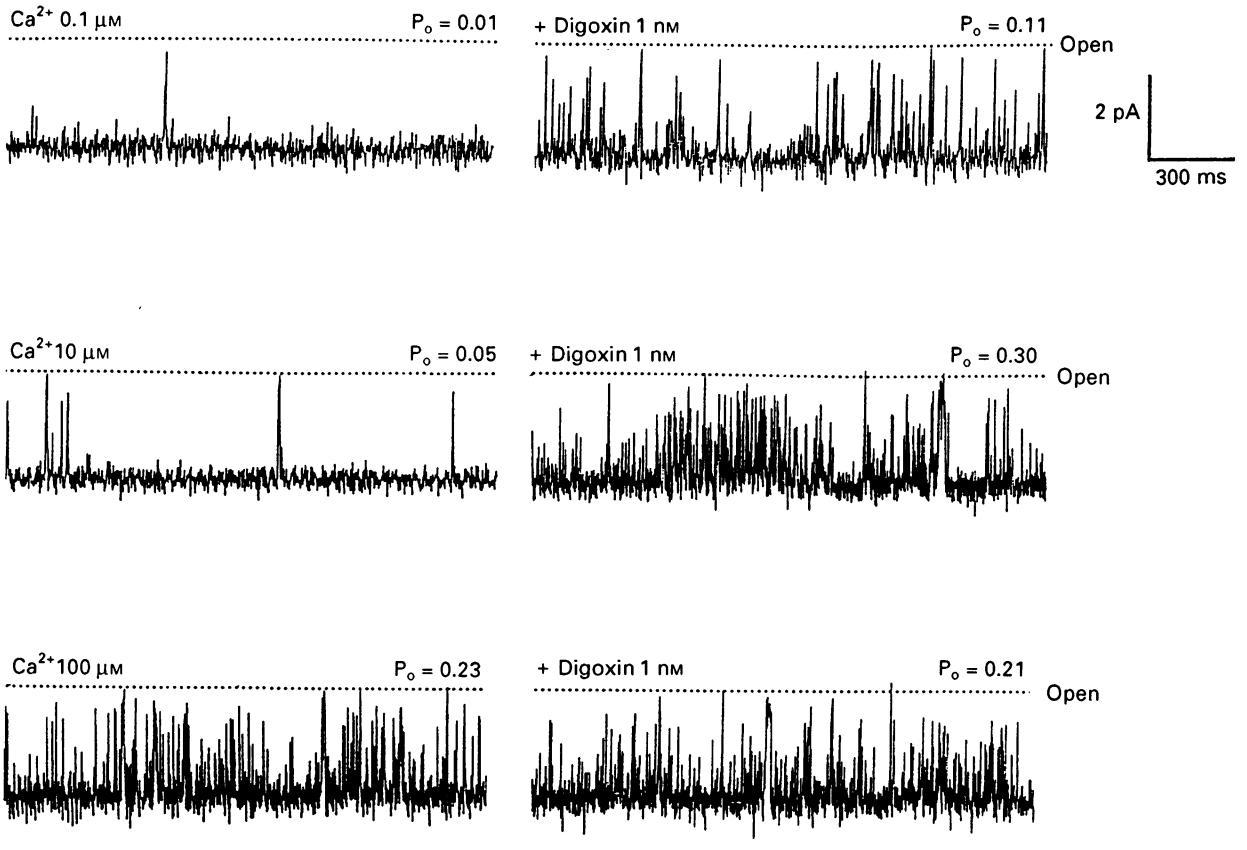


Figure 2 Representative portions of data from single SR Ca^{2+}-release channels held at 0 mV. Channels were first activated by Ca^{2+} and then 1 nM digoxin was added to the *cis* chamber. Single-channel P_o, determined from 3 min of continuous recordings, increased as cytosolic Ca^{2+} was raised. In the presence of 1 nM digoxin, P_o increased further at 0.1 μM and 10 μM Ca^{2+}, but not at 100 μM Ca^{2+}. Digoxin had no effect on single-channel conductance. Single-channel P_o values were calculated as described in the Methods section and are indicated above each respective portion of recording. Dotted lines indicate channel open level.

duration, only occurs at these higher concentrations of digoxin and therefore may be with a lower affinity site.

Activation of the cardiac SR Ca^{2+}-release channel was observed with similar concentrations of digitoxin (data not shown). However, ouabain was without effect at 1 nM. When the concentration of ouabain at the cytosolic side of the channel was increased to 10 nM, P_o increased from 0.040 ± 0.011 to 0.232 ± 0.061 at 10 μM Ca^{2+} ($\pm$ s.e.mean, $n = 3$) (Figure 5). Lifetime analysis of the channel open and closed states indicate that 10 nM ouabain activates the channel by the same mechanism as 1 nM digoxin (Figure 6) that is, it increases P_o mainly by a reduction in channel closed lifetimes. Activation by digoxin, digitoxin and ouabain was readily reversible on washout of the drug from the *cis* chamber.

Chlormadinone acetate, a progestin without inotropic activity, but which displaces [^{3}H]-ouabain from binding sites (LaBella *et al.*, 1979), had no effect on single channel P_o or conductance at up to 100 μM (not shown). It also did not affect the digoxin activation of the channel. Similarly spironolactone, which inhibits the Na^+/K^+ ATPase and also reduces digoxin binding to the ATPase (Musgrave *et al.*, 1977), had no effect on P_o or single-channel conductance. Like chlormadinone, it did not affect digoxin activation of the cardiac SR Ca^{2+}-release channel at up to 100 μM. These observations suggest that neither chlormadinone nor spironolactone bind to the cardiac SR Ca^{2+}-release channel.

Skeletal SR Ca^{2+}-release channels

Consistent with our findings that digoxin failed to enhance the rate or magnitude of Ca^{2+}-induced $^{45}Ca^{2+}$-release from sheep skeletal muscle SR vesicles, we observed no equivalent effect of digoxin on skeletal muscle SR Ca^{2+}-release channels: P_o at 0.1 μM $Ca^{2+} = 0.009 \pm 0.002$; P_o at 0.1 μM Ca^{2+} + 1 nM digoxin $= 0.011 \pm 0.004$ ($\pm$ s.e.mean, $n = 8$). There was no increase in P_o at concentrations up to 1 μM digoxin. Chlormadinone and spironolactone were also without effect on skeletal channels.

Discussion

Our data demonstrate a correlation between the effect of digoxin on rapid Ca^{2+}-induced $^{45}Ca^{2+}$ efflux from cardiac SR vesicles and its effect on single SR Ca^{2+}-release channel activity. Since other cardiac glycosides also activate the cardiac SR Ca^{2+}-release channel in the nM range, we propose that there is a high affinity binding site on this channel protein for cardiotonic steroids.

The mechanisms involved in the activation of the cardiac SR Ca^{2+}-release channel by digoxin are qualitatively similar to those seen with the positive inotropic phosphodiesterase inhibitors, caffeine (Sitsapesan & Williams, 1990) and sulmazole (Williams & Holmberg, 1990). In all cases low concentrations of the compounds (i.e. 1 nM digoxin) appear to increase channel P_o by sensitizing the channel to Ca^{2+} and hence increase the frequency of channel opening. This proposal is supported by the finding that 1 nM digoxin is without effect in the absence of activating Ca^{2+} and that following a digoxin-induced increase, P_o is reduced by the addition of Mg^{2+}. The observation that 1 nM digoxin, when added to channels activated by 10 μM Ca^{2+}, is capable of producing values of P_o in excess of those seen with 100 μM Ca^{2+} or 100 μM Ca^{2+} plus 1 nM digoxin may appear somewhat paradoxical. However, it is consistent with the relation-

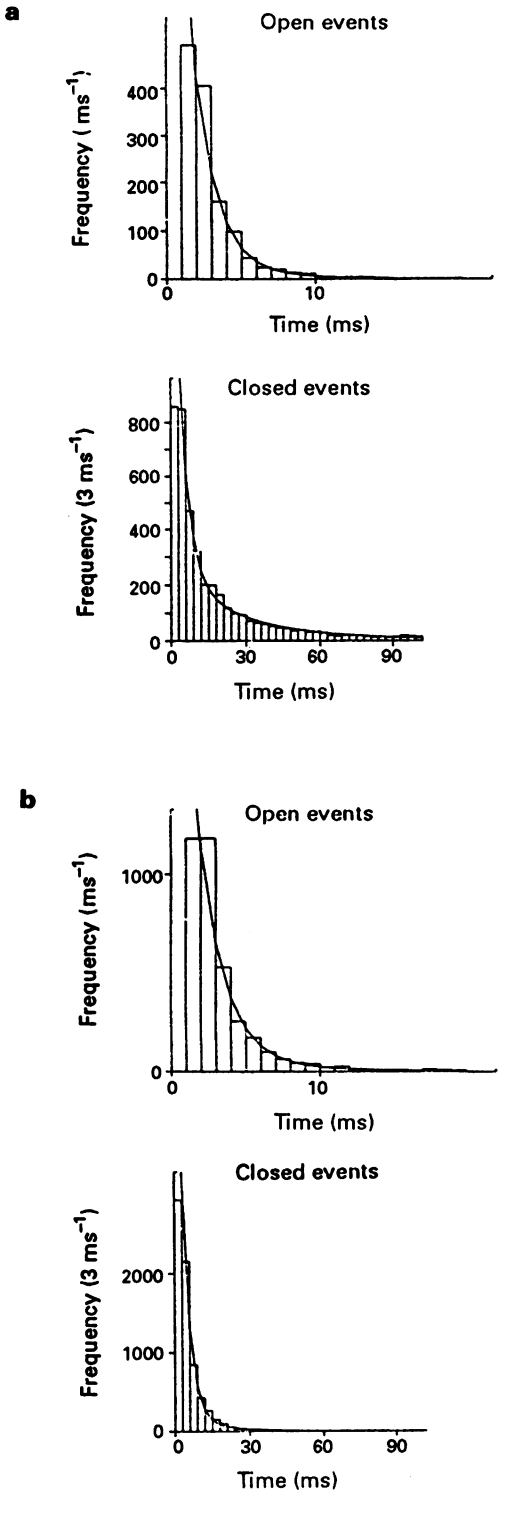


Figure 3 Open and closed lifetime histograms, with probability density functions obtained by maximum likelihood fitting to individual lifetimes for a single sheep cardiac SR Ca^{2+}-release channel activated by (a) Ca^{2+} 10 μM and (b) Ca^{2+} 10 μM + digoxin 1 nM on the cytosolic side of the channel. Lifetimes, obtained from continuous 3 min recordings, are displayed in non-cumulative histograms. Probability density functions, obtained by the method of maximum likelihood are drawn according to: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots\ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$. For open lifetimes most likely fits were obtained to double exponentials with (i) Ca^{2+} 10 μM: $a_1 = 93.37$, $a_2 = 6.63$, $\tau_1 = 1.32$, $\tau_2 = 12.51$ and (ii) Ca^{2+} 10 μM + digoxin 1 nM: $a_1 = 94.14$, $a_2 = 5.86$, $\tau_1 = 1.75$, $\tau_2 = 12.51$. For closed lifetimes, most likely fits were obtained to triple exponentials with (i) Ca^{2+} 10 μM: $a_1 = 21.91$, $a_2 = 56.67$, $a_3 = 21.42$, $\tau_1 = 4.67$, $\tau_2 = 28.09$, $\tau_3 = 162.21$ and (ii) Ca^{2+} 10 μM + digoxin 1 nM: $a_1 = 87.68$, $a_2 = 9.19$, $a_3 = 3.12$, $\tau_1 = 3.16$, $\tau_2 = 11.67$, $\tau_3 = 34.70$. Open and closed lifetime distributions are taken from the experiment shown in Figure 2.

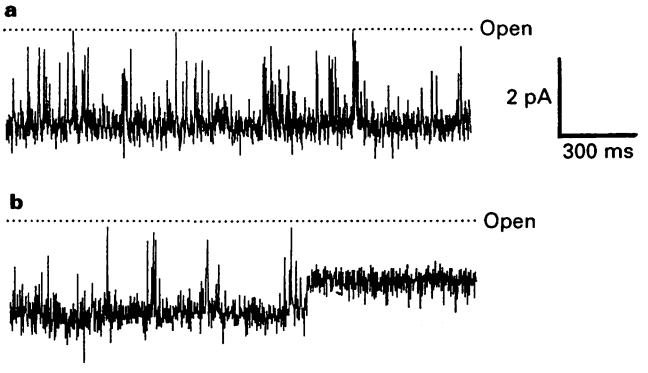


Figure 4 A single cardiac SR Ca^{2+}-release channel activated by digoxin (a) at Ca^{2+} 10 μM modified to a characteristic long-lasting open state of reduced conductance on addition of ryanodine 100 nM to the cytosolic side of the channel (b). The ryanodine-modified state has a conductance approximately 40% of the control value. Dotted lines indicate the fully open level of the channel.

Table 1 Summary of the effect of 1 nM digoxin on single-channel mean open and closed times and mean P_o at various concentrations of activating Ca^{2+}

	Mean open time (ms)	*Mean closed time* (ms)	*Mean* P_o
Ca^{2+} 0.1 μM	1.41 ± 0.45	138 ± 42	0.012 ± 0.003
+ digoxin 1 nM	1.36 ± 0.26	22 ± 3.87	0.110 ± 0.029
Ca^{2+} 10 μM	1.37 ± 0.20	34.80 ± 6.73	0.048 ± 0.012
+ digoxin 1 nM	1.73 ± 0.09	7.05 ± 1.54	0.281 ± 0.065
Ca^{2+} 100 μM	2.18 ± 0.38	14.35 ± 2.73	0.160 ± 0.043
+ digoxin 1 nM	2.05 ± 0.62	14.93 ± 3.14	0.143 ± 0.038

Values are mean ± s.e.mean.
For experiments at 0.1 μM Ca^{2+} the results are the mean of eight different single channels; at 10 μM Ca^{2+} the results are the mean of four channels and at 100 μM Ca^{2+}, the mean of three different channels. At 0.1 μM Ca^{2+} and 10 μM Ca^{2+} the increase in P_o caused by addition of 1 nM digoxin is mainly due to a reduction in the mean closed time of the channel, resulting in an increased frequency of channel opening. Mean open and closed lifetimes and mean P_o were calculated as described in the Methods section.

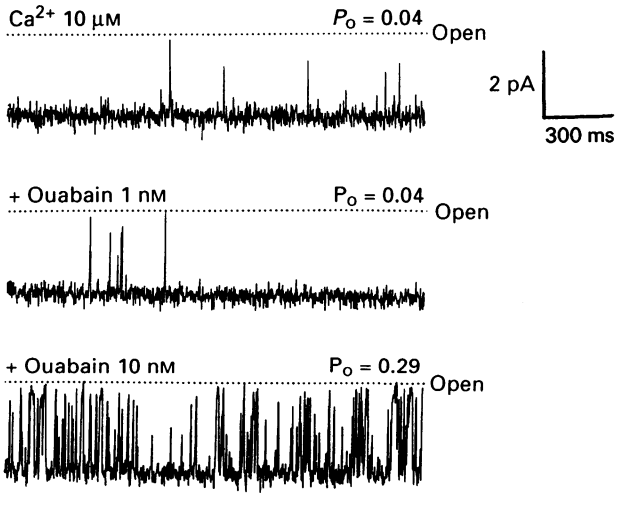


Figure 5 A single cardiac SR Ca^{2+}-release channel held at 0 mV, activated by Ca^{2+} and by Ca^{2+} and ouabain. The channel was activated by Ca^{2+} 10 μM added to the *cis* chamber, before addition of ouabain. Single-channel P_o was not affected by ouabain 1 nM, but was increased significantly on addition of ouabain 10 nM to the *cis* chamber. Ouabain, 1 nM and 10 nM, had no effect at Ca^{2+} 100 μM (not shown). Single-channel conductance was unaffected by ouabain. P_o values, indicated above each respective portion of data, were calculated as described in the Methods section. Dotted lines indicate channel open level.

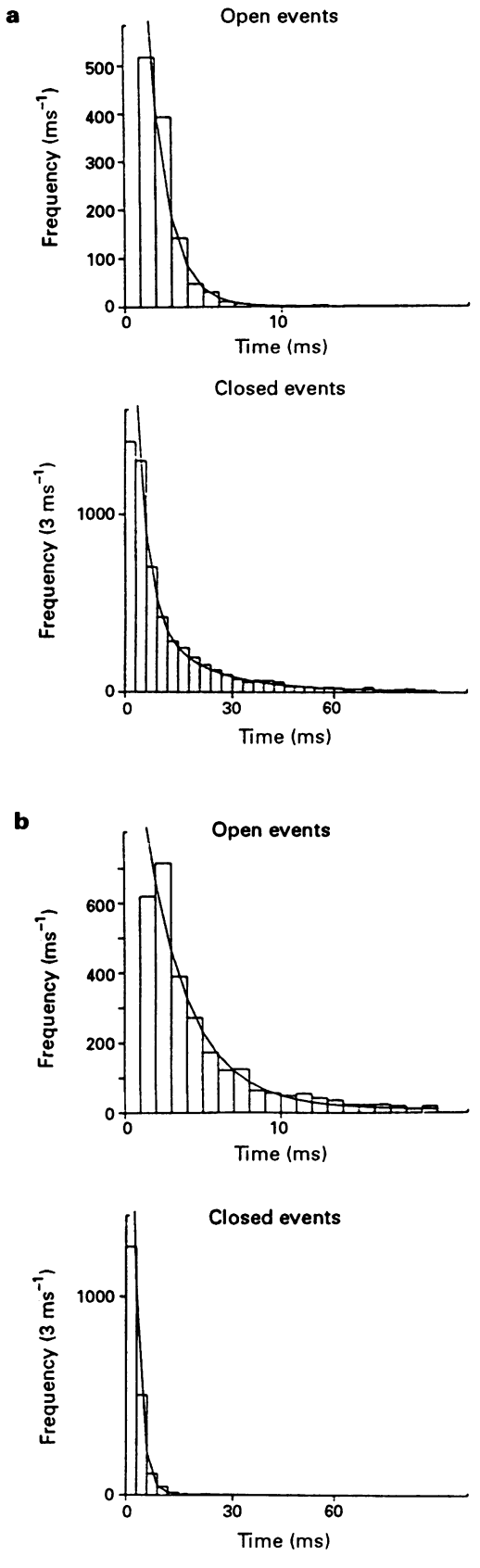


Figure 6 Single sheep cardiac SR Ca^{2+}-release channel open and closed lifetime histograms, with probability density functions obtained by maximum likelihood fitting to individual lifetimes. Open and closed lifetime distributions are taken from the experiment shown in Figure 5. Here, (a) corresponds to the channel activated by Ca^{2+} 10 μM and (b) to the channel activated by Ca^{2+} 10 μM + ouabain 10 nM. Lifetimes were obtained from 3 min of continuous recordings and are displayed in non-cumulative histograms. Probability density functions are drawn according to: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots\ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$. For open lifetimes most likely fits were obtained to double exponentials with (i) Ca^{2+} 10 μM: $a_1 = 98.09$, $a_2 = 1.91$, $\tau_1 = 1.24$, $\tau_2 = 14.40$ and (ii) Ca^{2+} 10 μM + ouabain 10 nM: $a_1 = 86.42$, $a_2 = 13.58$, $\tau_1 = 1.71$, $\tau_2 = 21.91$. For closed lifetimes, most likely fits were obtained to triple exponentials with (i) Ca^{2+} 10 μM: $a_1 = 51.67$, $a_2 = 38.27$, $a_3 = 10.06$, $\tau_1 = 3.28$, $\tau_2 = 14.83$, $\tau_3 = 96.89$ and (ii) Ca^{2+} 10 μM + ouabain 10 nM: $a_1 = 97.43$, $a_2 = 1.23$, $a_3 = 1.34$, $\tau_1 = 1.86$, $\tau_2 = 9.85$, $\tau_3 = 40.18$.

ship between SR Ca^{2+}-release channel P_o and activating Ca^{2+} concentration, which is complex and varies somewhat between individual channels. As the Ca^{2+} concentration at the cytosolic face of the channel is increased in the μM range P_o increases, reaches a maximum and then tends to decline as the concentration of Ca^{2+} at the cytosolic face of the channel is further increased (Sitsapesan *et al.*, 1991). Ca^{2+} does not fully activate the channel when it is present as the only activating ligand and the Ca^{2+} concentration producing maximal P_o will differ slightly from channel to channel. In the channels described here it appears probable that a Ca^{2+} concentration of 100 μM was greater than that required for maximal activation. The observation that P_o is unaffected or possibly slightly lowered by 1 nM digoxin at an activating Ca^{2+} concentration of 100 μM is, therefore, entirely consistent with Ca^{2+} sensitization.

The elevation of channel P_o by high concentrations of digoxin (10–20 nM) is associated with an increase in channel open lifetimes indicating a second mechanism of action of digoxin at these concentrations. A similar observation has been made with both caffeine (Sitsapesan & Williams, 1990) and sulmazole (Williams & Holmberg, 1990).

Since low concentrations of caffeine activate both the cardiac and skeletal SR Ca^{2+}-release channel by a similar mechanism to activation by digoxin of the cardiac channel, it may be suggested that these different compounds act at the same site on these channels. If this were so, digoxin would be expected to activate the skeletal, as well as the cardiac channel, however this does not occur. This presumably reflects the lack of a recognition site on the skeletal SR channel for digoxin, highlighting a functional difference in the two isoforms of this protein.

That chlormadinone does not affect the cardiac SR Ca^{2+}-release channel is an interesting finding. As it has no positive inotropic activity, it would not be expected to activate the channel. It acts as an antagonist of ouabain at the Na^+/K^+ ATPase yet it has no such effect on glycoside-induced effects on the cardiac SR Ca^{2+}-release channel, even at high concentrations. In view of the results obtained with digoxin and ouabain, these observations could be interpreted as confirming the findings of LaBella *et al.* (1979) that although chlormadinone antagonized ouabain binding to the ATPase, it did not affect the positive inotropy induced by the glycoside. The authors in that sutdy concluded that ouabain increased contraction of cardiac muscle by acting at a chlormadinone-insensitive site distinct from the ATPase. This chlormadinone-insensitive site may have been the SR Ca^{2+}-release channel. Similarly, the lack of effect of spironolactone on digoxin-induced inotropy, despite spironolactone inhibiting digoxin binding to the ATPase, was interpreted by Musgrave *et al.* (1977) as there being another site of action for digoxin in inducing inotropy, possibly the SR. Again, as with chlormadinone, although spironolactone antagonized glycoside binding to the Na^+/K^+ ATPase, it did not affect glycoside-induced inotropy. These observations again suggest that digoxin increases contraction of cardiac muscle by acting at a site distinct from the ATPase. This lends further credence to the theory, arising from the results presented here, that activation of the cardiac SR Ca^{2+}-release channel by glycosides, and therefore increased release of intracellular Ca^{2+}, contributes to their positive inotropic effect. Addi-

tionally, Musgrave *et al.* (1977) noted that spironolactone decreased digoxin toxicity by antagonizing digoxin binding to the ATPase. Since we contend that positive inotropy may, in part, occur as a result of digoxin binding to a site on the cardiac SR Ca^{2+}-release channel, this observation is even more interesting as it would seem to suggest that inhibition of the Na^+/K^+ ATPase by glycosides, possibly thereby inducing Ca^{2+} overload, contributes to glycoside toxicity, if not inotropy.

Structurally, chlormadinone and spironolactone are similar to digoxin and ouabain with respect to their steroid nucleus. However, neither possesses sugar residues attached to the hydroxyl at the C3 position of the molecule and chlormadinone does not contain a lactone ring. This may indicate that only aglycones or steroids with these sugar attachments may bind to the activation site on the cardiac SR Ca^{2+}-release channel and that the presence or composition of these sugar residues may be determinants of activity. The presence of the lactone ring on the steroid would appear not to be necessary for activity.

The concentration of digoxin achieved therapeutically in the blood is in the range of 0.75–2 nM, taking into account binding to plasma proteins (Noble, 1980). Given that digoxin and other glycosides are able to cross the sarcolemma (Dutta *et al.*, 1968; Fricke & Klaus, 1978) and that we calculate an EC_{50} of <1 nM for digoxin, we feel that the results presented here provide compelling evidence that activation of the SR Ca^{2+}-release channel by glycosides is a contributory factor to the positive inotropic effect seen with these drugs. This may not, however, be the case with all cardiotonic steroids, especially those that are more lipophobic than digoxin e.g. ouabain, or those which show a consistent correlation between Na^+/K^+ ATPase inhibition and positive inotropy. This raises the possibility of the existence of different mechanisms of action for therapeutic concentrations of different cardiac glycosides, with the lipid solubility of the drug (and therefore the ability to reach the SR Ca^{2+}-release channel) the determinant of which mechanism induces inotropy. The more lipophilic drugs such as digoxin and digitoxin may increase contraction of cardiac muscle by direct activation of SR Ca^{2+}-release, since they could easily gain access intracellularly, whereas relatively more lipophobic compounds like ouabain, although able to activate the SR Ca^{2+}-release channel, may indirectly increase contraction by inhibition of the Na^+/K^+ ATPase, as it is unlikely that ouabain could gain access to the cytosol unless it was actively transported. These observations support the suggestion that there may be several mechanisms by which cardiac glycosides induce positive inotropy. It is possible that these different mechanisms interact to increase intracellular Ca^{2+} and cardiac muscle contraction. However, as high concentrations of digoxin also activate the cardiac SR Ca^{2+}-release channel, it is also possible that toxic concentrations of digoxin in the blood i.e. >2 nM may contribute to Ca^{2+} overload and glycoside toxicity by acting at this site.

Glycoside-induced release of Ca^{2+} from the cardiac SR would result in only a transient positive inotropic effect if it occurred in the absence of processes which would produce an overall increase in cytosolic and hence SR Ca^{2+}. As glycosides induce a long-lasting increase in cardiac contraction, this may indicate the importance of other effects of these compounds on cellular Ca^{2+} entry and/or removal mechanisms. In support of this argument, Marban & Tsien (1982) concluded that glycosides increased the transient inward current. If this were to occur at the same time as the enhancement of Ca^{2+} release from the SR, this might account for an elevation of the steady state cytosolic Ca^{2+} concentration and a sustained increase in inotropy. Again it seems probable that there are several mechanisms by which the inotropic state of cardiac muscle may be increased by glycosides. Prolonged increases in cytosolic Ca^{2+} and hence contraction, may require that more than one of these mechanisms be activated by the drug.

As glycosides increase single Ca^{2+}-release channel open probability and also inhibit the Na^+/K^+ ATPase there appears to be a site on both proteins with a similar affinity for this class of drugs. However, there are differences in the glycoside binding sites between these proteins, as chlormadinone and spironolactone bind to the ATPase but not to the glycoside site on the channel. As we contend that activation of the SR Ca^{2+}-release channel by lipophilic glycosides contributes to, or is responsible for, the positive inotropy seen with these drugs, there exists a rationale for the development of drugs which are agonists at the glycoside site on the release channel but which have minimal or no ability to inhibit the ATPase. This would constitute a new class of positively inotropic drugs. Additionally, given the suggestion of Musgrave *et al.* (1977) that inhibition of the ATPase by glycosides contributes to toxicity but not to inotropy, then this new class of compound could be positively inotropic with less toxic side effects, due to lack of ATPase inhibition. These new drugs would be potentially more safe due to their decreased toxicity. However, as there may be more than one mechanism by which cardiac glycosides can increase contraction, it is equally feasible that these drugs may cause toxic side effects by more than one mechanism e.g. by excessive activation of the SR Ca^{2+}-release channel, as suggested above.

In conclusion, the present study has three main findings: First, nanomolar concentrations of cardiac glycosides activate the cardiac SR Ca^{2+}-release channel. The increase in P_o induced by therapeutic concentrations of the glycosides is Ca^{2+}-dependent and is achieved by increasing the frequency of channel opening. Secondly, there is no equivalent effect of glycosides on skeletal muscle SR Ca^{2+}-release channels, reflecting a difference in the cardiac and skeletal isoforms of the channel. Thirdly, as direct activation of the cardiac channel occurs within the range of concentrations of glycosides encountered therapeutically, we propose that activation of the cardiac SR Ca^{2+}-release channel by digoxin and related compounds contributes to the positive inotropy seen with these drugs.

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References

ALLEN, D.G., EISNER, D.A. & ORCHARD, C.H. (1984). Factors affecting free intracellular calcium concentration in quiescent ferret ventricular muscle. *J. Physiol.*, **350**, 615–630.

ALLEN, D.G., EISNER, D.A., PIROLO, J.S. & SMITH, G.L. (1985). The relationship between intracellular calcium and contraction in calcium-overloaded ferret papillary muscles. *J. Physiol.*, **364**, 169–182.

ASHLEY, R.H. & WILLIAMS, A.J. (1990). Divalent cation activation and inhibition of single calcium-release channels from sheep cardiac sarcoplasmic reticulum. *J. Gen. Physiol.*, **95**, 981–1005.

BERS, D.M. & BRIDGE, J.H.B. (1988). Effect of acetylstrophanthidin on twitches, microscopic tension fluctuations and cooling contractions in rabbit ventricle. *J. Physiol.*, **404**, 53–69.

BLATZ, A.L. & MAGLEBY, K.L. (1986). Quantitative description of three modes of activity of fast chloride channels from rat skeletal muscle. *J. Physiol.*, **378**, 141–174.

BOYETT, M.R., HART, G. & LEVI, A.J. (1986). Dissociation between force and intracellular sodium activity with strophanthidin in isolated sheep purkinje fibres. *J. Physiol.*, **381**, 311–331.

COHEN, I., DAUT, J. & NOBLE, D. (1976). An analysis of the actions of low concentrations of ouabain on membrane currents in purkinje fibres. *J. Physiol.*, **260**, 75–103.

COLQUHOUN, D. & SIGWORTH, F. (1983). Fitting and statistical analysis of single-channel recording. In *Single-Channel Recording*. ed. Sakmann, B. & Neher, E. pp. 191–263. New York: Plenum Press.

DUPONT, Y. (1984). A rapid-filtration technique for membrane fragments or immobilised enzymes: measurements of substrate binding or ion fluxes with a few-millisecond time resolution. *Anal. Biochem.*, **142**, 504–510.

DUTTA, S., GOSWAMI, S., DATTA, D.K., LINDOWER, J.O. & MARKS, B.H. (1968). The uptake and binding of six radiolabelled cardiac glycosides by guinea-pig hearts and by isolated sarcoplasmic reticulum. *J. Pharmacol. Exp. Ther.*, **164**, 10–21.

EISNER, D.A. (1990). Intracellular sodium in cardiac muscle: effects on contraction. *J. Exp. Physiol.*, **75**, 437–457.

EISNER, D.A. & LEDERER, W.J. (1979). The role of the sodium pump in the effects of potassium-depleted solutions on mammalian cardiac muscle. *J. Physiol.*, **294**, 279–301.

FATT, P. & GINSBORG, B.L. (1958). The ionic requirements for the production of action potentials in crustacean muscle fibres. *J. Physiol.*, **142**, 516–543.

FRICKE, U. & KLAUS, W. (1978). Sodium-dependent cardiac glycoside binding: experimental evidence and hypothesis. *Br. J. Pharmacol.*, **62**, 255–257.

GHYSEL-BURTON, J. & GODFRAIND, T. (1979). Low potassium or ouabain inotropy in cardiac muscle. *J. Physiol.*, **265**, 52–53P.

GODFRAIND, T. & GYSEL-BURTON, J. (1977). Binding sites related to ouabain-induced stimulation or inhibition of the sodium pump *Nature*, **265**, 165–166.

HART, G., NOBLE, D. & SHIMONI, Y. (1983). The effects of low concentrations of cardiotonic steroids on membrane currents and tension in sheep purkinje fibres. *J. Physiol.*, **334**, 103–131.

LABELLA, F.S., BIHLER, I. & KIM, R.S. (1979). Progesterone derivative binds to cardiac ouabain receptor and shows dissociation between sodium pump inhibition and increased contractile force. *Nature*, **278**, 571–573.

MARBAN, E. & TSIEN, R.W. (1982). Enhancement of calcium current during digitalis inotropy in mammalian heart: positive feed-back regulation by intracellular calcium? *J. Physiol.*, **329**, 589–614.

MEISSNER, G. & HENDERSON, J. (1987). Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide, and calmodulin. *J. Biol. Chem.*, **262**, 3065–3073.

MILLER, C. (1982). Open-state substructure of single chloride channels from *Torpedo electroplax*. *Phil. Trans. R. Soc.*, **B299**, 401–411.

MORGAN, J.P. (1985). The effects of digitalis on intracellular calcium transients in mammalian working myocardium as detected by aequorin. *J. Mol. Cell. Cardiol.*, **17**, 1065–1075.

MOUTIN, M.J. & DUPONT, Y. (1988). Rapid filtration studies Ca^{2+}-induced Ca^{2+} release from skeletal sarcoplasmic reticulum. Role of monovalent ions. *J. Biol. Chem.*, **263**, 4228–4235.

MUSGRAVE, G.E., BORN, C.K., DAVIDSON, P. & HAMRICK, M.E. (1977). Interaction of spironolactone and digoxin in dogs. *J. Pharmacol. Exp. Ther.*, **202**, 696–701.

NOBLE, D. (1980). Mechanism of action of therapeutic levels of cardiac glycosides. *Cardiovasc. Res.*, **14**, 495–514.

SITSAPESAN, R. & WILLIAMS, A.J. (1990). Mechanisms of caffeine activation of single calcium-release channels of sheep cardiac sarcoplasmic reticulum. *J. Physiol.*, **423**, 425–439.

SITSAPESAN, R., BORASO, A. & WILLIAMS, A.J. (1991). High concentrations of calcium and ATP reduce the open probability of the sheep cardiac sarcoplasmic reticulum calcium-release channel. *Biophys. J.*, **59**, 199a.

SMITH, J.S., CORONADO, R. & MEISSNER, G. (1985). Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. *Nature*, **316**, 446–449.

TOMLINS, B., HARDING, S.E., KIRBY, M.S., POOLE-WILSON, P.A. & WILLIAMS, A.J. (1986). Contamination of a cardiac sarcolemmal preparation with endothelial plasma membrane. *Biochim. Biophys. Acta*, **856**, 137–143.

WEINGART, R., KASS, R.S. & TSIEN, R.W. (1978). Is ditigalis inotropy associated with enhanced slow inward current? *Nature*, **273**, 389–392.

WIER, W.G. & HESS, P. (1984). Excitation-contraction coupling in cardiac purkinje fibres. Effects of cardiotonic steroids on the intracellular $[Ca^{2+}]$ transient, membrane potential, and contraction. *J. Gen. Physiol.*, **83**, 395–415.

WILLIAMS, A.J. (1992). Ion conduction and discrimination in the sarcoplasmic reticulum ryanodine receptor/calcium-release channel. *J. Musc. Res. Cell. Motil.*, **13**, 7–26.

WILLIAMS, A.J. & HOLMBERG, S.R.M. (1990). Sulmazole (AR-L 115BS) activates the sheep cardiac muscle sarcoplasmic reticulum calcium-release channel in the presence and absence of calcium. *J. Memb. Biol.*, **115**, 167–178.

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Characterization of the inhibitory prostanoid receptors on human neutrophils

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1 We have evaluated the effects of various prostanoid agonists on the release of leukotriene B_4 (LTB_4) and superoxide anions (O_2^-) from human neutrophils stimulated with opsonized zymosan (OZ) and formyl-methionyl-leucyl-phenylalanine (FMLP), respectively.

2 Prostaglandin E_2 (PGE_2) and PGD_2 inhibited both OZ-induced LTB_4 release (EC_{50} 0.72 μM and 0.91 μM respectively), and FMLP-induced O_2^- release (EC_{50} 0.42 μM and 0.50 μM respectively). $PGF_{2\alpha}$, the TP-receptor agonist, U46619, and the IP-receptor agonist, iloprost, were also active, but were all at least an order of magnitude less potent than PGE_2 and PGD_2.

3 The EP_2/EP_3-receptor agonist, misoprostol, and the selective EP_2-agonist, AH13205, were both effective inhibitors of LTB_4 release, being approximately equipotent with and 16-times less potent than PGE_2, respectively. In contrast, the EP_1/EP_3-receptor agonist, sulprostone, had no inhibitory activity at concentrations of up to 10 μM.

4 The selective DP-receptor agonist, BW245C, inhibited LTB_4 release, (EC_{50} 0.006 μM) being approximately 50 times more potent than PGD_2. BW245C also inhibited O_2^- release, and this inhibition was antagonized competitively by the DP-receptor blocking drug, AH6809 (pA_2 6.6).

5 These data indicate the presence of both inhibitory EP- and DP-receptors on the human neutrophil. The rank order of potency of EP-receptor agonists suggest that the EP-receptors are of the EP_2-subtype.

Keywords: Prostanoids; neutrophil; EP_2-receptor; DP-receptor.

Introduction

Neutrophils are primarily involved in host-defence. The ingestion of microbes by phagocytosis, and the release of toxic products such as superoxide anion, O_2^-, and lysosomal enzymes into the resultant phagolysosome enables the neutrophil to be an effective killer of invading pathogens. In certain inflammatory diseases, this physiological function becomes over-vigorous or inappropriate, and products of activated neutrophils cause or exacerbate the inflammatory lesion (Olson & Venge, 1980).

Prostanoids have a wide range of biological actions. Recently, evidence has accumulated which indicates that many of these effects are mediated by specific membrane-bound receptors. The classification of prostanoid receptor subtypes has been based upon the activities of natural and synthetic agonists (Coleman *et al.*, 1990).

It has been shown previously that certain prostanoids can inhibit various functions of the human neutrophil (Zurier *et al.*, 1974; Lad *et al.*, 1985). It has been suggested that inhibition by endogenously generated prostaglandins is one mechanism by which the pro-inflammatory effects of neutrophils are limited (Gryglewski *et al.*, 1987). As yet however, there has been no systematic attempt to determine which type(s) of protanoid receptor are involved in the inhibition of human neutrophil activity. In order to address this question, we now describe the effects of a range of receptor-selective prostanoid agonists on two functions of the human neutrophil.

Methods

Isolation of neutrophils

Venous blood was drawn from the forearm of healthy human volunteers, and anti-coagulated with 10 u heparin ml^{-1} blood. The blood was mixed with Plasmagel (0.67 times the volume of blood) and left to sediment at 37°C for 35–40 min. The supernatant buffy coat was removed by aspiration. Ficoll-paque (1/5 volume of buffy coat) was introduced under the buffy coat and centrifuged at 400 *g* for 20 min at 22°C. The granulocyte pellet was washed twice in calcium-free Tris buffer composition (mM): NaCl 122, KCl 2.7, Tris 25, glucose 5.6, $MgCl_2$ 10 and bovine serum albumin 0.1% w/v, pH 7.4, washed again in Tris buffer containing $CaCl_2$, 1 mM then resuspended in this buffer.

Aliquots of the suspension were taken to determine total white blood cell numbers (Medonic Cell Analyser CA480). Cells were stained with chromotrope 2 (to stain eosinophils) or Leishman's stain. Granulocyte populations comprising > 95% neutrophils were used.

Cells were resuspended to a concentration of $1-2 \times 10^6$ cells ml^{-1}. In all experiments, indomethacin (3 μM) and adenosine deaminase (1 u ml^{-1}) were incorporated into the buffer to inhibit the endogenous production of prostaglandins and remove any adenosine released by the cells (Schmeichel & Thomas, 1987).

Opsonization of zymosan

Zymosan was boiled for 5 min in distilled water then sedimented (~250 *g* for 5 min) and washed once with phosphate buffered saline (PBS, pH 7.4). Following a further sedimentation, the zymosan was resuspended at a concentration of 5 mg ml^{-1} in previously frozen (−20°C) human serum and incubated for 1 h at 37°C. The opsonized zymosan was then washed twice in PBS and finally resuspended at a concentration of 10 mg ml^{-1} in Tris buffer containing $CaCl_2$, 1 mM.

Leukotriene B_4 release

Aliquots (400 μl) of cells were incubated with prostanoid or vehicle (50 μl) at 37°C for 10 min prior to the addition of

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opsonized zymosan (OZ, 50 μl, 10 mg ml^{-1}). After a further 20 min, the cell suspension was centrifuged at 200 *g* for 5 min and the cell-free supernatants, assayed for LTB_4 by specific radioimmunoassay (Amersham International).

O_2^- release

The reduction of ferricytochrome C with a consequent increase in absorption at 550 nm was used to assay the release of superoxide anion. Cytochalasin b (10 μM) was incorporated into the buffer to enhance the amount of anion released. Aliquots (400 μl) of cells suspended in Tris buffer containing cytochrome C (0.1 mM) were incubated with prostanoid or vehicle (50 μl) for 10 min prior to the addition of FMLP (50 μl, 2 μM). Ten min later, the cells were centrifuged at 200 *g* for 5 min, and 300 μl of supernatant transferred to a 96 flat-blottomed well microtitre plate. Absorption at 550 nm was determined (Anthos Labtec microplate reader) using an extinction coefficient of 21.2 mM^{-1} cm^{-1} at 550 nm. O_2^- release was calculated as nmol of cytochrome C reduced/10^6 cells.

Data analysis

Each incubation was carried out in triplicate. After allowing for spontaneous release of products from cells incubated in the absence of stimulus, results were expressed as percentage inhibitions to the formula:

$$\text{Percentage inhibition} = \frac{(a-b) \times 100}{(a)}$$

Where a = release of product in stimulated cells in the absence of prostanoids, b = release of product in stimulated cells in the presence of prostanoids.

Equipotent concentrations (EPC) values were calculated by dividing the concentration of test agonist required to produce 50% of the maximum effect (EC_{50}) by the EC_{50} for the standard agonist (PGE_2 or PGD_2) in the same experiment.

Data are expressed as means with confidence limits or standard error of mean of (*n*) experiments performed in triplicate.

Materials

Plasmagel (Roget Bellon Labs, France), Ficoll-Paque (Pharmacia, UK), adenosine deaminase (Boerhinger Mannheim, Germany). PGE_2 and $PGF_{2\alpha}$ (Upjohn, UK) U46619 (9α, 11α-dideoxymethanoepoxy prostaglandin $F_{2\alpha}$, Cayman Chemicals, USA), iloprost (Schering, Germany), BW245C (3-[3-cyclohexyl - 3 - hydroxypropyl] - 2, 5 - dioxo - 4 - imidazolidine heptanoic acid; Burroughs-Wellcome, UK), PGD_2, sulprostone, AH13205 (trans-2-[4-(1-hydroxyhexyl)phenyl]-5-oxocyclo pentaneheptanoic acid; a selective EP_2 receptor agonist, Nials *et al.*, 1991) and AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid; a DP-receptor blocking drug, Keery & Lumley, 1988), were synthesized at Glaxo Group Research, Ware. All other materials were purchased from Sigma, UK.

Results

The effects of prostanoid receptor agonists

Inhibition of LTB_4 release Stimulation of granulocyte suspensions with OZ, induced the release of immunoreactive LTB_4 (0.65 ± 0.18 ng/10^6 cells, $n = 8$). Both PGE_2, EC_{50} 0.72 (0.39–1.30) μM, $n = 36$, and PGD_2, EC_{50} 0.91 (0.21–4.07) μM, $n = 11$, inhibited the release of LTB_4 in a concentration-dependent manner (Figure 1a). $PGF_{2\alpha}$ (1–10 μM), and the synthetic mimetics of PGI_2, iloprost (1–10 μM), and TxA_2, U46619 (1–10 μM), were also inhibitory, but were at least an order of magnitude weaker than PGE_2 and PGD_2 (Figure 1a).

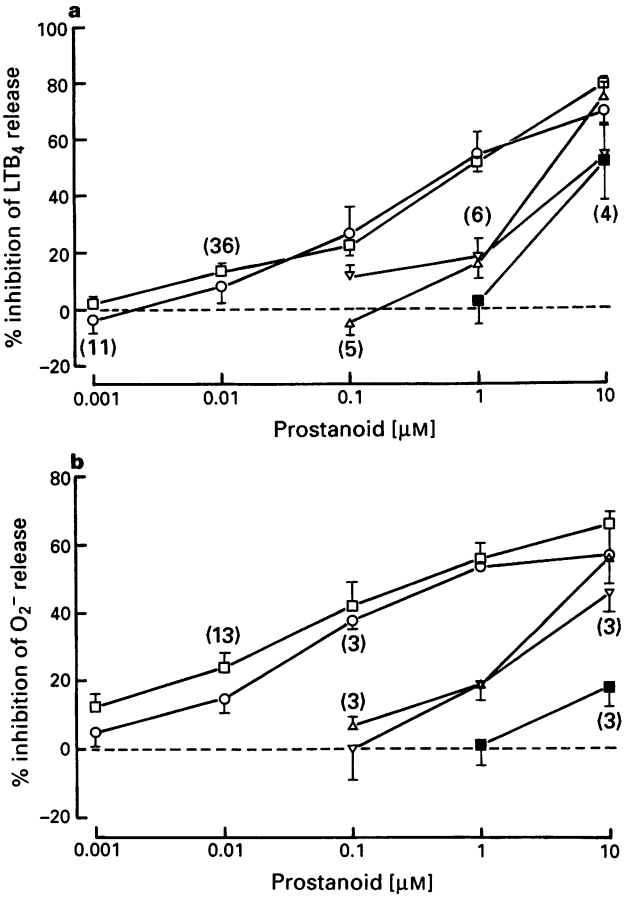


Figure 1 Mean inhibitory concentration-effect curves for prostaglandin E_2 (PGE_2, □), PGD_2 (○), $PGF_{2\alpha}$ (△), iloprost (▽), and U46619 (■) against (a) OZ-induced release of LTB_4 and (b) FMLP-induced release of O_2^- from human neutrophils. The number of observations obtained for each curve is indicated in parentheses.

Inhibition of O_2^- release FMLP, (0.2 μM) in the presence of cytochalasin b (10 μM), induced the release of O_2^- (15.0 ± 1.7 nmol/10^6 cells, $n = 10$). As for inhibition of LTB_4 release, PGE_2, EC_{50} 0.42 (0.1–1.74) μM, $n = 13$, and PGD_2, EC_{50} 0.50 (0.29–0.84) μM, $n = 3$ were the most potent inhibitors of O_2^- release (Figure 1b). The TxA_2 mimetic, U46619 (up to 10 μM), had little or no inhibitory activity. $PGF_{2\alpha}$ (1–10 μM) and the PGI_2 mimetic, iloprost (1–10 μM) did inhibit O_2^- release, but were at least an order of magnitude less potent than PGE_2 and PGD_2 (Figure 1b).

The effects of selective EP-receptor subtype agonists

The EP_2/EP_3-receptor agonist, misoprostol (0.1–10 μM), and the selective EP_2-receptor agonist,AH13205 (0.1–10 μM), both caused concentration-related inhibition of OZ-induced LTB_4 release. Misoprostol was about equipotent with PGE_2,

Table 1 Opsonized zymosan (OZ)-induced release of leukotriene B_4 (LTB_4) from human neutrophils: effects of selective EP-receptor subtype agonists

Agonist	*EC_{50} (μM)*	*Equipotent concentration* ($PGE_2 = 1$)	n
PGE_2	0.72 (0.39–1.30)	1	(36)
Misoprostol	1.47 (0.725–2.99)	0.75 (0.30–1.92)	(11)
AH13205	6.88 (2.92–16.2)	15.9 (6.7–37.5)	(6)
Sulprostone	ND*	–	(12)

*ND = not determined-sulprostone enhanced release at 0.01–10 μM.

Data are expressed as geometric means with 95% confidence limits in parentheses derived from *n* separate experiments.

and AH13205 about 16 times less potent that PGE_2 (Table 1). In contrast, the EP_1/EP_3-receptor agonist, sulprostone did not inhibit OZ-induced LTB_4 release. Indeed, sulprostone (0.01–10 μM) significantly ($P<0.05$) enhanced release to a small degree (maximum of 31.4 ± 7.7% at 1 μM, $n = 12$). The selective EP-receptor agonists were not tested against FMLP-induced O_2^- release.

The effects of a selective DP-receptor agonist

Inhibition of LTB_4 and O_2^- release by BW245C The highly potent, selective DP-receptor agonist, BW245C (0.1 nM–100 nM), caused concentration-related inhibition of both OZ-induced LTB_4 release (Figure 2) and FMLP-induced O_2^- release (Figure 3). Of the prostanoids tested, BW245C was the most potent inhibitor of both markers of neutrophil activation, having an EPC of 0.018 (with 95% confidence limits of 0.003–0.124, $PGD_2 = 1$). Thus BW245C was found to be about 50 times more potent than PGD_2.

Antagonism of the effect of BW245C by AH6809 Preincubation of cells with the DP-receptor, antagonist, AH6809 (1–10 μM), caused progressive rightward shifts of concentration-inhibition curves of BW245C against O_2^- release induced by FMLP (Figure 3). Arunlakshana & Schild (1959) plots of data derived from two separate experiments suggest that antagonism is competitive, and pA_2 values of 6.5 (slope −1.2) and 6.6 (slope −1.1) were determined.

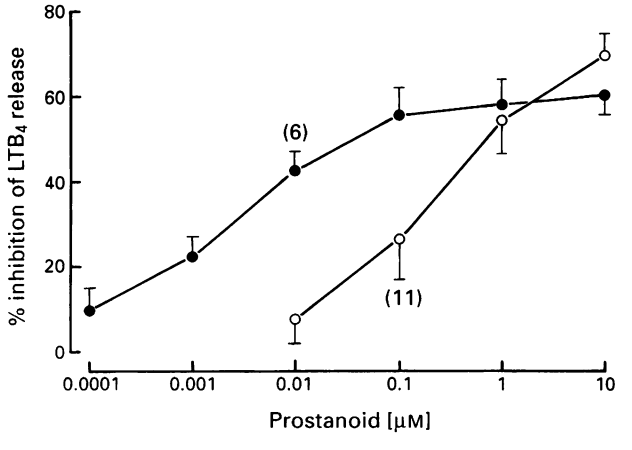


Figure 2 Opsonized zymosan (OZ)-induced release of leukotriene B_4 from human neutrophils. Mean inhibitory concentration-effect curves for prostaglandin D_2 (○, EC_{50} 0.91 μM with 95% confidence limits of 0.21–4.07) and BW245C (●, EC_{50} 0.006 μM with 95% confidence limits of 0.002–0.017). The number of observations for each curve is indicated in parentheses.

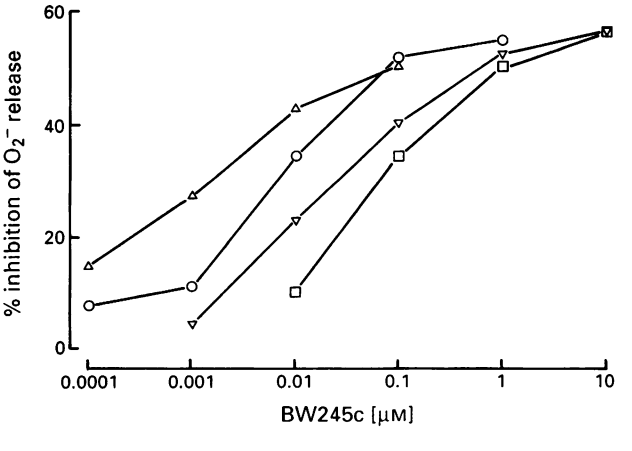


Figure 3 FMLP-induced release of O_2^- from human neutrophils. Antagonism by AH6809 of the inhibitory activity of BW245C, mean inhibitory concentration-effect curves from 2 independent experiments for BW245C in the absence of (△) and presence of AH6809, 1 μM (○), 3 μM (▽) and 10 μM (□).

Discussion

We have investigated the effect of prostanoids on two aspects of neutrophil function, opsonized zymosan-induced release of LTB_4 (the major 5-lipoxygenase product of the human neutrophil) and FMLP-induced release of O_2^-. The release of LTB_4 (which itself can activate neutrophils) and O_2^- have been suggested to be important in neutrophil-dependent inflammation (Ham *et al.*, 1983). Of the prostanoids selected as representative of agonists active at each of the five major prostanoid receptors, PGE_2 and PGD_2 were the most potent. This agrees with previous work (Gryglewski *et al.*, 1987), and others have shown that, PGE_2 and PGD_2 also inhibit lysosomal enzyme release (Zurier *et al.*, 1974; Ham *et al.*, 1983; Lad *et al.*, 1985; Ney & Schrör, 1991). Such results raise the possibility that human neutrophils possess inhibitory DP- and EP-receptors.

It is interesting to note that Ham *et al.* (1983) showed that although rat neutrophils were sensitive to inhibition by PGE_2, PGD_2 was ineffective, illustrating that neutrophils from different species may possess different prostanoid receptors.

In the present study, the relative potencies of the prostanoid receptor agonists were similar, irrespective of the stimulus being used or of the function being measured. The most likely explanation for this is that prostanoids inhibit a common pathway.

The fact that the EP_2/EP_3-receptor agonist, misoprostol, inhibited LTB_4 release, whereas the EP_1/EP_3-agonist, sulprostone, had no inhibitory activity suggests the involvement of EP_2-receptors. This is supported by the observed activity of AH13205, which was 16 times less potent than PGE_2, which is within the potency range derived from tissues previously classified as containing EP_2-receptors (Coleman *et al.*, 1990; Nials *et al.*, 1991). Unfortunately, at present, no EP_2-receptor antagonist exists that we can use to confirm this deduction. The observation that the EP_1/EP_3-agonist, sulprostone, consistently enhanced release is consistent with the findings of Armstrong, (1992) and suggests that neutrophils possess either stimulatory EP_1 or EP_3-receptors. More selective agonists (or antagonists) are needed to confirm this deduction.

The potencies of the DP-receptor selective agonists, BW245C and PGD_2-were similar, both in absolute and relative terms, to those seen on other DP-receptor containing preparations, such as human platelets (Keery & Lumley, 1988) and human eosinophils (Butchers & Vardey, 1990), where the prostanoids cause inhibition of aggregation and degranulation, respectively. Moreover, inhibition by BW245C was antagonized competitively with the DP-receptor antagonist, AH6809. The pA_2 for AH6809 of 6.6 falls within the range expected for interactions at DP-receptors (Keery & Lumley, 1988).

As prostanoids can clearly inhibit neutrophil activation induced by a range of stimuli, it is possible that they represent an endogenous mechanism designed to modulate neutrophil function under physiological and pathological conditions (Gryglewski *et al.*, 1987). It is interesting to note that two of the major prostanoids produced by neutrophils are PGD_2 and PGE_2 (McGuire & Sun, 1980; Gemsa *et al.*, 1981), and PGD_2 is the major cyclo-oxygenase product of activated mast cells (Lewis *et al.*, 1981; Peters *et al.*, 1984). Thus the release of endogenous prostaglandins may be a process by which cells involved in inflammation both modulate their own function and that of other inflammatory cell types.

Prostaglandins of the E-series have been shown to have anti-inflammatory activity *in vivo* (Zurier & Quagliata, 1971). Systemic administration of PGE_1 or its stable analogue, 15-(S)-15-dimethyl PGE_1, inhibits neutrophil-dependent immune

complex tissue injury (Kunkel *et al.*, 1979). In addition, intravenous infusion of PGE_1 into man inhibits FMLP-induced lysosomal enzyme release from neutrophils (Fantone *et al.*, 1981), and has been claimed to have therapeautic benefit in the treatment of victims of adult respiratory distress syndrome (Holcroft *et al.*, 1986). Although it is possible that these effects of PGE_1 are associated with other properties of the compound, it is tempting to speculate that direct inhibitory effects on neutrophils are responsible.

In summary, the present study has shown that occupation of EP_2 or DP protanoid receptors inhibits certain functions of the activated neutrophil. Both DP- and EP_2-prostanoid receptors are therefore potential targets for novel anti-inflammatory agents. While DP-receptor agonists have, perhaps, the greater potential, particularly as both human platelets and eosinophils also possess inhibitory DP-receptors (Keery & Lumley, 1988; Butchers & Vardey, 1990) the undesirable vascular side-effects of such compounds may limit their usefulness (Orchard *et al.*, 1983; Heavey *et al.*, 1984).

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References

ARMSTRONG, R.A. (1992). PGE_2 and EP_3 agonists induce chemotaxis of human neutrophil *in vitro*. *Br. J. Pharmacol.*, **105**, 45P.

ARUNLAKSHANA, D. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BUTCHERS, P.R. & VARDEY, C.J. (1990). The effect of prostanoids on the function of human eosinophils. *Agents Actions*, Suppl, **31**, 103–112.

COLEMAN, R.A., KENNEDY, I., HUMPHREY, P.P.A., BUNCE, K. & LUMLEY, P. (1990). Prostanoids and their receptors. In *Comprehensive Medicinal Chemistry*, Vol. 3, *Membranes and their Receptors*. ed. Hansch, C., Sammes, P.G. & Taylor, J.B. pp. 643–676. Oxford: Pergamon Press.

FANTONE, J.C., KUNKEL, S.L., WARD, P.A. & ZURIER, R.B. (1981). Suppression of human polymorphonuclear leukocyte function after intravenous infusion of PGE_1. *Prostaglandins Med.*, **2**, 195–198.

GEMSA, D., LESER, H.G., SEITZ, M., DIEMANN, W. & BARLIN, E. (1982). Membrane perturbation and stimulation of arachidonic acid metabolism. *Mol. Immunol.*, **19**, 1287–1296.

GRYGLEWSKI, R.J., SZCZEKLIK, A. & WANDZILAK, M. (1987). The effect of six prostaglandins, prostacyclin and iloprost on generation of superoxide anions by human polymorphonuclear leukocytes stimulated by zymosan or formyl-methionyl-leucyl-phenylalanine. *Biochem. Pharmacol.*, **36**, 4209–4213.

HAM, E.A., SODERMAN, D.D., ZANETTI, M.E., DOUGHERTY, H.W., MACAULEY, E. & KUEHL, Jr. F.A. (1983). Inhibition by prostaglandins of leukotriene B_4 release from activated neutrophils. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 4343–4353.

HEAVEY, D.J., LUMLEY, P., BARROW, S.E., MURPHY, M.B., HUMPHREY, P.P.A. & DOLLERY, C.T. (1984). Effects of intravenous infusions of prostaglandin D_2 in man. *Prostaglandins*, **28**, 755–767.

HOLCROFT, J.W., VASSAR, M.J. & WEBER, C.J. (1986). Prostaglandin E_1 and survival in patients with the Adult Respiratory Distress Syndrome. *Ann. Surg.*, **203**, 371–378.

KEERY, R.J. & LUMLEY, P. (1988). AH6809, a prostaglandin DP-receptor blocking drug on human platelets. *Br. J. Pharmacol.*, **94**, 745–754.

KUNKEL, S.L., THRALL, R.T., KUNKEL, R.G., WARD, P.A. & ZURIER, R.B. (1979). Suppression of immune complex vasculitis in rats by prostaglandins. *J. Clin. Invest.*, **64**, 1525–1529.

LAD, P.M., GOLDBERG, B.J. SMILEY, P.A. & OLSON, C.V. (1985). Receptor-specific threshold effects of cyclic AMP are involved in the regulation of enzyme release and superoxide production from human neutrophils. *Biochim. Biophys. Acta.*, **846**, 286–295.

LEWIS, R.A., HOLGATE, S.T., ROBERTS, L.J., OATES, J.A. & AUSTEN, K.F. (1981). Preferential generation of prostaglandin D_2 by rat and human mast cells. In *Biochemistry of the Acute Allergic Reactions*. ed. Thaber, T.A., Wintroub, B.V. & Simon, A.S. pp.239–254. New York: Alan Liss.

MCGUIRE, J.C. & SUN, F.F. (1980). Metabolism of arachidonic acid and prostaglandin endoperoxide by assorted leukocytes. *Adv. Prostaglandin Thromboxane Res.*, **8**, 1665–1667.

NEY, P. & SCHRÖR, K. (1991). PGD_2 and its mimetic ZK110.841 are potent inhibitors of receptor-mediated activation of human neutrophils. *Eicosanoids*, **4**, 21–28.

NIALS, A.T., COLEMAN, R.A., HARTLEY, D. & SHELDRICK, R.L.G. (1991). AH13205 - A novel selective prostanoid EP_2 agonist. *Br. J. Pharmacol.*, **102**, 24P.

OLSON, I. & VENGE, P. (1980). The role of the human neutrophil in the inflammatory reaction. *Allergy*, **35**, 1–13.

ORCHARD, M.A., RITTER, J.M., SHEPHERD, G.L. & LEWIS, P.J. (1983). Cardiovascular and platelet effects in man of BW245C, a stable mimic of epoprostenol (PGI_2). *Br. J. Clin. Pharmacol.*, **15**, 509–511.

PETERS, S.P., MACGLASHAN, D.W., SCHLUMAN, E.S., SCHLEIMER, R.P., HAYES, E.C., ROKACH, J., ADKINSON, N.F. & LICHTENSTEIN, L.M. (1984). Arachidonic acid metabolism in purified human lung mast cells. *J. Immunol.*, **132**, 1972–1979.

SCHMEICHEL, C.J. & THOMAS, L.L. (1987). Methylxanthine bronchodilators potentiate multiple human neutrophil functions. *J. Immunol.*, **138**, 1896–1903.

ZURIER, R.B. & QUAGLIATA, F. (1971). Effects of prostaglandin E_1 on adjuvant arthritis. *Nature*, **234**, 304–305.

ZURIER, R.B., WEISSMAN, G., HOFFSTEIN, S., KAMMERMAN, S. & TAI, H.H. (1974). Mechanisms of lysosomal enzyme release from human leukocytes II. Effects of cAMP and cGMP, autonomic agonists and agents which influence microtubule function. *J. Clin. Invest.*, **153**, 297–309.

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Effects of dicentrine on haemodynamic, plasma lipid, lipoprotein level and vascular reactivity in hyperlipidaemic rats

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1 The effects of dicentrine on haemodynamic, plasma lipid, lipoprotein level and vascular reactivity were investigated in Wistar-Kyoto (WKY) and spontaneously hypertensive (SH) rats, fed a high fat-high cholesterol diet.

2 In high fat-high cholesterol (HF-HC) diet fed WKY and SH rats, oral administration of dicentrine (5 and 10 mg kg^{-1}, twice a day) for 4 weeks caused significant reductions in total plasma cholesterol (CE) by reducing the low density lipoprotein (LDL) fraction, and reductions in total plasma triglyceride (TG) by reducing the very low density lipoprotein (VLDL) fraction.

3 Dicentrine therapy was associated with increased high density lipoprotein (HDL)-cholesterol levels; thus the ratio of total plasma cholesterol to HDL-cholesterol was improved.

4 In HF-HC diet fed conscious WKY and SH rats, oral administration of dicentrine (5 and 10 mg kg^{-1}, twice a day) also evoked dose-related decreases in mean arterial pressure (MAP) which were of greater magnitude in SH rats. Neither dose of dicentrine caused a significant change in heart rate (HR).

5 The aortic arches from SH rats fed the HF-HC diet for 8 weeks were significantly more affected by the atherosclerotic lesions than the abdominal aortae and renal arteries of WKY and SH rats. Oral administration of dicentrine (5 and 10 mg kg^{-1}) for 4 weeks did not diminish the atherosclerotic lesion areas in WKY and SH rats.

6 In aortae of the hyperlipidaemic rats, significantly attenuated EC_{50} values and augmented maximal responses for phenylephrine-induced contraction were obtained. Endothelium-dependent relaxation to acetylcholine was abolished, while endothelium-independent relaxation to nitroprusside was well preserved. Dicentrine therapy caused significantly augmented EC_{50} values and attenuated maximal responses for phenylephrine-induced contraction in hyperlipidaemic rats. However, dicentrine neither prevented the impaired relaxation to acetylcholine, nor affected the relaxation to nitroprusside during atherosclerosis progression.

7 It is concluded that dicentrine decreases MAP, plasma CE, LDL-CE, plasma TG, VLDL-TG, vascular hyperreactivity to phenylephrine and increases HDL-CE levels. Dicentrine may thus hold potential for the reduction of two of the major risk factors, hypertension and hyperlipidaemia, for cardiovascular disease.

Keywords: Dicentrine; lipid; lipoprotein; mean arterial pressure; vascular reactivity; hyperlipidaemia

Introduction

Hypertension and abnormal plasma lipid levels are two of the major risk factors for cardiovascular diseases. Traditional antihypertensive therapy with β-adrenoceptor blockers or diuretics is now known to affect adversely the plasma lipid profile (Weinberger, 1986; William *et al.*, 1989). Several long-term prospective clinical studies have implicated these changes in plasma lipids as a significant factor in the failure of antihypertensive therapy to reduce the incidence of ischaemia heart diseases and related mortality (William *et al.*, 1989). Recent data demonstrate that α- and β-adrenoceptor blockade can influence lipid metabolism, suggesting that sympathetic activity may play a role in lipid metabolism. Preliminary studies suggest that α-blockade may decrease total plasma cholesterol (CE), low density lipoprotein (LDL)-cholesterol, triglyceride (TG) and increase high density lipoprotein (HDL)-cholesterol (Miller, 1987).

Recently, we found that dicentrine, an aporphine derivative isolated from the plant *Lindera megaphylla*, possessed α_1-adrenoceptor antagonistic action in isolated vessels (Teng *et al.*, 1991) and antihypertensive effect in spontaneously hypertensive (SH) rats (Yu *et al.*, 1992). In this study, we have evaluated the effects of dicentrine on haemodynamic, plasma lipid, lipoprotein and vascular reactivity in SH and WKY rats fed with high fat-high cholesterol (HF-HC) diet.

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Methods

Rat model of hyperlipidaemia

Male SH rats weighing 230–250 g (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) (mean arterial pressure (MAP): 160 ± 9 mmHg; heart rate (HR): 365 ± 12 beats min^{-1}) and age-matched WKY control animals weighing 230–260 g (Biological Research Laboratories, Fullinsdorf, Switzerland) (MAP: 102 ± 4 mmHg; HR: 357 ± 9 beats min^{-1}) were used in this study. The rats were housed in their own cages and given food (as follows) and water *ad libitum*. WKY and SH rats were fed either normal rat chow (No. 5001, Ralston Purina, St. Louis, MO, U.S.A.) or chow supplemented with 20% olive oil and 1% cholesterol (HF-HC diet) for 8 weeks prior to the study and during the dicentrine treatment period. No significant difference in food intake (g/day) was observed among the normal chow, HF-HC chow and dicentrine-treated groups. Since the 1% cholesterol and 20% olive oil diets produce a relatively mild hyperlipidaemia

in the rat (DeLamatre & Roheim, 1981; Krause & Newton, 1985), we added the additional bile acid to increase cholesterol absorption so that we might obtain more marked differences in plasma cholesterol between groups.

The rats were randomly assigned to one of the three following treatment groups: controls, HF-HC chow and dicentrine (5 and 10 mg kg^{-1}, orally, twice a day)/HF-HC chow. At the end of the period of receiving the HF-HC chow and dicentrine treatment/HF-HC chow, the rats MAP and HR were monitored with an electronic analyzer. Rats were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.), a blood sample was obtained for determination of lipoprotein analysis after 16 h overnight fast, and then the rats were killed and various vessels (aortic arch, abdominal aorta and renal artery) were removed.

Measurement of plasma lipids and lipoproteins

Plasma lipoproteins were separated by a one-step density gradient ultracentrifugation technique (Demacker *et al.*, 1983). The very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL) fractions correspond to the density intervals < 1.006 g ml^{-1}, 1.006–1.063 g ml^{-1} and 1.063–1.21 g ml^{-1} respectively. Total plasma cholesterol and the cholesterol concentrations in each lipoprotein fraction were assayed by an enzymatic colorimetric technique with Boehringer Mannheim GmbH diagnostic kits (Roschlau *et al.*, 1974) and triglycerides were measured with Biomerieux kits (Takayama *et al.*, 1977). Apolipoprotein quantitation was performed with the radial immunodiffusion assay described by Chong *et al.* (1987).

Histological examinations

Segments of the three arteries obtained from control and hyperlipidaemic rats, were opened longitudinally and stained with $AgNO_3$. Briefly, the opened segments were mounted on pieces of cork and stained in the dark with $AgNO_3$ (24 mM) in the presence of glucose (233 mM) and HEPES buffer (20 mM) at pH 7.4 for 60 s. After being rinsed with glucose solution, the tissues were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The fixed tissues were dehydrated, embedded in DPX, and their luminal surface was examined by light microscopy.

Segments of the three arteries were opened longitudinally and put into 2.5% glutaraldehyde for fixation. After fixation for at least 8 h, the specimen was dehydrated in 50, 60, 70, 80, 90 and 95% alcohol orderly for 20 min in each concentration. Then, it was put in amyl acetate for 10 min and critical point drying was carried out with liquid carbon dioxide. The specimen was coated with gold and was observed under scanning electron microscope (sEM, JXA-840, JEOL, Japan).

Pharmacological measurements

Rats were anaesthetized with sodium pentobarbitone, and aortic arches or abdominal aortae were isolated and cleaned of surrounding tissue. Aortic rings of about 3 mm in length and mounted in organ baths containing 5 ml Krebs solution (composition mM: NaCl 118, KCl 4.0, $CaCl_2$ 1.9, $MgSO_4$ 1.2, $NaHPO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.7) and equilibrated at 37°C with 95% O_2:5% CO_2 gas mixture. Two stainless steel hooks were inserted into the aortic wall, one was fixed while the other was connected to a transducer. Aortae were equilibrated in the medium for 60 min with three changes of Krebs solution and maintained under an optimal tension of 1 g before specific experimental protocols were initiated. Contractions were recorded isometrically via a force-displacement transducer connected to a Grass polygraph. The cumulative concentration-response curve was obtained with phenylephrine (0.01–30 μM) for 24 min at 3 min intervals. Aortic rings were contracted with 3 μM phenylephrine for 15 min and then subsequently relaxed by the cumulative addition of acetylcholine or nitroprusside. In some experiments, the endothelium was removed mechanically by rubbing the intimal surface with filter paper moistened with the buffer. Relaxation values were expressed as percentage decreases of the phenylephrine (3 μM)-induced contraction.

Data analysis

The experimental results are expressed as the mean ± s.e. mean and accompanied by the number of observations. Statistical significance was assessed by Student's t test and P values less than 0.05 were considered significant.

Drugs

(+)-Dicentrine was prepared according to the method described previously by Chen *et al.* (1991). The following drugs were used: phenylephrine, acetylcholine, nitroprusside, cholesterol and bile acid, all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dicentrine was suspended in arabic gum and distilled water. Control rats were given 1 ml kg^{-1} of the vehicle.

Results

Plasma lipid and lipoprotein profiles

The plasma lipid and lipoprotein profiles of WKY and SH rats fed either normal or high fat-high cholesterol (HF-HC) chow are summarized in Table 1. Total plasma CE, VLDL-, LDL-CE, total plasma TG, VLDL-, LDL- and HDL-TG levels were markedly elevated in HF-HC chow fed groups compared with the normal chow fed rats. However, the HDL-CE was decreased in HF-HC chow rats (Table 1). The increases of plasma CE and TG levels were time-dependent (Figure 1). The increase in total plasma CE appeared generally to plateau out whereas the TG level appeared to increase over the whole time period of the study. Although the data also showed that higher levels of CE and TG were achieved in SH rats than in WKY rats, there were no statistical differences.

Table 1 Plasma lipid and lipoprotein profiles of WKY and SH rats treated with either normal or high fat-high cholesterol chow

		Cholesterol (mg dl^{-1})					*Triglyceride* (mg dl^{-1})			
Rat		*Total*	*VLDL*	*LDL*	*HDL*	*CE/HDL*	*Total*	*VLDL*	*LDL*	*HDL*
WKY	Normal chow	55 ± 1	8 ± 2	28 ± 2	17 ± 1	3.23 ± 0.8	114 ± 14	91 ± 4	12 ± 4	8 ± 3
	HF-HC chow	218 ± 6**	38 ± 2**	170 ± 4**	9 ± 1**	24.22 ± 2	231 ± 10**	180 ± 8**	22 ± 5	26 ± 2**
SH	Normal chow	60 ± 1	9 ± 2	36 ± 2	19 ± 3	3.16 ± 0.5	97 ± 6	76 ± 3	9 ± 2	12 ± 1
	HF-HC chow	244 ± 3**	40 ± 1**	195 ± 3**	9 ± 1*	27.11 ± 2	271 ± 12**	200 ± 5**	35 ± 7*	30 ± 4**

Values are means ± s.e. mean for n observations ($n = 9$). VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; HF-HC, high fat-high cholesterol.
*$P < 0.01$ and **$P < 0.001$ denote significant differences between normal and HF-HC chow values in each compared group of WKY or SH rats.

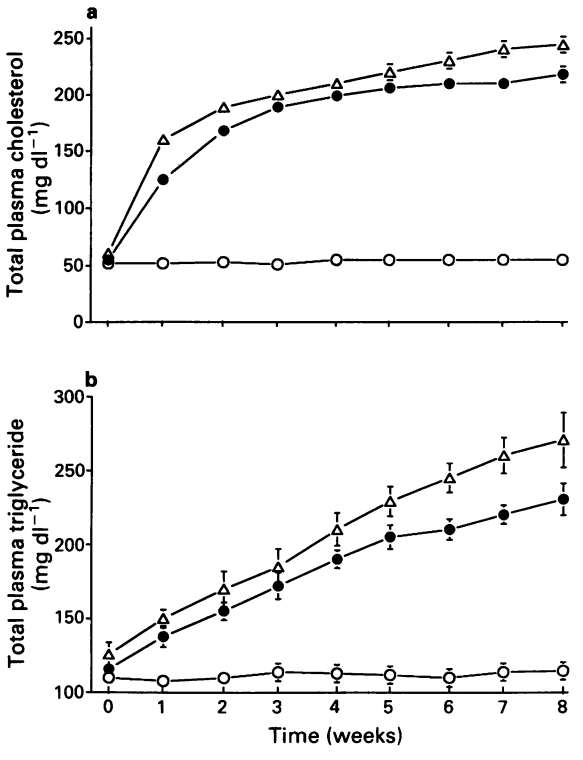


Figure 1 Total plasma cholesterol (a) and triglyceride (b) levels of normal chow fed rats (○), HF-HC chow fed rat (WKY ●, SH rats △) during experimental periods. Each point represents the mean and vertical bars show s.e. mean ($n = 15-20$).

Effects of dicentrine on haemodynamic, plasma lipid and lipoprotein profiles in hyperlipidaemia rats

Oral administration of dicentrine (5 and 10 mg kg^{-1}, twice a day) for 4 weeks affected plasma lipid, lipoprotein levels and as well as mean arterial pressure (MAP) in WKY and SH rats treated with HF-HC chow (Table 2). Dicentrine evoked a dose-related hypotensive effect, which was of greater magnitude in SH rats (5 mg vs 10 mg kg^{-1} of dicentrine-evoked hypotensive effect was not statistically different in WKY groups, whereas $P<0.01$ in SH groups). A higher dose of dicentrine (10 mg kg^{-1}) did not cause significant changes in heart rate (HR) of WKY and SH rats (Table 2). Both in WKY and SH rats receiving the HF-HC diet, dicentrine caused dose-dependent reductions in total plasma CE by reducing the LDL fraction, and reductions in total plasma TG by reducing VLDL fraction (Figure 2). Dicentrine treatment was associated with increased HDL-CE levels, thus the ratio of total plasma CE to HDL-CE was improved (Figure 3).

Histological examination of the intimal surface of the hyperlipidaemic arteries

No visible atherosclerotic lesions were detected in any vessels removed from control rats. Light microscopic study of vessels from HF-HC fed WKY and SH rats indicated that the aortic arch had grossly visible dots of lipid deposition. The thickness of the fatty streaks significantly augmented with the time rats were fed the HF-HC diet (data not shown). The aortic arches from SH and WKY rats fed the HF-HC diet for 8 weeks were significantly more affected by the atherosclerotic lesions than the abdominal aortae and renal arteries of WKY and SH rats. However, the lesions of aortic arches of SH rats were more severe than in WKY rats fed with the same HF-HC diet.

Effects of dicentrine on the vascular reactivity in hyperlipidaemic rats

Increasing concentrations of phenylephrine (0.01–30 μM) evoked concentration-dependent contraction in control segments of the aortic arch and abdominal aorta (Figure 4). Both in the aortic arch and abdominal aorta of WKY and SH rats fed HF-HC diet for 8 weeks, significantly attenuated EC_{50} values and augmented maximal responses for phenylephrine were obtained (Table 3, Figure 4). Oral administration of dicentrine (5 and 10 mg kg^{-1}, twice a day) for 4 weeks, inhibited the contraction caused by phenylephrine in a concentration-dependent manner in aortic arch and abdominal aorta of hyperlipidaemic rats (Figure 4). Dicentrine (5 and 10 mg kg^{-1}) caused significantly augmented EC_{50} values and attenuated maximal responses for phenylephrine-induced contraction in hyperlipidaemic WKY and SH rats. In SH rats, the augmented EC_{50} values and attenuated maximal responses of phenylephrine caused by dicentrine were more pronounced than in WKY (Table 3).

During contractions caused by phenylephrine (3 μM) for 15 min, cumulative addition of acetylcholine (0.01–30 μM) evoked concentration-dependent relaxation in control segments of the aortic arch and abdominal aorta (Figure 5). However, whilst the concentration-dependent relaxation to acetylcholine was significantly attenuated, that to nitroprusside was unaffected in hyperlipidaemic WKY and SH rats (Table 3, Figure 5). Oral administration of dicentrine (5 and 10 mg kg^{-1}) for 4 weeks, neither prevented the impaired relaxation to acetylcholine, nor affected the relaxation to nitroprusside in hyperlipidaemic rats (Figure 5).

Table 2 Effects of dicentrine on haemodynamic, plasma lipid, lipoprotein and glucose levels in WKY and SH rats treated with high fat-high cholesterol chow

	WKY			*SH rats*		
	Control	*Dicentrine*		*Control*	*Dicentrine*	
		5 mg kg^{-1}	10 mg kg^{-1}		5 mg kg^{-1}	10 mg kg^{-1}
MAP (mmHg)	100 ± 3	87 ± 2**	81 ± 4***	160 ± 3	119 ± 5***	102 ± 3***
HR (beats min^{-1})	357 ± 9	349 ± 11	347 ± 12	365 ± 14	358 ± 10	356 ± 14
CE (mg dl^{-1})	218 ± 6	166 ± 6***	94 ± 4***	244 ± 3	146 ± 5***	79 ± 6***
TG (mg dl^{-1})	231 ± 10	142 ± 12***	122 ± 10***	271 ± 12	136 ± 18***	110 ± 10***
Apo-B (mg dl^{-1})	20 ± 3	15 ± 2	10 ± 1**	23 ± 4	13 ± 2*	9 ± 3**
Glucose (mg dl^{-1})	142 ± 11	143 ± 7	144 ± 8	136 ± 8	140 ± 5	147 ± 5

Values are means ± s.e. mean for n observations ($n = 9-15$). MAP, mean arterial pressure (mmHg); HR, heart rate (beats min^{-1}); CE, cholesterol (mg dl^{-1}); TG, triglyceride (mg dl^{-1}); Apo-B, apolipoprotein B (mg dl^{-1}).
*$P<0.05$; **$P<0.01$ and ***$P<0.001$ denote significantly different from the respective control values.

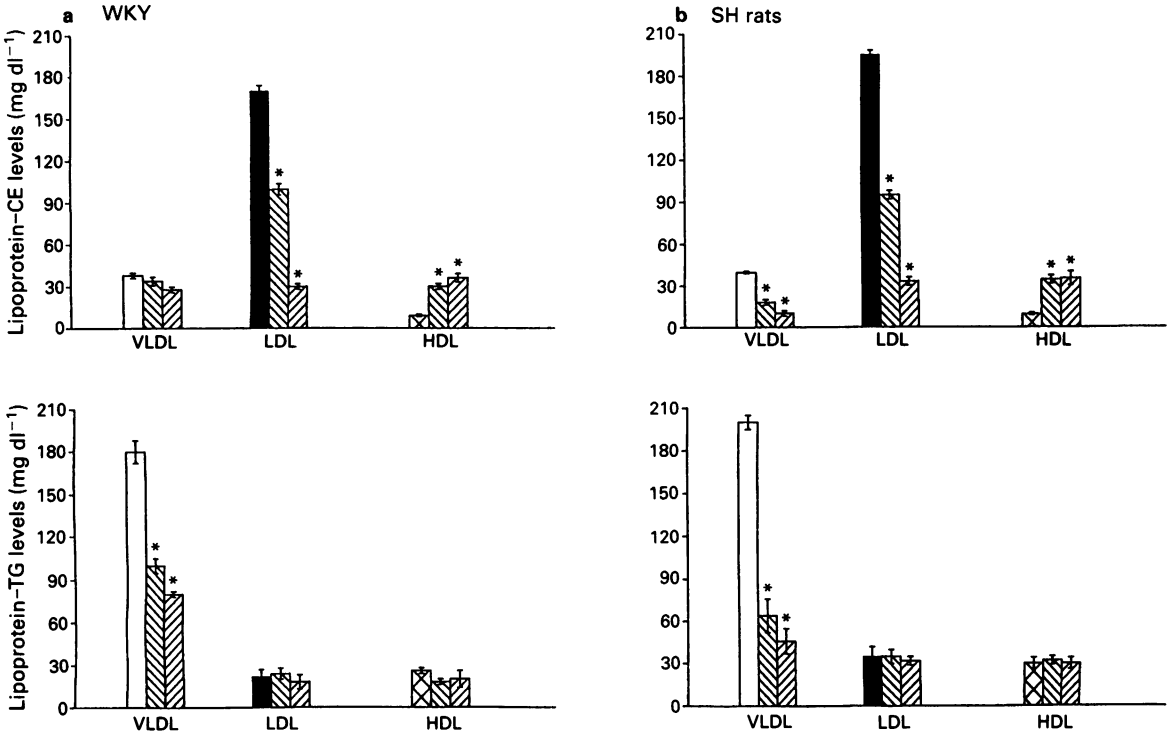


Figure 2 Changes in plasma cholesterol (CE) and triglyceride (TG) concentrations in each lipoprotein fraction (□, VLDL; ■, LDL; ⊠, HDL) produced by dicentrine (▨, 5; ▨ 10 mg kg^{-1}, p.o. twice a day) treatment for 4 weeks in HF-HC diet fed WKY (a) and SH (b) rats. VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein. Each column represents the mean and vertical bars show s.e.mean ($n = 8-10$). *$P < 0.001$ different from untreated (control) rats.

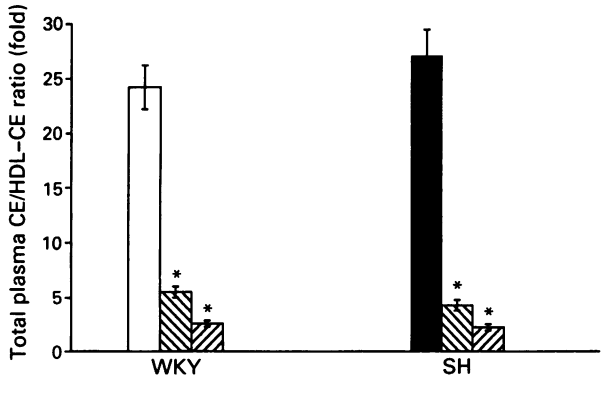


Figure 3 Changes in total plasma cholesterol (CE)/high density lipoprotein (HDL)-CE ratio produced by dicentrine (▨, 5; ▨, 10 mg kg^{-1}, p.o.) treatment for 4 weeks in HF-HC diet fed WKY (□) and SH rats (■). Each column represents the mean and vertical bars show s.e. mean ($n = 8-10$). *$P < 0.001$ different from untreated (control) rats.

Discussion

The results of this study indicate that when fed a HF-HC diet, WKY and SH rats have elevated total plasma CE, TG, decreased HDL-CE levels and dramatically increased CE/HDL-CE ratio as compared with normal chow-fed rats. These data are consistent with those of Uchida *et al.* (1978), who reported that rats hyperrespond to HF-HC feeding. There is evidence from epidaemiological studies that increases in plasma CE, TG and decreases in HDL-CE levels are risk factors for coronary artery disease (Miller & Miller, 1975). The important finding of this study is that dicentrine administration results in reduction of plasma CE, TG, LDL-CE, VLDL-TG and apolipoprotein-B levels while increasing HDL-CE. A strong positive relationship exists between the severity of atherosclerosis and plasma levels of total CE, LDL-CE and its principal apolipoprotein, apolipoprotein-B (Castelli & Anderson, 1986). Plasma TG and VLDL-TG levels have been positively associated with the development of coronary heart disease (CHD) (Krzesinski *et al.*, 1988). On the other hand, a strong inverse relationship has been demonstrated between severity of atherosclerosis and plasma levels of HDL-CE (Wallace *et al.*, 1986). Although several studies show that lowering LDL-CE concentrations reduces the incidence of atherosclerotic disease, it has not been demonstrated that raising HDL-CE concentrations alone will reduce the risk of future disease (Chobanian, 1983; Dall'Aglio *et al.*, 1983). The present study demonstrates that dicentrine not only results in reduction of LDL-CE, but also produces an increase in HDL-CE concentrations, so that higher levels are achieved in dicentrine-treated groups. However, the mechanisms of action of dicentrine-increased HDL-CE levels need further investigation. Thus, in contrast to diuretics and β-blockers which may increase plasma CE, LDL-CE, TG and reduce HDL-CE levels, dicentrine can be used to lower blood pressure in hypertension without the disadvantage of offsetting alterations in the lipoprotein profile. Selective α_1- and β-blockers affect plasma lipid and lipoprotein levels differently (Weinberger, 1986). In general, selective α_1-blockers lead to a more favourable lipid profile than β-blockers. How adrenergic blockade affects these changes is largely unknown. Whether the decrease of plasma TG and CE by dicentrine is due to direct increase of lipoprotein lipase and decrease of HMG-Co A reductase activity needs further investigation.

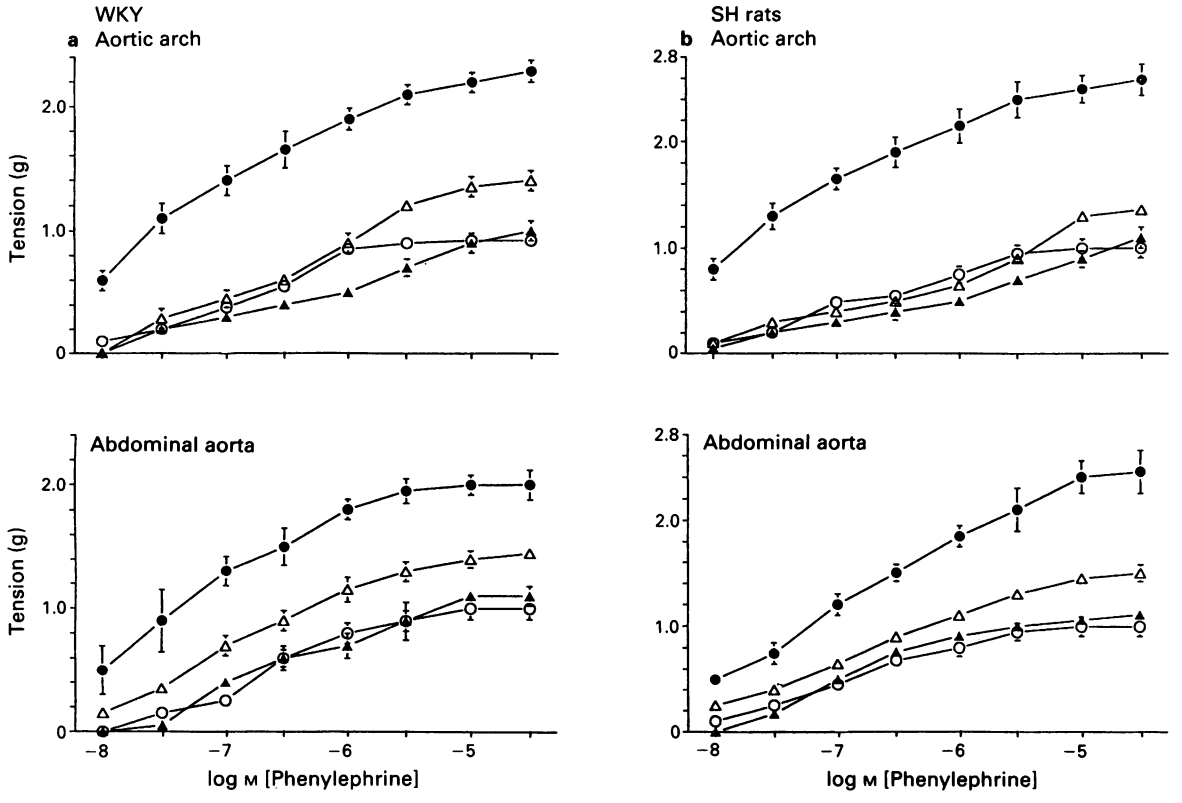


Figure 4 Cumulative concentration-response curves to phenylephrine in aortic arch and abdominal aorta obtained from normal chow (○), HF-HC chow fed rats (WKY, a; SH rats, b) (●) and dicentrine (△, 5; ▲, 10 mg kg^{-1}, p.o.) treatment for 4 weeks in hyperlipidaemic WKY and SH rats. Each point represents the mean and vertical bars show s.e. mean ($n = 6-8$).

Table 3 Effects of dicentrine on the vascular reactivity in aortic arch of WKY and SH rats

		Maximum response			EC_{50} (× 10^{-7} M)		
		PE (g)	*ACh* (% relaxation)	*NP* (% relaxation)	*PE*	*ACh*	*NP*
WKY	Normal chow	0.92 ± 0.09	74.01 ± 4.21	94.01 ± 4.00	2.00 ± 0.12	2.79 ± 0.05	1.59 ± 0.08
	HF-HC chow	2.29 ± 0.15	15.00 ± 3.61	95.01 ± 2.22	0.36 ± 0.08	3.00 ± 0.15	1.55 ± 0.06
	HF-HC chow + Dicentrine 5 mg kg^{-1}	1.4 ± 0.12*	12.00 ± 2.31	93.12 ± 3.00	8.87 ± 0.06*	3.85 ± 0.06	1.45 ± 0.08
	10 mg kg^{-1}	1.0 ± 0.12*	11.00 ± 0.09	96.11 ± 3.61	12.50 ± 0.07*	4.94 ± 0.05*	1.40 ± 0.06
SH	Normal chow	1.00 ± 0.08	70.21 ± 3.65	96.51 ± 3.59	1.79 ± 0.23	3.10 ± 0.06	1.58 ± 0.07
	HF-HC chow	2.59 ± 0.14	11.00 ± 3.62	89.00 ± 3.00	0.32 ± 0.03	3.10 ± 0.04	1.89 ± 0.09
	HF-HC chow + Dicentrine 5 mg kg^{-1}	1.36 ± 0.08*	9.51 ± 0.08	93.61 ± 2.33	11.70 ± 0.12*	3.86 ± 0.05	1.98 ± 0.06
	10 mg kg^{-1}	1.09 ± 0.14*	11.01 ± 2.00	95.00 ± 2.41	16.75 ± 0.14*	4.86 ± 0.04*	1.99 ± 0.07

Values are means ± s.e. mean for n observations ($n = 9$). PE, phenylephrine; ACh, acetylcholine; NP, nitroprusside; HF-HC, high fat-high cholesterol.
*$P < 0.001$ denotes significantly different from data obtained from high fat-high cholesterol chow dicentrine-untreated animals.

Results from several studies have demonstrated that there is a greater sympathetic activity and noradrenaline concentration in SH rats (Yu *et al.*, 1992). This may explain why oral administration of dicentrine evoked greater decreases in MAP and contraction to phenylephrine in SH rats and supports the concept that dicentrine acts via inhibition of α-adrenoceptor activity. Hyperlipidaemia is known to enhance the sensitivity of the circulatory system to adrenergic stimulus (Rosendorff *et al.*, 1981; Heistad *et al.*, 1984); Broderick & Tulenko (1984) for example, reported that dose-response changes in coronary vascular resistance with noradrenaline was shifted to the left in hyperlipidaemic rats. They suggested that this could be due to an increase in either the number or affinity of the α-adrenoceptors. The results of the present study show an enhanced vasoconstrictor responsiveness to phenylephrine in rats with high levels of plasma lipid. Although the mechanism of these responses remains to be determined, the data suggest that adrenergic vascular control is altered in the presence of high levels of plasma lipid. The vascular sensitivity to phenylephrine was increased in hyperlipidaemic rats, thus α_1-adrenoceptor blockade with dicentrine produced significantly attenuated maximal responses and augmented EC_{50} values for phenylephrine.

In hyperlipidaemic rat aortae, endothelium-dependent re-

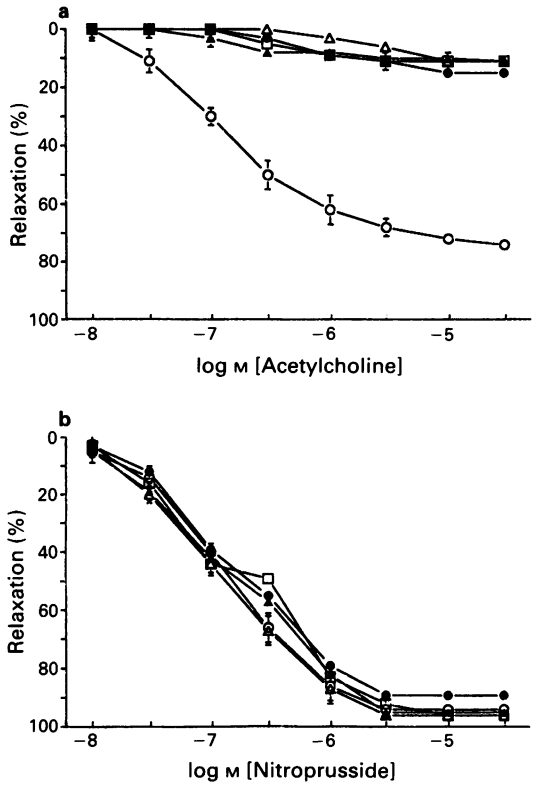


Figure 5 Endothelium-dependent relaxation to acetylcholine (a) and endothelium-independent relaxation to nitroprusside (b) in aortic arch obtained from normal chow (○), HF-HC chow fed rats (WKY ●; SH rats △) and dicentrine (▲, 5; □, 10 mg kg^{-1}, p.o.) treatment for 4 weeks in hyperlipidaemic WKY and SH rats. Each point represents the mean and vertical bars show s.e. mean ($n = 6-8$).

laxation to acetylcholine was decreased and was progressively attenuated during atherosclerotic progression, while relaxation to nitroprusside was well preserved. There are a number of reports which demonstrate that endothelium-dependent relaxation is impaired in atherosclerotic arteries obtained from animals and man (Hirata *et al.*, 1992). There are several possible mechanisms for the impairment of endothelium-dependent relaxation in atherosclerotic arteries. First, the production and/or release of endothelium-derived relaxing factor (EDRF) may be decreased. Second, the thickened intima may reduce the diffusion of EDRF, the half-life of which is extremely short. Third, the characteristics of smooth muscle cells and their sensitivity to EDRF may be changed. The last possibility is unlikely because the relaxation to nitroprusside, which induces relaxation through activation of guanylate cyclase in smooth muscle cells as does EDRF, was well maintained in atherosclerotic arteries. Recently, Kolodgie *et al.* (1990) have demonstrated that endothelium-mediated relaxation is reduced in hyperlipidaemic rabbits because of a loss of endothelial cells. At present, precise mechanisms for the attenuation of EDRF-mediated relaxation in atherosclerotic arteries are still debated.

In conclusion, dicentrine decreases MAP, plasma CE, LDL-CE, apolipoprotein-B, plasma TG, VLDL-TG, vascular hyperreactivity to phenylephrine and increases HDL-CE levels. Dicentrine may thus hold potential for the reduction of two of the major risk factors, hypertension and hyperlipidaemia, for cardiovascular disease.

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References

BRODERICK, R. & TULENKO, T.N. (1984). Mechanism of cholesterol-induced arterial supersensitivity [Abstract]. *Fed. Proc.*, **43**, 350.

CASTELLI, W.P. & ANDERSON, K. (1986). A population at risk. Prevalence of high cholesterol levels in hypertensive patients in the Framingham study. *Am. J. Med.*, **80**, (suppl 2A), 23–32.

CHEN, C.C., HUANG, Y.L., OU, J.C., SU, M.J., YU, S.M. & TENG, C.M. (1991). Bioactive principles from the roots of *Lindera megaphylla*. *Planta Medica*, **57**, 406–408.

CHOBANIAN, A. (1983). The influence of hypertension and other haemodynamic factors in atherogenesis. *Prog. Cardiovasc. Dis.*, **26**, 177–196.

CHONG, K.S., NICOLOSI, R.J. & RODGER, R.F. (1987). Effect of dietary fat saturation on plasma lipoproteins and high density lipoprotein metabolism of the rhesus monkey. *J. Clin. Invest.*, **79**, 675–683.

DALL'AGLIO, E., CHANG, H. & REAVEN, G. (1983). Disparate effects of prazosin and propranolol on lipid metabolism in a rat model. *Metabolism*, **32**, 510–513.

DE LAMATRE, J.G. & ROHEIM, P.S. (1981). Effect of cholesterol feeding on apo B and apo E concentrations and distributions in euthyroid and hypothyroid rats. *J. Lipid Res.*, **22**, 297–306.

DEMACKER, P.N., VAN SOMMEREN-ZONDAG, D.F., STALENHOEF, A.F., STUYT, P.M. & VANT LAAR, A. (1983). Ultracentrifugation in swinging-bucket and fixed-angle rotors evaluated for isolation and determination of high density lipoprotein subfractions HDL_2 and HDL_3. *Clin. Chem.*, **29**, 656–663.

HEISTAD, D.D., ARMSTRONG, M.L., MARCUS, M.L., PIEGORS, D.J. & MARK, A.L. (1984). Augmented responses to vasoconstrictor stimuli in hypercholesterolemic and atherosclerotic monkeys. *Circ. Res.*, **54**, 711–718.

HIRATA, K.I., AKITA, H., YOKOYAMA, M. & WATANABE, Y. (1992). Impaired vasodilatory response to atrial natriuretic peptide during atherosclerosis progression. *Arteriosclerosis*, **12**, 99–103.

KOLODGIE, F.D., VIRMANI, R., RICE, H.E. & MERGNEG, W.J. (1990). Vascular reactivity during the progression of atherosclerotic plaque: a study in Watanabe heritable hyperlipidemic rabbits. *Circ. Res.*, **66**, 1112–1117.

KRAUSE, B.R. & NEWTON, R.S. (1985). Apolipoprotein responses associated with the plasma lipid-regulating effect of gemfibrozil in cholesterol-fed rats. *J. Lipid Res.*, **26**, 940–949.

KRZESINSKI, J.M., CARLIER, P.G. & RORIVE, G.L. (1988). Interrelationship of hypertension, plasma lipids and atherosclerosis. *Drug*, **36** (suppl 2), 18–26.

MILLER, G.J. & MILLER, N.E. (1975). Plasma high density lipoprotein concentration and development of ischemic heart disease. *Lancet*, **i**, 16–19.

MILLER, N.E. (1987). Effects of adrenoceptor-blocking drugs on plasma lipoprotein concentrations. *Am. J. Cardiol.*, **60**, 17E–23E.

ROSCHLAU, P., BERNT, E. & GRUBER, W. (1974). Enzymatic determination of total cholesterol in serum using peroxidase as indicating enzyme. *J. Clin. Chem. Clin. Biochem.*, **12**, 226.

ROSENDORFF, C., HOFFMAN, J.I.E., VERRIER, E.D., ROULEAU, J. & BOERBOOM, L.E. (1981). Cholesterol potentiates the coronary artery response to norepinephrine in anaesthetized and conscious dogs. *Circ. Res.*, **48**, 320–329.

TAKAYAMA, J., ITOH, S., NAGASAKI, T. & TANIMIZU, I. (1977). A new enzymatic method for determination of serum choline-containing phospholipids. *Clin. Chem. Acta*, **79**, 93–98.

TENG, C.M., YU, S.M., KO, F.N., CHEN, C.C., HUANG, Y.L. & HUANG, T.F. (1991). Dicentrine, a natural vascular α_1-adrenoceptor antagonist, isolated from *Lindera megaphylla*. *Br. J. Pharmacol.*, **104**, 651–656.

UCHIDA, K., NORMURA, Y., KADOWAKI, M., TAKASE, H. & TAKEUCHI, N. (1978). Disturbance of cholesterol and bile acid metabolism in spontaneously hypertensive rats (SHR). *J. Biochem.*, **84**, 1113–1118.

WALLACE, R.B., HUNNINGHAKE, D.B., CHAMBLESS, L.E., HEISS, G., WAHL, P. & BARETT-CONNOR, E. (1986). A screening survey of dyslipoproteinemias associated with prescription drug use. The Lipid Research Clinics Program Prevalence Study. *Circulation*, **73** (suppl 1), I-70–I-79.

WEINBERGER, M.H. (1986). Antihypertensive therapy and lipids. Paradoxical influences on cardiovascular disease risk. *Am. J. Med.*, **80** (suppl 2A), 64–70.

WILLIAM, H., KARGE III, B.S., EDWARD, J.W., MARK, C.K., ROBERT, M.G. & ROBERT, J. (1989). Effects of prazosin on hemodynamics, hemostasis, and serum lipid and lipoprotein levels in normal and hypercholesterolemic monkeys. *Am. J. Med.*, **86** (suppl 1B), 19–23.

YU, S.M., HSU, S.Y., KO, F.N., CHEN, C.C., HUANG, Y.L., HUANG, T.F. & TENG, C.M. (1992). Haemodynamic effects of dicentrine, a novel α_1-adrenoceptor antagonist: comparison with prazosin in spontaneously hypertensive and normotensive Wistar-Kyoto rats. *Br. J. Pharmacol.*, **106**, 797–801.

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Effects of nifedipine and ryanodine on adrenergic neurogenic contractions of rat vas deferens: evidence for a pulse-to-pulse change in Ca^{2+} sources

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1 The effects of nifedipine and ryanodine on the adrenergic component of neurogenic contractions of the rat isolated vas deferens were studied in an attempt to identify the sources of Ca^{2+} mediating the contraction. The tissue was electrically stimulated by single pulses or pairs of widely spaced pulses. The purinergic component of contraction was suppressed by the presence of 300 μM suramin.

2 In Mg^{2+}-free medium, nifedipine (0.01–10 μM) reduced the first and, to a greater extent, the second twitch elicited by two pulses 3 s apart. This pattern of inhibition was observed both in the absence of rauwolscine (when twitch 2 was smaller than twitch 1) and in the presence of 0.1 μM rauwolscine (when, due to interruption of prejunctional α_2-adrenoceptor-mediated autoinhibition, twitch 2 was of similar height to twitch 1). Nifedipine reduced only twitch 2 but not twitch 1 in medium containing 1.2 mM Mg^{2+}.

3 Single pulses of increasing current strength elicited increasing contraction. Nifedipine reduced contractions by about the same absolute extent at all current strengths, so that the relative contribution of the nifedipine-sensitive component decreased with increasing current strength.

4 When the pulse interval in a pair was increased from 5 to 60 s, the inhibition by nifedipine of the second twitch was most marked at an interval of 5 s and declined as the interval increased.

5 In contrast to nifedipine, 20 μM ryanodine reduced the first twitch of a pair to a greater extent than a second twitch 5 s later, so that twitch 2 became greater than twitch 1. The inhibition by ryanodine of twitch 2 increased with increasing pulse interval.

6 In vasa deferentia preincubated with [^{3}H]-noradrenaline, 1 μM nifedipine and 20 μM ryanodine did not change the electrically evoked overflow of tritium, whereas 10 μM nifedipine increased it.

7 It is concluded that, when the sympathetic axons of the vas deferens are stimulated by a single pulse (or the first pulse of a pair) in Mg^{2+}-free medium, both Ca^{2+} mobilization inside the smooth muscle cells and Ca^{2+} entry contribute to the ensuing adrenergic contraction. The relative contribution of Ca^{2+} entry is small at maximal stimulus strength but increases with decreasing stimulus strength. When a second pulse follows the first after an appropriate interval, a switch of Ca^{2+} sources occurs: intracellular Ca^{2+} mobilization is decreased during twitch 2, whereas Ca^{2+} entry is increased.

Keywords: Rat vas deferens; nifedipine; ryanodine; α_1-adrenoceptors; Ca^{2+} entry; Ca^{2+} mobilization; prejunctional α_2-autoinhibition

Introduction

The contraction of the rat vas deferens elicited by a single electric pulse, adjusted to stimulate exclusively neural elements, is biphasic, consisting of a rapid and a slow phase (McGrath, 1978). The response to trains of pulses depends on the frequency. When the frequency is low, e.g. 0.5 Hz, each pulse elicits a discrete twitch, with a characteristic development of the fast and slow phases; the fast phase remains relatively constant, whereas the slow phase declines and can become undetectable in the second twitch. When the frequency is high, e.g. 5 Hz, the responses to the individual pulses merge to a tetanic contraction which reaches an initial peak within 1 s (transient contraction), then falls off, and finally rises again (sustained contraction; Swedin, 1971; Brown *et al.*, 1983; Amobi & Smith, 1987).

These various contraction phenomena differ both in the transmitters involved and in the sources of Ca^{2+} used by the smooth muscle cells. The slow phase of single pulse-induced contractions is abolished by α-adrenoceptor antagonists and is therefore mediated by noradrenaline; the fast phase, in contrast, is resistant to α-adrenoceptor antagonists. The tetanus elicited by high-frequency trains contains both an adrenergic and an α-adrenoceptor antagonist-resistant component throughout its duration (Swedin, 1971; McGrath, 1978; Brown *et al.*, 1983; Amobi & Smith, 1987). Experiments with the P_2-purinoceptor desensitizing agent α,β-methylene ATP (Meldrum & Burnstock, 1983) and the P_2-purinoceptor antagonist, suramin (Dunn & Blakeley, 1988) have now shown that the non-adrenergic components are mediated by ATP (Mallard *et al.*, 1989; Bourreau *et al.*, 1991; for review see Burnstock, 1990; von Kügelgen & Starke, 1991). The marked fall of the slow adrenergic but not the fast purinergic phase during low-frequency trains probably reflects a preferential prejunctional α_2-adrenergic autoinhibition of the adrenergic phase (Brown *et al.*, 1983; cf. for mouse vas deferens von Kügelgen *et al.*, 1989; Bültmann *et al.*, 1991).

Even before the availability of P_2-purinoceptor antagonists or desensitizing agents, the calcium channel blocker nifedipine was found to suppress selectively the fast phase of the response of the vas deferens to single or low-frequency pulses while leaving the slow phase unaffected (French & Scott, 1981; Brown *et al.*, 1983; Hay & Wadsworth, 1983; see also Amobi & Smith, 1987; Mallard *et al.*, 1992). In the tetanic response to high frequency, nifedipine reduced the transient initial peak but abolished the sustained contraction (Brown *et al.*, 1983; Amobi & Smith, 1987; Mallard *et al.*, 1992). In

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conjunction with present knowledge about cotransmission, these findings have been interpreted to indicate that the purinergic contraction components are nifedipine-sensitive and, therefore, due to Ca^{2+} entry from the extracellular space; that the adrenergic component of the late sustained tetanic contraction is also nifedipine-sensitive and due to Ca^{2+} entry; whereas the adrenergic slow phase of single pulse-evoked twitches is nifedipine-resistant and presumably due to intracellular Ca^{2+} mobilization. The nifedipine sensitivity of the purinergic components is well established (e.g. Bourreau *et al.*, 1991). It has also been shown that the adrenergic component of the late sustained tetanus, when isolated by treatment with α,β-methylene ATP, is in fact abolished by nifedipine (Bourreau *et al.*, 1991). However, when we isolated the adrenergic slow phase of low frequency-induced contractions by treatment with suramin in Mg^{2+}-free medium we observed that, unexpectedly, the first twitch and, to an even greater extent, the subsequent twitches were reduced by nifedipine and thus seemed to contain a component of Ca^{2+} entry, in contrast to previous views (Brown *et al.*, 1983; Amobi & Smith, 1987). These observations are described here, together with the effect of ryanodine, which blocks intracellular Ca^{2+} mobilization, a second possible source of Ca^{2+} for contraction (Iino, 1990). Some results have been reported previously in abstract form (Bültmann & Starke, 1992).

Methods

Male Wistar rats were decapitated and the vasa deferentia removed and cleaned of adherent tissue. Unless stated otherwise, experiments were carried out on tissues from rats 6 to 7 weeks old (150–180 g, 'young' rats). For several later series animals at least 12 weeks old were used (280–340 g; 'mature' rats). The vasa deferentia from mature rats displayed less post-stimulus oscillations in tension (see Results) which, especially in the presence of ryanodine, interfered with the quantification of neurogenic contractions.

Unless stated otherwise, the medium used for incubation and superfusion 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. It was saturated with 95% O_2/5% CO_2 and kept at 37°C.

Contraction

The vasa deferentia were suspended vertically in an organ bath containing 5.7 ml medium. The lower end was fixed and the upper end attached to an isometric force transducer (K30; Hugo Sachs Elektronik, Hugstetten, Germany) under an initial tension of 9.8 mN. The medium was replaced every 15 min. Suramin, 300 μM, was added in all experiments from the beginning in order to suppress the purinergic component of contractions. Solutions of drugs were added to the organ bath in aliquots not exceeding 30 μl.

During a 60 min equilibration period the tissues were allowed to relax to a resting tension of approximately 3 mN. This final resting tension remained constant during the course of the experiment. A Stimulator II (Hugo Sachs Elektronik) operating in the constant current mode was used for electrical stimulation. The platinum electrodes were located at the top and the bottom of the organ bath (0.3 ms pulse width, current strength 100 mA or as indicated). Contractions were recorded on a Graphtec thermal pen recorder (Ettlingen, Germany). In some experiments responses were digitized at a frequency of 30 Hz by means of an analog-digital converter and stored on a microcomputer for later replay.

The IC_{50} value of nifedipine was interpolated as the concentration producing 50% of the inhibition obtained with nifedipine 1 μM, which produced the maximal inhibition and was the highest concentration that did not alter the evoked release of [^{3}H]-noradrenaline (see Results).

Drug and solvent experiments were always carried out in parallel. Whenever drug-induced changes were calculated, they were corrected for the mean change occurring in the corresponding solvent group.

Other experimental details are explained in the Results section.

Tritium overflow

The medium did not contain suramin in [^{3}H]-noradrenaline experiments. Vasa deferentia were split open longitudinally. Four pieces of about 10 mg were cut from the prostatic portions. The eight pieces were incubated for 30 min in medium with reduced Ca^{2+} ($CaCl_2$ 0.2 mM; see Limberger *et al.*, 1992) containing 0.2 μM (-)-[^{3}H]-noradrenaline, specific activity 43.7 Ci mmol^{-1}. They were then rinsed and one piece was transferred to each of six 0.16 ml superfusion chambers (two pieces were discarded). The tissue was held by a polypropylene mesh between platinum wire electrodes 6 mm apart and superfused with [^{3}H]-noradrenaline-free medium at 2 ml min^{-1} ($CaCl_2$ 2.5 mM). A Stimulator I (Hugo Sachs Elektronik) operating in the constant voltage mode was used for electrical stimulation. An initial stimulation period (180 pulses, 1 Hz, 1 ms pulse width, 24 V cm^{-1} voltage drop between the electrodes of each chamber, yielding a current of 40 mA) was applied after 30 min of superfusion; it was not used for determination of tritium overflow. The tissues were then stimulated six times (S_1 to S_6) after 78, 96, 114, 184, 202 and 220 min of superfusion. Each of the six stimulation periods consisted either of one train of 6 pulses at a frequency of 100 Hz, or of two trains of 6 pulses/100 Hz, train interval 3 s, or of two trains of 6 pulses/100 Hz, train interval 10 s. The superfusate was collected in 2, 10 or 30 min periods from 74 min of superfusion onwards. Drugs and solvents were added to the superfusion medium after S_3, 64 min before S_4. At the end of the experiment, the tissue pieces were solubilized in 1 ml Soluene (Packard Instrument, Frankfurt am Main, Germany). Tritium was measured in superfusate samples and solubilized tissues by liquid scintillation spectrometry.

The outflow of tritium was calculated as a fraction of the amount of tritium in the tissue at the start of the respective collection period (fractional rate of outflow, min^{-1}). The stimulation-evoked overflow was calculated as the difference between the total outflow of tritium in the 4 min following the start of a stimulation period, and the estimated basal outflow during this time (all in nCi); the basal outflow was assumed to decline linearly from the 2 min interval before, to the interval 4–6 min after the start of stimulation; the difference (total minus basal) was expressed as a percentage of the amount of tritium in the tissue (in nCi) at the start of the stimulation period. For further evaluation of the basal efflux, ratios were calculated of the fractional rate of outflow in the 2 min before S_4 over that in the 2 min before S_3. For further evaluation of the stimulation-evoked overflow, the sum of the overflow elicited by S_1 to S_3 was formed for each experiment ('S_{1-3}'), as was the sum of the overflow elicited by S_4 to S_6 ('S_{4-6}'), and ratios S_{4-6}/S_{1-3} were then calculated.

Materials

The following drugs were used: suramin hexasodium salt (Bayer, Wuppertal, Germany); ryanodine (Biotrend, Köln, Germany); (-)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 43.7 Ci mmol^{-1} (Du Pont, Dreieich, Germany); prazosin HCl (Pfizer, Karlsruhe, Germany); rauwolscine HCl (Roth, Karlsruhe, Germany); nifedipine, tetrodotoxin (Sigma, Deisenhofen, Germany). Stock solutions of nifedipine and ryanodine were prepared in absolute ethanol and methanol, respectively; maximal final concentrations of ethanol and methanol were below 0.1 and 0.2%, respectively. Suramin,

prazosin and rauwolscine were dissolved in distilled water. Tetrodotoxin was dissolved in 0.1 M sodium acetate buffer pH 4.85. Experiments with nifedipine were carried out in near darkness or under sodium light.

Statistics

The arithmetic mean and s.e.mean are given throughout, except for IC_{50} values which are geometric means with 95% confidence intervals (c.i.). Differences between means were tested for statistical significance by the Mann-Whitney test. Differences with error probabilities of $P<0.05$ were taken to be statistically 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).

Results

Nature of contractions

The contractions elicited by electric stimulation in medium containing suramin (300 μM) were monophasic and peaked 460–660 ms after the stimulation pulse. Tetrodotoxin (0.5 μM) when added at the end of several experiments of the series described below, abolished the contractions. Prazosin was added at two increasing concentrations (10 followed by 100 nM) in 4 experiments using the pulse pair protocol of Figure 1 below, but with 60 min exposure to either concentration. Prazosin 10 nM greatly reduced, and 100 nM abolished, both twitch 1 and twitch 2 of the pair. The responses, hence, were neurogenic and adrenergic and represented the slow phase of the discrete twitches evoked in the vas deferens by single or low-frequency pulses.

Pairs of pulses

Two pulses, 3 s apart, elicited separate contractions; the tension returned to baseline between the twitches (Figure 1). The second twitch was smaller than the first (Figure 1, upper tracings, and Figure 2a). In control experiments in which the pair of pulses was applied every 5 min and solvent was administered, contractions remained approximately constant (left-hand panel of Figure 1, open symbols in Figure 2a). Increasing concentrations of nifedipine (0.01–10 μM) reduced both twitches, but reduced the second twitch to a much greater extent than the first (right-hand panel of Figure 1, solid symbols in Figure 2a). The IC_{50} values of nifedipine were 42 (95% c.i. 18–98) nM for the first twitch of the pair and 59 (12–288) nM for the second twitch. Nifedipine (1 μM) reduced the first twitch by 2.5 ± 0.4 mN and the second twitch by 6.2 ± 0.7 mN ($P<0.05$ for the difference between first and second twitch).

One possible reason for the greater inhibition of twitch 2 than of twitch 1 was the smaller size of twitch 2 which is mainly due to prejunctional α_2-adrenoceptor-mediated autoinhibition (see Introduction). In order to examine this possibility, experiments were carried out in the presence of the α_2-adrenoceptor antagonist rauwolscine which prevents autoinhibition. In fact, when 0.1 μM rauwolscine was present in the medium from the beginning, the two contractions of the pair were of similar height (and shape; '0' values in Figure 2b). In control experiments, the contractions remained approximately constant (open symbols in Figure 2b). Nifedipine reduced both twitches, but again reduced the second twitch to a much greater extent than the first, so that the second became smaller than the first (solid symbols in Figure 2b). The IC_{50} values of nifedipine were 54 (20–143) and 82 (57–119) nM for the first and second twitches, respectively. Nifedipine 1 μM reduced the first twitch by 1.5 ± 0.3 mN and the second twitch by 4.3 ± 0.7 mN ($P<0.05$).

The protocol of Figure 1 was used in three additional groups of experiments: on mature rats, in Mg^{2+}-containing medium, and with replacement of the second pulse by two pulses, 10 ms apart.

Responses of vasa deferentia from mature rats differed from those of young rats (Figures 1 and 2) in two ways. Stimulation-evoked twitches were of greater magnitude (see Figure 4 below) and in tissues from young animals, 'spontaneous' contractions always followed the neurogenic contractions proper; nifedipine abolished the spontaneous contractions (Figure 1). No, or much smaller, oscillations developed after the neurogenic twitches in vasa deferentia from mature rats. Despite these differences, nifedipine (0.01–10 μM) also reduced the first and, to a greater extent, the second of the twitches elicited by two pulses 3 s apart in vasa deferentia from mature rats. Nifedipine (1 μM) reduced the first twitch by 2.3 ± 0.4 mN and the second twitch by 6.1 ± 0.6 mN ($P<0.05$; $n=4$ for solvent and nifedipine).

The experiments described so far were carried out in Mg^{2+}-free medium. In medium containing 1.2 mM Mg^{2+} (young rats), contraction heights were only about 1/3 to 2/3 of those obtained in the absence of Mg^{2+}. Nifedipine (0.01–10 μM) did not significantly reduce the first twitch but did reduce the second, by 2.1 ± 0.5 mN at a concentration of 1 μM ($n=6$ for solvent and nifedipine).

When the second pulse of the pair was replaced by two pulses 10 ms apart, the ensuing response was monophasic and similar in shape and height to twitch No. 1 (so that the pair looked like pairs obtained in the presence of rauwolscine). Nifedipine (0.01–10 μM) reduced both twitches, but again reduced the second twitch more than the first. Nifedipine 1 μM reduced the first twitch by 2.0 ± 0.4 mN and the second twitch by 4.8 ± 0.5 mN ($P<0.05$; $n=4$ for solvent and nifedipine).

For all subsequent experiments nifedipine was used at a concentration of 1 μM since this concentration was maximally effective (Figure 2) and since 10 μM nifedipine enhanced the electrically evoked overflow of tritium in experiments with [^{3}H]-noradrenaline (see below).

Current strength

Single pulses of increasing current strength elicited twitches of increasing height. A maximum was reached at 80 mA. When the stimulation cycle was repeated under control conditions, all contractions were increased (Figure 3a). Comparison of these controls with the nifedipine group (Figure 3b) shows that 1 μM nifedipine depressed the entire current strength-response curve. The absolute inhibition caused by nifedipine (mN) was similar for all current strengths (Figure 3c) and averaged 3.2 ± 0.3 mN ($n=35$; all current strengths pooled).

Similar results were obtained with tissues taken from mature rats ($n=4$ for solvent and nifedipine).

Pulse interval

The second twitch of a pair of twitches, 3 s apart, was smaller than the first twitch (Figures 1 and 2a). The same was true for a pulse interval of 5 s (Figure 4, upper panel; pulse intervals less than 3 s were not used in order to keep the two twitches well separated). As the interval was prolonged from 5 to 60 s, the height of the second twitch increased progressively, but did not attain the height of twitch 1. This dependence on the pulse interval was observed both before and after (Figure 4, upper panel and Figure 5, open diamonds) the addition of solvent.

Nifedipine reduced the second twitch in a pair, 3 s apart, to a much greater extent than the first twitch (Figures 1 and

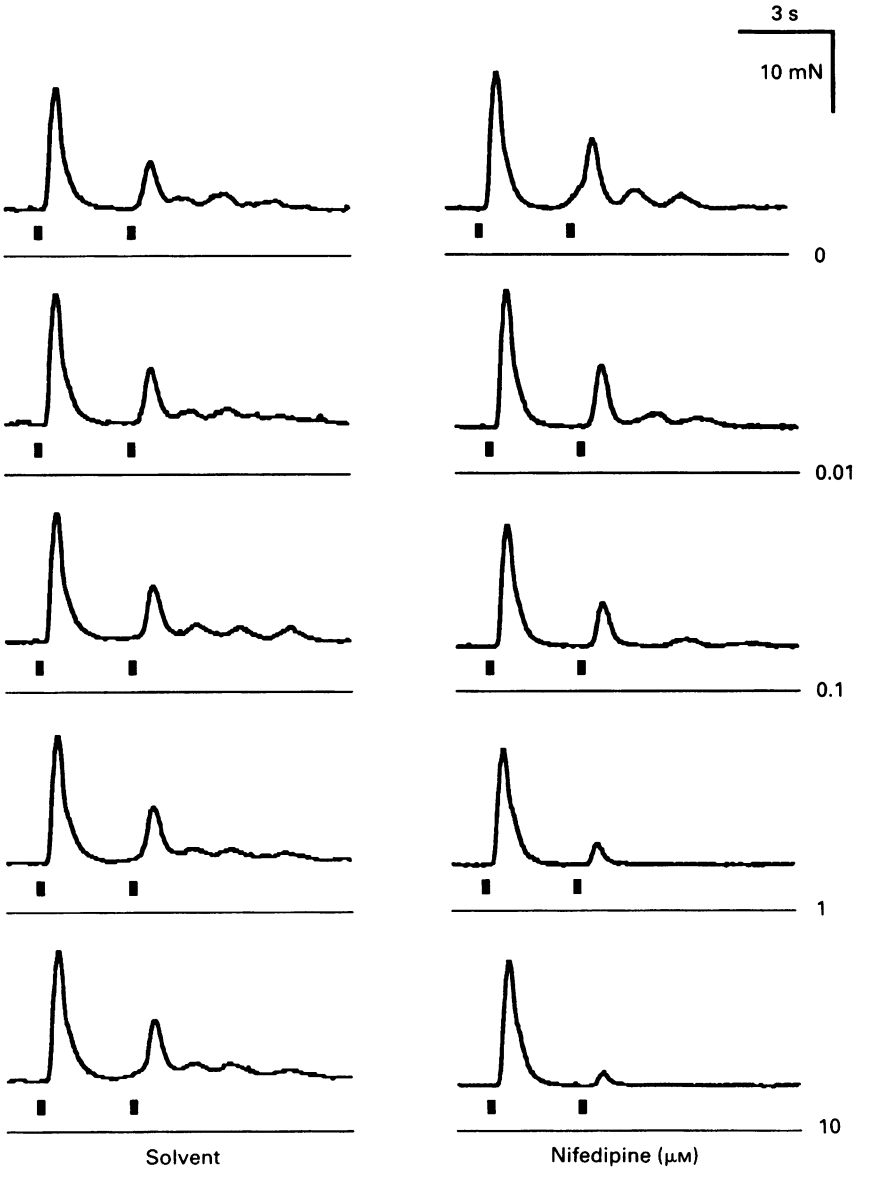


Figure 1 Effect of nifedipine on adrenergic twitches elicited by two pulses 3 s apart: representative tracings. The adrenergic component was isolated by the presence of suramin (300 μM). Tissues were stimulated every 5 min. Each stimulation period consisted of two pulses 3 s apart (■). After 30 min of stimulation in nifedipine-free medium, a cumulative concentration-response curve was determined by stepwise increases of the concentration of nifedipine (0.01–10 μM) every 30 min (right-hand panel). Control experiments with increasing concentrations of solvent were run in parallel (left-hand panel). Shown are the last responses before addition of solvent or nifedipine (top tracings) and the last responses in the presence of each concentration of solvent or nifedipine (i.e. after 30 min of exposure to the respective concentration). The uneven appearance of the tracings is due to the resolution of the computer storage system. Representative tracings from 4 experiments each.

2), and the same was true for an interval of 5 s. When the interval was prolonged, the inhibition caused by 1 μM nifedipine declined (Figures 4 and 5). At the 30 s and 60 s intervals, the inhibition of twitch 2 (by 5.0 ± 0.7 mN and 3.9 ± 0.8 mN) no longer exceeded that of twitch 1 (by 3.7 ± 0.7 mN and 3.6 ± 0.7 mN, respectively; $P > 0.05$ for the difference between first and second twitch).

Ryanodine (20 μM) augmented the spontaneous contraction waves following the neurogenic twitches proper (compare top and bottom tracings of Figure 4). Moreover, ryanodine attenuated both neurogenic twitches of the pairs. The first twitch was very markedly reduced (Figure 4). The effect on the second twitch depended on the pulse interval in a manner opposite to that observed with nifedipine: at short intervals, the inhibition was relatively small so that a substantial part of the second twitch persisted and twitch 2 became greater than twitch 1; the inhibition then increased with increasing pulse interval to become indistinguishable from the inhibition of twitch 1 after 30 s (Figures 4 and 5). There was a significant inverse correlation between the degree of inhibition caused by ryanodine and that caused by nifedipine (Figure 5 inset).

Responses remaining in the presence of 20 μM ryanodine were abolished by subsequent addition of 1 μM nifedipine (nifedipine was added in the ryanodine experiments of Figures 4 and 5 in a third stimulation cycle; $n = 4$).

Tritium overflow

Release of [^{3}H]-noradrenaline was measured as overflow of total tritiated compounds. Since the overflow elicited by single electric pulses was too low to be measurable, trains of 6 pulses/100 Hz were applied. Nevertheless the evoked overflow of tritium was rather small. For this reason, three stimulation

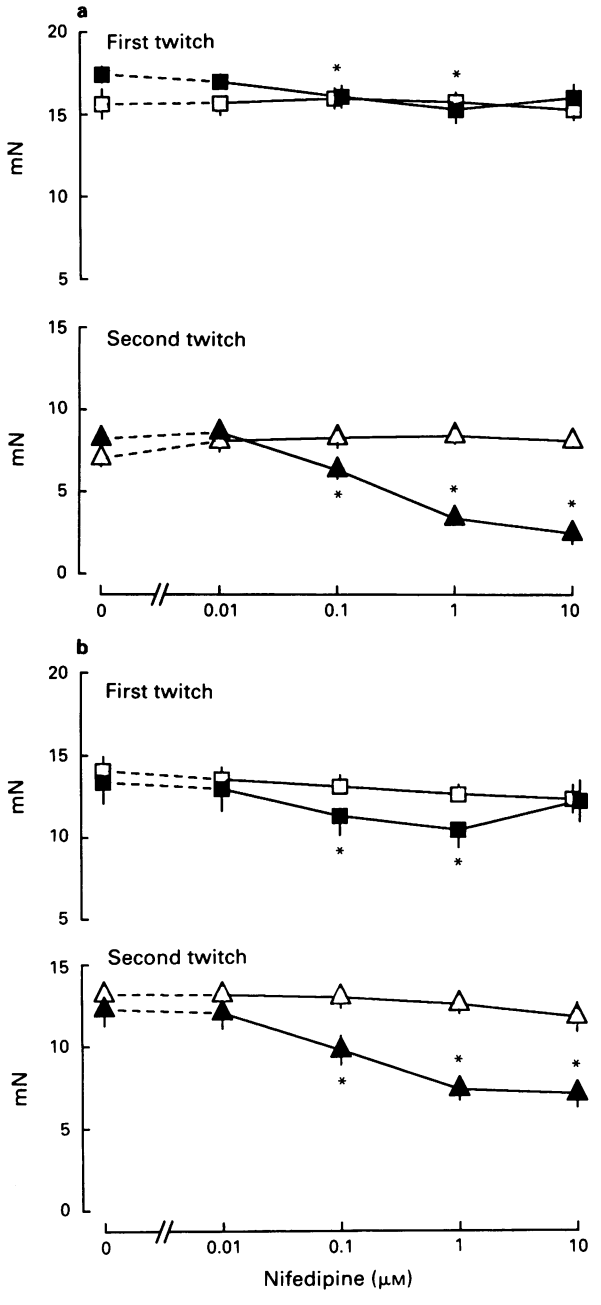


Figure 2 Effect of nifedipine on adrenergic twitches elicited by two pulses 3 s apart: statistical evaluation. Protocol as in Figure 1. (a) Shows responses in the presence of 300 μM suramin; (b) responses in the presence of 300 μM suramin and 0.1 μM rauwolscine. Upper panels show responses (mN) to the first, lower panels responses to the second of the two pulses; note difference in ordinate scales. Open symbols show responses in the presence of increasing concentrations of solvent, solid symbols responses in the presence of increasing concentrations (abscissae) of nifedipine. The last responses before addition of solvent or nifedipine ('0' on abscissae) and the last responses in the presence of each concentration of solvent or nifedipine (i.e. after 30 min of exposure to the respective concentration) were evaluated. *Denotes a significant difference between nifedipine and solvent, based on changes from pre-solvent and pre-nifedipine contractions ($P<0.05$). Means and s.e.mean from 4 (a) or 6 (b) experiments.

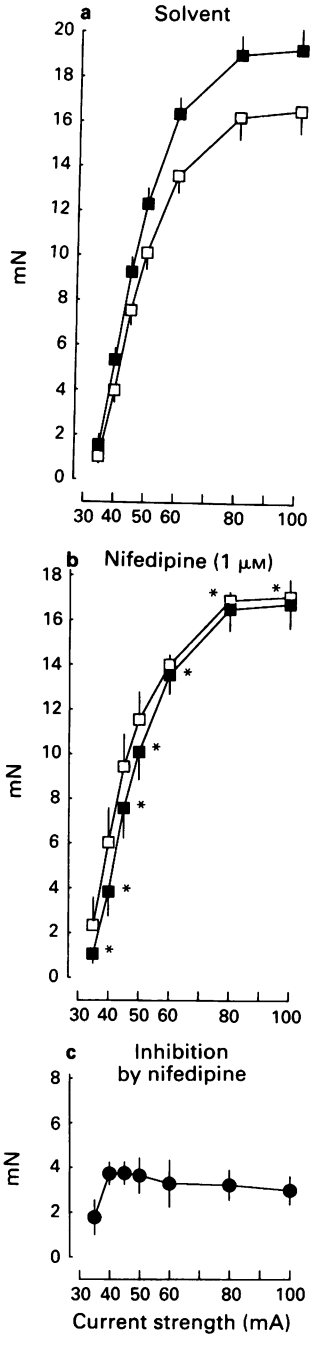


Figure 3 Effect of nifedipine on adrenergic twitches: influence of current strength. The adrenergic component was isolated by the presence of suramin (300 μM). Tissues were stimulated every 5 min by single pulses of either increasing (35 to 100 mA) or decreasing (100 to 35 mA) current strength. When the first stimulation cycle was completed, either nifedipine (1 μM) or solvent was added and kept in the medium for the remainder of the experiment. The stimulation cycle was repeated 60 min later. Results obtained with increasing and decreasing current strength were pooled. (□) in (a) and (b) represents current strength-response curves before, and (■) represents curves after addition of solvent (a) or nifedipine (b). (c) shows the inhibition by nifedipine; it was calculated as the difference between responses obtained before and those obtained after addition of nifedipine, corrected for the mean change occurring in solvent experiments. *Denotes a significant difference between nifedipine and solvent, based on changes from pre-solvent and pre-nifedipine contractions ($P<0.05$). Means and s.e.mean from 5 experiments.

periods were applied before and after the addition of drugs, and the sums of the overflow from S_1 to S_3 (before drug) as well as from S_4 to S_6 (in presence of drug) were calculated. In solvent experiments, the overflow of tritium was slightly lower at S_4 to S_6 than at S_1 to S_3 (Table 1).

Tetrodotoxin (0.5 μM) almost abolished the overflow elicited by any of the three stimulation protocols (Table 1). Rauwolscine (0.1 μM) changed neither the overflow evoked

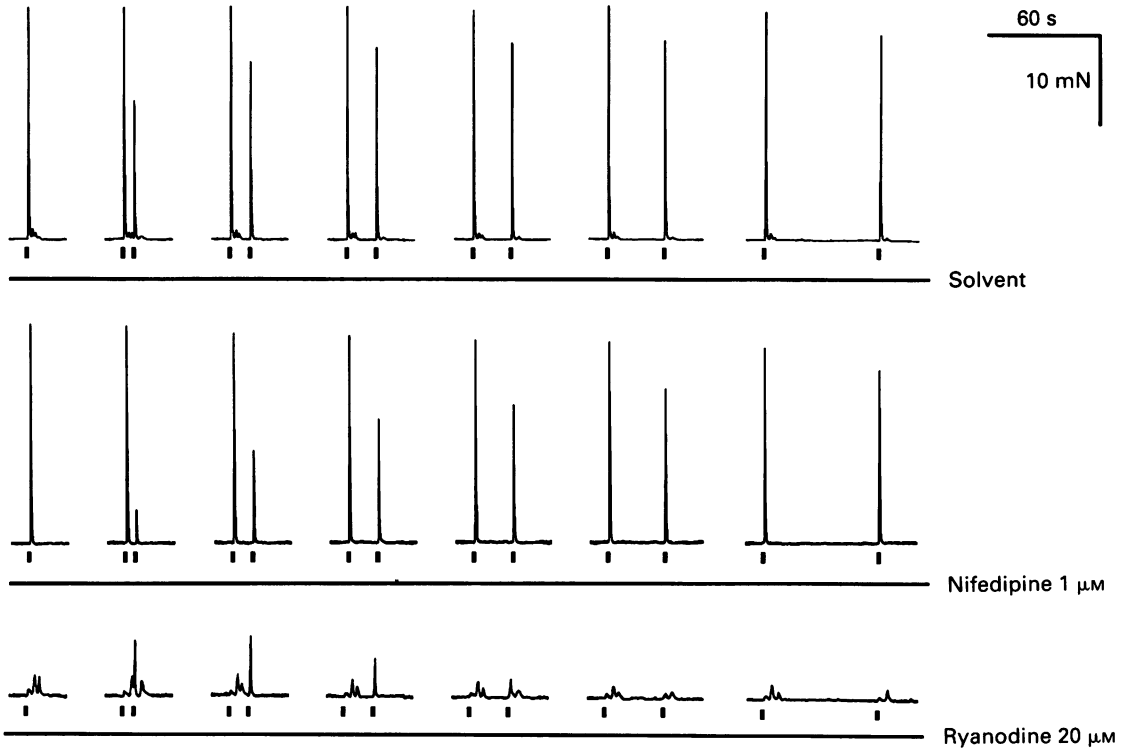
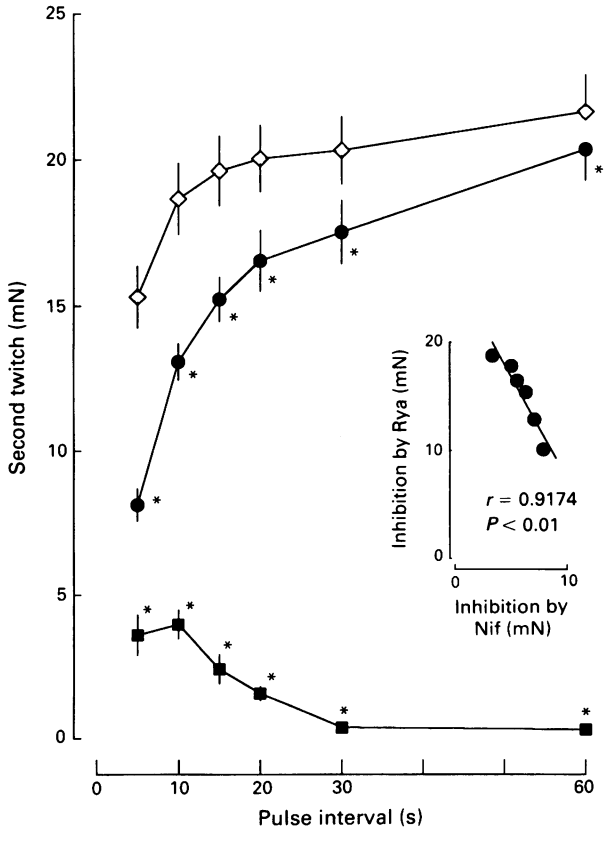


Figure 4 Effect of nifedipine and ryanodine on adrenergic twitches elicited by two pulses of varying interval: representative tracings. The adrenergic component was isolated by the presence of suramin (300 μM). Tissues from mature rats were stimulated every 5 min. The first stimulation period consisted of a single pulse, the following periods of two pulses of increasing interval: 5, 10, 15, 20, 30 and 60 s (▮). When the first stimulation cycle was completed, either 1 μM nifedipine, 20 μM ryanodine or the respective solvent was added and kept in the medium for the remainder of the experiment. The stimulation cycle was repeated 60 min later. All tracings shown were obtained during the second stimulation cycle. Top tracings show responses in the presence of nifedipine solvent (ethanol), middle tracings responses in the presence of nifedipine, and bottom tracings responses in the presence of ryanodine. Note that ryanodine increased the oscillatory contractions that followed the neurogenic twitches proper; the second neurogenic twitch of the 5 s interval pair is superimposed on an oscillatory contraction. Representative tracings from 4–5 experiments each.



by a single train of 6 pulses nor that evoked by two trains 10 s apart, but increased the overflow evoked by two trains 3 s apart. Nifedipine (1 μM) and ryanodine (20 μM) caused no change. Nifedipine at the higher concentration of 10 μM increased the evoked overflow by more than 60% for all three stimulation protocols (Table 1). The increase in noradrenaline release may explain why 10 μM nifedipine depressed stimulation-evoked contractions less than did 1 μM nifedipine in several experimental groups (see Figure 2a and b, upper panels).

None of the drugs changed the basal outflow of tritium.

Discussion

Neither nifedipine nor ryanodine reduced the electrically evoked overflow of tritium after preincubation with [^{3}H]-

Figure 5 Effect of nifedipine and ryanodine on adrenergic twitches elicited by the second of two pulses delivered at varying interval: statistical evaluation. Protocol as in Figure 4. Tissues from mature rats. Shown are contractions obtained in the second stimulation cycle, in the presence of 1 μM nifedipine (●), 20 μM ryanodine (■) or solvent (◇; nifedipine and ryanodine solvent pooled); these three groups did not differ statistically in contraction heights obtained during the first stimulation cycle. *Denotes a significant difference between nifedipine or ryanodine and solvent, based on changes from pre-solvent and pre-nifedipine contractions ($P<0.05$). Means and s.e.mean from 5 (nifedipine), 4 (ryanodine) or 9 (solvent) experiments. Inset shows correlation between inhibition by nifedipine (Nif) and inhibition by ryanodine (Rya). The inhibition was calculated as the difference between responses obtained before and those obtained after addition of nifedipine or ryanodine, corrected for the mean change occurring in solvent experiments.

Table 1 Effects of drugs on evoked tritium overflow from rat vasa deferentia preincubated with [^{3}H]-noradrenaline

Drug present from S_4 to S_6	S_{4-6}/S_{1-3} for stimulation periods consisting of: One train of 6 pulses/100 Hz	Two trains of 6 pulses/100 Hz, 3 s interval	Two trains of 6 pulses/100 Hz, 10 s interval
Solvent	0.86 ± 0.04 (5)	0.91 ± 0.06 (5)	0.92 ± 0.07 (6)
Tetrodotoxin 0.5 μM	0.10 ± 0.02* (4)	0.06 ± 0.02* (5)	0.04 ± 0.02* (4)
Rauwolscine 0.1 μM	0.94 ± 0.09 (4)	1.44 ± 0.16* (4)	1.17 ± 0.08 (4)
Solvent	0.89 ± 0.04 (5)	0.98 ± 0.09 (6)	0.98 ± 0.06 (5)
Nifedipine 1 μM	0.98 ± 0.11 (6)	1.13 ± 0.06 (5)	1.05 ± 0.09 (6)
Solvent	0.82 ± 0.09 (5)	0.79 ± 0.03 (6)	0.84 ± 0.07 (5)
Nifedipine 10 μM	1.38 ± 0.10* (4)	1.46 ± 0.09* (6)	1.37 ± 0.11* (4)
Solvent	0.92 ± 0.09 (7)	Not determined	0.83 ± 0.06 (7)
Ryanodine 20 μM	0.81 ± 0.07 (5)	Not determined	0.93 ± 0.05 (5)

Tissues from mature rats. After preincubation, tissues were superfused. There were six periods of electric stimulation (S_1-S_6). Each period consisted either of one train of 6 pulses/100 Hz, or of two trains of 6 pulses/100 Hz, train interval 3 s, or of two trains of 6 pulses/100 Hz, train interval 10 s. Solvent or drug was added 64 min before S_4 and kept for the remainder of the experiment. Values are ratios of the overflow elicited by S_4 to S_6 (S_{4-6}) and the overflow elicited by S_1 to S_3 (S_{1-3}). S_{1-3} amounted to 0.134 ± 0.007% (one train per stimulation period; $n = 45$), 0.168 ± 0.007% (two trains 3 s apart per stimulation period; $n = 37$) and 0.142 ± 0.004% (two trains 10 s apart per stimulation period; $n = 46$), respectively. Means and s.e.mean from (n) tissues.
*Denotes a significant difference from solvent ($P < 0.05$).

noradrenaline, indicating that neither inhibited the release of noradrenaline (see also Hyland *et al.*, 1984; Starke *et al.*, 1984). In reducing contractions, both acted on the smooth muscle rather than the sympathetic nerves, and at the concentrations used, only their major modes of action, i.e. blockade of voltage-dependent Ca^{2+} channels for nifedipine and prevention of the mobilization of intracellular Ca^{2+} for ryanodine, require consideration (see Iino, 1990; Tsien & Tsien, 1990). The main question to be discussed on this basis is: what do the effects of nifedipine and ryanodine tell us about the origins of the Ca^{2+} that mediates the adrenergic component of the smooth muscle response to nerve stimulation?

Single twitches

The adrenergically mediated contractions evoked by single pulses of maximal current strength (in most experiments the first pulses of pulse pairs, after a period of rest of at least 5 min) were very markedly reduced by ryanodine (Figure 4). The decrease shows directly, as far as is possible with pharmacological tools, that the response is largely due to intracellular Ca^{2+} mobilization (Amobi & Smith, 1987; Mallard *et al.*, 1992). However, these adrenergic contractions were also reduced by nifedipine, although only in Mg^{2+}-free medium, indicating that Ca^{2+} entry also plays some role. The nifedipine-sensitive, Ca^{2+} entry-mediated, part of the contractions, when expressed in absolute terms (mN), was similar at all stimulus strengths (Figure 3c). On the other hand, the nifedipine-resistant contraction which presumably is due to mobilization of intracellular Ca^{2+} increased with the strength of the neural stimulus (Figure 3b). The *relative* roles played by Ca^{2+} entry and Ca^{2+} mobilization, hence, depend on the stimulus: Ca^{2+} entry contributes little to adrenergic contractions elicited by the maximal neural stimuli used throughout most of the present study but contributes more to contractions elicited by weak stimuli. Exogenous noradrenaline activates Ca^{2+} entry into smooth muscle cells at lower concentrations than are required for Ca^{2+} mobilization (Casteels *et al.*, 1977; van Breemen *et al.*, 1981; Han *et al.*, 1987). Our observations may indicate that this relationship also holds true for neurally released noradrenaline.

The second twitch

At appropriate pulse intervals, the effects of nifedipine and ryanodine on a second twitch differed quantitatively from their effects on the first twitch. The inhibition by nifedipine of the second twitch was greater than the inhibition of the first twitch, indicating a greater entry of Ca^{2+} during twitch 2 than during twitch 1 (Figures 1 and 2a, and compare top and middle panels of Figure 4). The greater inhibition by nifedipine was not due to the smaller size of the second twitch under control conditions, as it was also observed when twitch 2 was brought to the height of twitch 1 by interruption of α_2-adrenoceptor-mediated autoinhibition (Figure 2b) or by replacement of the second pulse by two pulses 10 ms apart. Conversely, the inhibition by ryanodine of the second twitch was much smaller than the inhibition of the first twitch, indicating less mobilization of intracellular Ca^{2+} during twitch 2 than during twitch 1 (compare top and bottom panels of Figure 4).

If nifedipine blocks Ca^{2+} entry and ryanodine prevents intracellular Ca^{2+} mobilization, the contraction component *abolished* by one of the two should behave like the contraction component *remaining* in the presence of the other. In fact, like the contraction component suppressed by nifedipine, the contraction remaining in the presence of ryanodine was much greater in the case of twitch 2 than of twitch 1 (bottom panel of Figure 4). Conversely, like the contraction component suppressed by ryanodine, the contraction remaining in the presence of nifedipine was smaller in the case of twitch 2 than of twitch 1; again, this was so even when twitch 2 was brought to the height of twitch 1 by interruption of α_2-adrenergic autoinhibition (Figure 2b) or the application of two pulses 10 ms apart.

All of these complementary observations indicate that the first adrenergic neurogenic twitch triggers a change in the Ca^{2+} sources used for contraction. Ca^{2+} entry during a second twitch, evoked after an appropriate interval, is enhanced; conversely, the mobilization of intracellular Ca^{2+} is reduced. As indicated by the effects of nifedipine and ryanodine, the change is pronounced when pulse 2 follows pulse 1 after 3, 5 or 10 s; it then fades within about 30 s (Figures 4 and 5).

The basis for the switch in Ca^{2+} sources is not known. The increase in Ca^{2+} entry and the decrease in intracellular Ca^{2+} mobilization during twitch 2 appeared and faded in a closely parallel manner, yielding a close inverse correlation between inhibition by nifedipine and inhibition by ryanodine and suggesting some connection between the two phenomena (Figure 5 inset). One explanation could be that Ca^{2+} mobilized from intracellular stores during twitch 1 inhibits the entry of Ca^{2+} during twitch 1, that the primary change in

twitch 2 is a decrease in intracellular Ca^{2+} mobilization, and this then leads to disinhibition of Ca^{2+} entry during twitch 2. There is some evidence for this speculation. Inositol-1,4,5-trisphosphate (IP_3) is the second messenger that links α_1-adrenoceptor activation to intracellular Ca^{2+} mobilization (see Ruffolo *et al.*, 1991). In rabbit intestinal smooth muscle cells, release of IP_3 from its 'caged' precursor by flash photolysis rapidly and markedly inhibited voltage-dependent Ca^{2+} entry, and apparently the inhibition was due to Ca^{2+} mobilized from intracellular stores (Komori & Bolton, 1991; see also Nakazawa *et al.*, 1987). It is also known that the mobilization of Ca^{2+} by IP_3 is subject to rapid tachyphylaxis. In isolated heptocytes, for example, the second of two applications of IP_3, a few seconds apart, mobilized much less intracellular Ca^{2+} than the first, a depression that faded with a time course very similar to the depression of Ca^{2+} mobilization in the present experiments (Ogden *et al.*, 1990).

α_2-Adrenoceptor-mediated autoinhibition

The first pulse of a train can exert an additional influence on subsequent pulses: prejunctional α_2-adrenergic autoinhibition. The α_2-adrenoceptor antagonist rauwolscine did not change the overflow of tritium, and presumably the release of [^{3}H]-noradrenaline, elicited by a single train of 6 pulses/100 Hz (release evoked by such brief trains resembles single pulse-evoked release: Singer, 1988; Limberger *et al.*, 1992) and also did not change the release elicited by two trains 10 s apart; however, rauwolscine significantly increased the release elicited by two trains 3 s apart. The single 50 ms train, hence, was free from autoinhibition, as expected (see Story *et al.*, 1981; Starke, 1987; Singer, 1988). Autoinhibition operated 3 s later, and then declined. This time frame agrees with that found previously with contraction measurements by French & Scott (1983). The shift in Ca^{2+} sources was basically independent of prejunctional autoinhibition: the decrease in Ca^{2+} mobilization from twitch 1 to twitch 2 persisted in the presence of rauwolscine (Figure 2b), and the increase in Ca^{2+} entry cannot of course be due to a decrease in noradrenaline release.

Calcium in pulse trains

In conjunction with reports cited in the Introduction, our results suggest a more complete picture of the contribution of different calcium sources to the adrenergic response of the vas deferens to trains of pulses. As shown by the effect of nifedipine, Ca^{2+} entry can contribute to a single twitch. The contribution of Ca^{2+} entry increases markedly upon the next stimulus, and simultaneously the contribution of Ca^{2+} mobilization decreases. It is presumably this change in Ca^{2+} sources which continues during subsequent appropriately spaced stimuli and then, late in high-frequency pulse trains, makes the entire adrenergic component susceptible to blockade by nifedipine and resistant to ryanodine (Brown *et al.*, 1983; Bourreau *et al.*, 1991). It should be noted that the switch in Ca^{2+} sources took place despite the fact that the two twitches were separated by an interval during which the tension returned to baseline or, in other words, the concentration of noradrenaline fell to sub-threshold levels. No continuous presence of the agonist is required.

It has been suggested that the nifedipine resistance of the adrenergic response to a single stimulus and the nifedipine-sensitivity of the adrenergic component of a sustained tetanus are due to an effect of noradrenaline on two different α_1-adrenoceptor populations: a single stimulus would release noradrenaline only onto receptors close to the sites of release and coupled to Ca^{2+} mobilization, whereas a pulse train would also (and mainly) release noradrenaline onto receptors far from the sites of release and coupled to Ca^{2+} entry (Brown *et al.*, 1983; Mallard *et al.*, 1992). Our findings argue against this view: the waves of noradrenaline causing the first and, a few seconds later, the second, separate twitch certainly reached the same receptors. The change from twitch 1 to twitch 2 seems to be a change in the transduction mechanism of one population of receptors.

Most of the present experiments were carried out in Mg^{2+}-free medium. In medium containing a physiological concentration of Mg^{2+}, contractions were smaller (compare Evans & Cunnane, 1992). Moreover, although second twitches were reduced by nifedipine, first twitches were not. There is, therefore, no conflict of evidence between the present findings and previous reports of nifedipine resistance of single pulse-evoked twitches in Mg^{2+}-containing medium (see Introduction). Mg^{2+}, like nifedipine, blocks voltage-dependent Ca^{2+} channels and in this manner may minimize the effect of nifedipine. In Mg^{2+}-containing medium the response of the vas deferens to the first pulse after a period of rest may in fact be practically exclusively due to Ca^{2+} mobilization. The switch to less Ca^{2+} mobilization and more Ca^{2+} entry upon the second pulse, however, also occurs at physiological Mg^{2+} concentrations.

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References

AMOBI, N.I.B. & SMITH, I.C.H. (1987). Adrenergic and 'non-adrenergic' contributions to the two-component tetanus in the rat vas deferens. *Eur. J. Pharmacol.*, **135**, 173–182.

BOURREAU, J.P., ZHANG, Z.D., LOW, A.M., KWAN, C.Y. & DANIEL, E.E. (1991). Ryanodine and the adrenergic, purinergic stimulation in the rat vas deferens smooth muscle: functional and radioligand binding studies. *J. Pharmacol. Exp. Ther.*, **256**, 1063–1071.

BROWN, D.A., DOCHERTY, J.R., FRENCH, A.M., MACDONALD, A., MCGRATH, J.C. & SCOTT, N.C. (1983). Separation of adrenergic and non-adrenergic contractions to field stimulation in the rat vas deferens. *Br. J. Pharmacol.*, **79**, 379–393.

BÜLTMANN, R. & STARKE, K. (1992). Pulse-to-pulse changes in the calcium sources for neurogenic adrenergic contractions of rat vas deferens. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **345**, R107.

BÜLTMANN, R., VON KÜGELGEN, I. & STARKE, K. (1991). Contraction-mediating α_2-adrenoceptors in the mouse vas deferens. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **343**, 623–632.

BURNSTOCK, G. (1990). Co-transmission. *Arch. Int. Pharmacodyn.*, **304**, 7–33.

CASTEELS, R., KITAMURA, K., KURIYAMA, H. & SUZUKI, H. (1977). Excitation-contraction coupling in the smooth muscle cells of the rabbit main pulmonary artery. *J. Physiol.*, **271**, 63–79.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

EVANS, R.J. & CUNNANE, T.C. (1992). Relative contributions of ATP and noradrenaline to the nerve evoked contraction of the rabbit jejunal artery. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **345**, 424–430.

FRENCH, A.M. & SCOTT, N.C. (1981). A comparison of the effects of nifedipine and verapamil on rat vas deferens. *Br. J. Pharmacol.*, **73**, 321–323.

FRENCH, A.M. & SCOTT, N.C. (1983). Feedback inhibition of responses of rat vas deferens to twin pulse stimulation. *Eur. J. Pharmacol.*, **86**, 379–383.

HAN, C., ABEL, P.W. & MINNEMAN, K.P. (1987). α_1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature*, **329**, 333–335.

HAY, D.W.P. & WADSWORTH, R.M. (1983). The effects of calcium channel inhibitors on twitches and noradrenaline contractions of the rat bisected vas deferens. *Eur. J. Pharmacol.*, **87**, 367–378.

HYLAND, L., WARNOCK, P. & DOCHERTY, J.R. (1984). An investigation of the actions of the calcium entry facilitator Bay K 8644 on the rat vas deferens. *Eur. J. Pharmacol.*, **104**, 363–367.

IINO, M. (1990). Calcium release mechanisms in smooth muscle. *Jpn. J. Pharmacol.*, **54,** 345–354.

KOMORI, S. & BOLTON, T.B. (1991). Inositol trisphosphate releases stored calcium to block voltage-dependent calcium channels in single smooth muscle cells. *Pflügers Arch.*, **418,** 437–441.

LIMBERGER, N., TRENDELENBURG, A.U. & STARKE, K. (1992). Pharmacological characterization of presynaptic α_2-autoreceptors in rat submaxillary gland and heart atrium. *Br. J. Pharmacol.*, **107,** 246–255.

MALLARD, N.J., MARSHALL, R.W., SITHERS, A.J. & SPRIGGS, T.L.B. (1992). Separation of putative α_{1A}- and α_{1B}-adrenoceptor mediated components in the tension response of the rat vas deferens to electrical field stimulation. *Br. J. Pharmacol.*, **105,** 727–731.

MALLARD, N.J., MARSHALL, R.W. & SPRIGGS, T.L.B. (1989). Suramin, a selective inhibitor of NANC transmission in rat vas deferens: comparison with nifedipine. *Br. J. Pharmacol.*, **98,** 618P.

MCGRATH, J.C. (1978). Adrenergic and 'non-adrenergic' components in the contractile response of the vas deferens to a single indirect stimulus. *J. Physiol.*, **283,** 23–39.

MELDRUM, L.A. & BURNSTOCK, G. (1983). Evidence that ATP acts as a co-transmitter with noradrenaline in sympathetic nerves supplying the guinea-pig vas deferens. *Eur. J. Pharmacol.*, **92,** 161–163.

NAKAZAWA, K., SAITO, H. & MATSUKI, N. (1987). Involvement of calcium in calcium-current inactivation in smooth muscle cells from rat vas deferens. *J. Membrane Biol.*, **100,** 13–19.

OGDEN, D.C., CAPIOD, T., WALKER, J.W. & TRENTHAM, D.R. (1990). Kinetics of the conductance evoked by noradrenaline, inositol trisphosphate or Ca^{2+} in guinea-pig isolated hepatocytes. *J. Physiol.*, **422,** 585–602.

RUFFOLO, R.R., NICHOLS, A.J., STADEL, J.M. & HIEBLE, J.P. (1991). Structure and function of α-adrenoceptors. *Pharmacol. Rev.*, **43,** 475–505.

SINGER, E.A. (1988). Transmitter release from brain slices elicited by single pulses: a powerful method to study presynaptic mechanisms. *Trends Pharmacol. Sci.*, **9,** 274–276.

STARKE, K. (1987). Presynaptic α-autoreceptors. *Rev. Physiol. Biochem. Pharmacol.*, **107,** 73–146.

STARKE, K., SPÄTH, L. & WICHMANN, T. (1984). Effects of verapamil, diltiazem and ryosidine on the release of dopamine and acetylcholine in rabbit caudate nucleus slices. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325,** 124–130.

STORY, D.F., MCCULLOCH, M.W., RAND, M.J. & STANDFORD-STARR, C.A. (1981). Conditions required for the inhibitory feeback loop in noradrenergic transmission. *Nature*, **293,** 62–65.

SWEDIN, G. (1971). Biphasic mechanical response of the isolated vas deferens to nerve stimulation. *Acta Physiol. Scand.*, **81,** 574–576.

TSIEN, R.W. & TSIEN, R.Y. (1990). Calcium channels, stores, and oscillations. *Ann. Rev. Cell Biol.*, **6,** 715–760.

VAN BREEMEN, C., HWANG, O.K. & MEISHERI, K.D. (1981). The mechanism of inhibitory action of diltiazem on vascular smooth muscle contractility. *J. Pharmacol. Exp. Ther.*, **218,** 459–463.

VON KÜGELGEN, I., BÜLTMANN, R. & STARKE, K. (1989). Effects of suramin and α,β-methylene ATP indicate noradrenaline-ATP co-transmission in the response of the mouse vas deferens to single and low frequency pulses. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **340,** 760–763.

VON KÜGELGEN, I. & STARKE, K. (1991). Noradrenaline-ATP co-transmission in the sympathetic nervous system. *Trends Pharmacol. Sci.*, **12,** 319–324.

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

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Pharmacological characterization of two distinct α_1-adrenoceptor subtypes in rabbit thoracic aorta

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1 α_1-Adrenoceptor subtypes in rabbit thoracic aorta have been examined in binding and functional experiments.

2 [^{3}H]-prazosin bound to two distinct populations of α_1-adrenoceptors ($pK_{D,high} = 9.94$, $R_{high} = 79.2$ fmol mg^{-1} protein; $pK_{D,low} = 8.59$, $R_{low} = 215$ fmol mg^{-1} protein). Pretreatment with chloroethylclonidine (CEC, 10 μM) almost inactivated the prazosin-high affinity sites and reduced the number of the low affinity sites without changing the pK_D value.

3 In the displacement experiments with CEC-untreated membranes, unlabelled prazosin, WB4101 and HV723 displaced the binding of 200 pM [^{3}H]-prazosin monophasically; the affinities for WB4101 ($pK_I = 8.88$) and HV723 (8.49) were about 10 times lower than that for prazosin (9.99). In the CEC-pretreated membranes also, the antagonists inhibited the binding of 1000 pM [^{3}H]-prazosin monophasically; the pK_I values for prazosin, WB4101 and HV723 were 9.09, 8.97 and 8.17, respectively. These results suggest that the prazosin-high and low affinity sites can be independently appraised in the former and latter experimental conditions. Noradrenaline, but not methoxamine, showed slightly higher affinity for the prazosin-high affinity site than for the low affinity site.

4 In the functional experiments, noradrenaline (0.001–100 μM) and methoxamine (0.1–100 μM) produced concentration-dependent contractions. Pretreatment with CEC inhibited the contractions induced by low concentrations of noradrenaline but without effect on the responses to methoxamine. Prazosin inhibited the concentration-response curves for noradrenaline in the CEC-untreated aorta in a manner which was not consistent with competitive antagonism at a single site, and two distinct affinity constants ($pK_B = 9.71$ and 8.74) were obtained. However, after CEC-pretreatment, Schild plots for prazosin were not significantly different from unity ($pK_B = 8.50$). WB4101 and HV723 competitively inhibited the noradrenaline-induced contraction with low pK_B values (approximately 8.30), irrespective of CEC-pretreatment. Methoxamine-induced contractions were competitively inhibited by prazosin, WB4101 and HV723 with low pK_B values similar to those obtained when noradrenaline was used as the agonist. These were not affected by CEC-pretreatment.

5 The affinity constant for noradrenaline ($pK_A = 6.40$) in CEC-untreated aorta was slightly greater than that obtained in CEC-pretreated aorta (5.78). On the other hand, methoxamine showed a similar affinity in CEC-untreated and pretreated aortae (pK_A = approximately 4.5).

6 Nifedipine (1 μM) slightly attenuated the contractile responses to noradrenaline and methoxamine in CEC-untreated and pretreated aortae, suggesting that nifedipine cannot discriminate between α_1-adrenoceptors involved in CEC-sensitive and -resistant contractions.

7 From these results it is suggested that in the rabbit thoracic aorta there are two distinct α_1-adrenoceptor subtypes (presumably α_{1B} and α_{1L} subtypes according to recently proposed subclassification), both of which are involved in noradrenaline-induced contraction. The α_{1L} subtype predominantly mediates the contraction induced by methoxamine.

Keywords: α_1-Adrenoceptor subtype; α_{1B}-adrenoceptor; α_{1L}-adrenoceptor; rabbit thoracic aorta; [^{3}H]-prazosin binding

Introduction

α_1-Adrenoceptors were originally subdivided into two subclasses (α_{1A} and α_{1B}) (Morrow & Creese, 1986; Minneman, 1988). However, subsequent functional and binding studies have suggested the possible existence of additional subtypes with different affinities for prazosin and other α_1-adrenoceptor antagonists (Flavahan & Vanhoutte, 1986; Mignot *et al.*, 1989; Muramatsu *et al.*, 1990a; 1991; Han & Minneman, 1991; Hiramatsu *et al.*, 1992). We previously subclassified α_1-adrenoceptors into three subgroups according to different affinities for prazosin and HV723 (α_{1H}: prazosin-high and HV723-low affinity site; α_{1L}: prazosin- and HV723-low affinity site; α_{1N}: prazosin-low but HV723-high affinity site) (Muramatsu *et al.*, 1990a) and subsequently suggested that the originally proposed α_{1A} and α_{1B} subtypes may be included in the α_{1H} group because of their high affinity for prazosin (Muramatsu *et al.*, 1991; Oshita *et al.*, 1991; Ohmura *et al.*, 1992).

Rabbit thoracic aorta is one of classical tissues used in pharmacological studies. Noradrenaline and other α_1-adrenoceptor agonists produce concentration-dependent contractions through α_1-adrenoceptors on the smooth muscle. Recently, we have found that the concentration-response curves for noradrenaline are displaced by prazosin in a manner which is not consistent with single competitive antagonism, suggesting the possible involvement of two distinct α_1-adrenoceptor subtypes in the contractile response to noradrenaline (Muramatsu *et al.*, 1990b). In the present study, we have characterized the α_1-adrenoceptor subtypes of rabbit thoracic aorta in more detail.

Methods

Binding experiments

The thoracic aortae were isolated from rabbits (2.5–3.5 kg) under pentobarbitone anaesthesia and homogenized in 20 vol

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of buffer (Tris HCl 50 mM, NaCl 100 mM, EDTA 2 mM, pH 7.4) with a polytron (setting 8, 15 s × 3) and centrifuged at 700 *g* for 10 min. The supernatant was filtered through four layers of cheese cloth and subjected to centrifugation at 10,000 *g* for 20 min; the supernatant was further centrifuged at 80,000 *g* for 30 min. The resulting pellet was suspended in assay buffer (Tris HCl 50 mM, EDTA 1 mM, pH 7.4), incubated at 37°C for 10 min, and again centrifuged under the same conditions mentioned above. The final pellet was resuspended in assay buffer and used for binding assay. The membranes were incubated with [^{3}H]-prazosin for 45 min at 30°C. Incubation volume was 1 ml (about 50 μg protein/tube). Reactions were terminated by rapid filtration through using a Brandel cell harvester with Whatman GF/C filters presoaked in 0.3% polyethylenimine for 5 min. The filters were then washed 4 times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and dried. The filter-bound radioactivity was determined by liquid scintillation counting. Nonspecific binding was defined as binding in the presence of 10 μM phentolamine or 0.1 μM prazosin. Assays were conducted in duplicate.

CEC treatment Membrane preparations were incubated for 20 min at 37°C with 10 μM CEC and centrifuged at 80,000 *g* for 30 min. The pellet was washed once with assay buffer before the binding experiment.

Saturation binding data were analyzed by the weighted least-squares iterative curve fitting programme LIGAND (Munson & Rodbard, 1980). The data were first fitted to a one- and then a two-site model, and if the residual sums of squares were statistically less for a two-site fit of the data than for a one-site, as determined by an *F*-test comparison, then the two-site model was accepted. *P* values less than 0.05 were considered significant. Displacement binding data were analyzed by the EBDA programme (McPherson, 1985; Biosoft, Elsevier) when the slope factor was close to unity and IC_{50} values were converted to K_i values with the Cheng-Prusoff approximation (1973). However, when the slope factor seemed to deviate from unity, the LIGAND programme was used to determine which of one- or two-site model fitted. Proteins were assayed according to the method of Bradford with bovine serum albumin used as standard (Bradford, 1976).

Functional experiments

Rabbits were killed under pentobarbitone anaesthesia and the thoracic aorta was rapidly removed. The aorta was cleaned of adherent connective tissue and cut helically under a dissecting microscope. In order to avoid the possible involvement of endothelium-derived relaxing factor in the mechanical response (Furchgott, 1981), the endothelial cells were removed by rubbing them with filter paper (Muramatsu *et al.*, 1990b). The strip was mounted vertically in an organ bath containing 20 ml Krebs-Henseleit solution of the following composition (mM): NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1.2 and glucose 11.5. Desmethylimipramine (0.1 μM), deoxycorticosterone (5 μM) and propranolol (3 μM) were added to the bathing solution to block neural and extraneural uptake of noradrenaline and to block β-adrenoceptors, respectively. The bath medium was maintained at 37°C, pH 7.4 and was equilibrated with a gas mixture consisting of 95% O_2 and 5% CO_2. A resting tension of 1.5 g was applied and the responses were recorded isometrically through force-displacement transducers. All preparations were equilibrated for 90 min before the experiments were begun.

Concentration-response curves for noradrenaline and methoxamine were obtained by adding the drug directly to the bathing medium in cumulative concentrations. The curves were obtained 6 times in the same strip at 90 min intervals and the third concentration-response curve was used as control. There was little change in agonist sensitivity between the third and sixth concentration-response curves (Muramatsu *et al.*, 1990a). Preparations were treated with α-adrenoceptor antagonists for 30 min before, and during, the construction of concentration-response curves. With chloroethylclonidine (CEC) treatment, the preparation were treated once for 20 min with 10 μM CEC, then washed with the drug-free solution and two different concentrations of antagonist were subsequently tested in each preparation.

The pK_B values for α_1-adrenoceptor antagonists were estimated according to Arunlakshana & Schild (1959). Data were plotted as the −log antagonist concentration (M) vs the log (concentration ratio-1, CR-1), and pA_2 values along mean slope and 95% confidence limit (95% CL) calculated and straight lines were drawn by least square linear regression. When the straight line yielded a slope with unity, the pA_2 value estimated was represented as the pK_B (Arunlakshana & Schild, 1959). When a part of the data deviated from a straight line with a slope of unity (see Figure 3, solid circles at low concentrations of prazosin), the pK_B value was also determined for a single concentration of antagonist by the concentration method (Furchgott, 1972).

Dissociation constants (K_A) of adrenoceptor agonists were determined by comparing equieffective concentrations of the agonist under control conditions and after partial irreversible blockade of adrenoceptors with phenoxybenzamine (Furchgott, 1966). In this case, the preparations were treated with 20 or 70 nM phenoxybenzamine for 30 min and then washed 90 min before agonist responses were re-established.

Statistical analysis

Experimental values are given as the mean ± s.e.mean. Results were analysed by Student's *t* test and a probabilty of less than 0.05 was considered significant.

Drugs

The following drugs were used: [^{3}H]-prazosin (specific activity 87.0 Ci mmol^{-1}, NEN, Boston, U.S.A.), (−)-noradrenaline bitartrate, desipramine hydrochloride, nifedipine, prazosin hydrochloride (Sigma, St. Louis, U.S.A.), phentolamine mesylate (Ciba, Basel, Switzerland), WB4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride), chloroethylclonidine dihydrochloride (CEC) (Research Biochemicals Inc., Natick, U.S.A.), HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxyl)ethyl)-amino)-propyl) benzeneacetonitrile fumarate) (Hokuriku Seiyaku, Katsuyama, Japan), deoxycorticosterone acetate, (±)-propranolol hydrochloride, phenoxybenzamine hydrochloride (Nacalai, Kyoto, Japan) and methoxamine hydrochloride (Nippon-Shinyaku, Kyoto, Japan).

Results

Binding experiments

[^{3}H]-prazosin at concentrations ranging from 20–6000 pM was used to label α_1-adrenoceptors of rabbit thoracic aorta. The specific binding was approximately 90% of the total binding at 200 pM [^{3}H]-prazosin. Scatchard plots of the binding data were curvilinear, suggesting more than a single class of binding site. LIGAND analysis fitted the data to a two site model. The high affinity site showed a B_{max} of 79.2 ± 9.4 fmol mg^{-1} protein (R_{high}) and an equilibrium dissociation constant ($pK_{D\ high}$) of 9.94 ± 0.07, while the low affinity site showed R_{low} of 215.4 ± 54.1 fmol mg^{-1} protein and $pK_{D\ low}$ of 8.59 ± 0.29 ($n = 5$, Figure 1a and b).

In contrast, Scatchard plots of the data obtained from the membranes pretreated with 10 μM CEC were apparently linear, and the data best fitted to a one-site model. The pK_D value (9.07 ± 0.08) estimated was close to the value for the low affinity site in control membranes but the B_{max} value

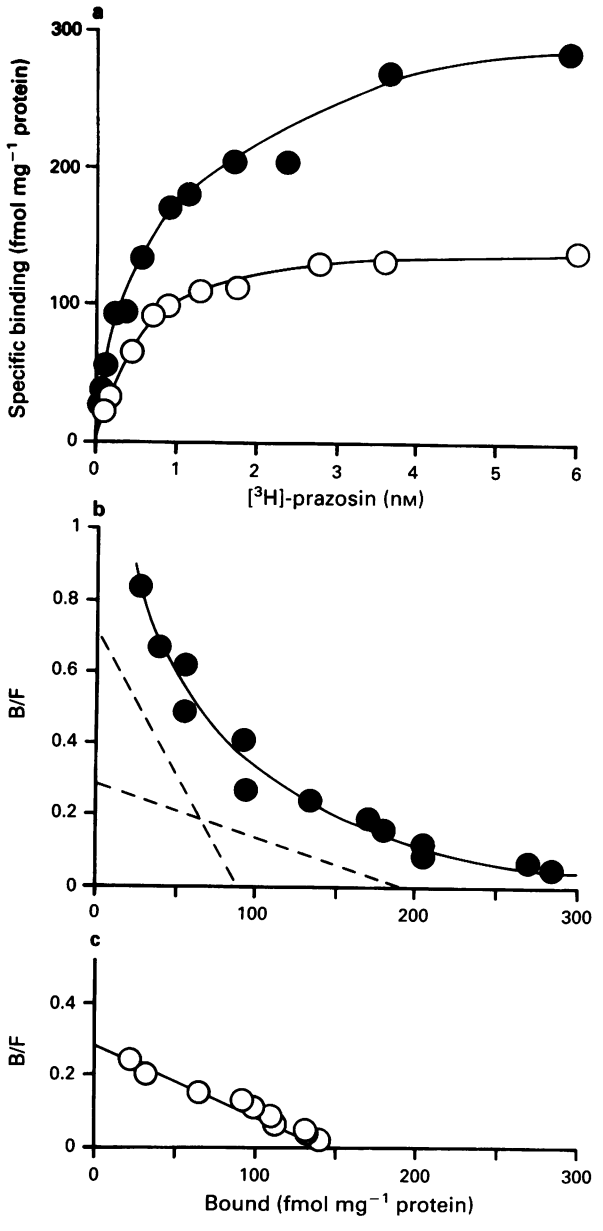


Figure 1 Saturation experiments of [^{3}H]-prazosin binding to rabbit thoracic aorta membranes. (a) Saturation curves of specific [^{3}H]-prazosin binding to chloroethylclonidine (CEC)-untreated (●) and CEC-pretreated membranes (○). (b) Scatchard analysis in CEC-untreated membranes. (c) Scatchard analysis in CEC-pretreated membranes. The data are obtained from a single experiment where each point is the mean of duplicate determinations.

(99.2 ± 15.6 fmol mg^{-1} protein, $n = 6$) was significantly lower than that of CEC-untreated membranes (Figure 1a and c).

In order to characterize the prazosin-high and low affinity sites, two kinds of displacement experiments were performed. Firstly, 200 pM [^{3}H]-prazosin binding sites in the CEC-untreated membranes were analyzed. Unlabelled prazosin displaced the binding monophasically with a high pK_i value (9.99). WB4101 and HV723 also antagonized the 200 pM [^{3}H]-prazosin binding. Computer analysis of the displacement curves gave a better fit to a one-site model, estimating more than 10 times lower affinities of WB4101 and HV723 as compared with that of prazosin (Figure 2a, Table 1).

Secondly, the low affinity sites for prazosin were characterized in the CEC-pretreated membranes, because prazosin-high affinity sites were found to be almost inactivated by

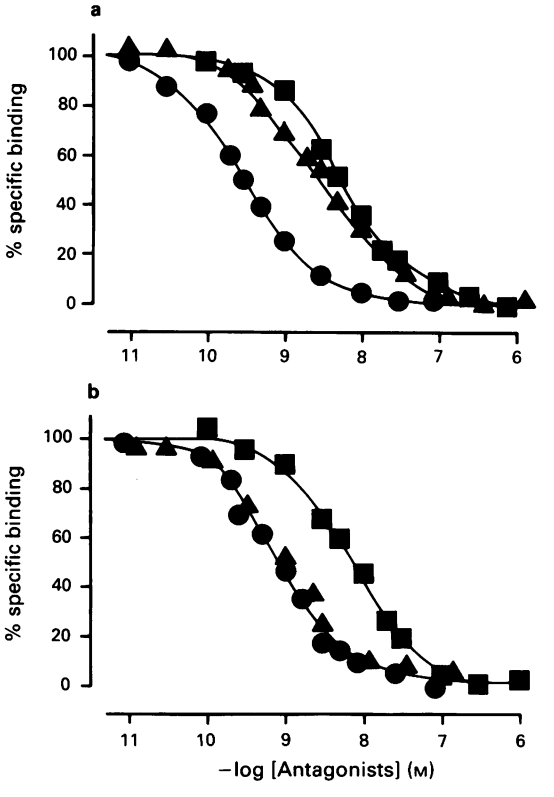


Figure 2 Displacement of [^{3}H]-prazosin binding from rabbit thoracic aorta membranes by prazosin (●), WB4101 (▲) and HV723 (■) in chloroethylclonidine (CEC)-untreated (a) and pretreated membranes (b). The concentrations of [^{3}H]-prazosin used were 200 pM in (a) and 1000 pM in (b).

Table 1 Inhibition of [^{3}H]-prazosin binding to α_1-adrenoceptors of rabbit thoracic aorta membranes by α-antagonists

	CEC-untreated			*CEC-pretreated*		
Drug	n	pK_i	*Slope factor*	n	pK_i	*Slope factor*
Prazosin	3	9.99 ± 0.05	1.05 ± 0.04	3	9.09 ± 0.03[a]	0.94 ± 0.03
WB4101	3	8.88 ± 0.05	0.88 ± 0.02	4	8.97 ± 0.06	0.92 ± 0.04
HV723	3	8.49 ± 0.10	1.07 ± 0.09	3	8.17 ± 0.11	1.03 ± 0.09
Noradrenaline	3	5.84 ± 0.06	0.91 ± 0.09	3	5.30 ± 0.05[a]	1.00 ± 0.04
Methoxamine	3	4.10 ± 0.06	0.86 ± 0.02	3	4.43 ± 0.08[a]	0.89 ± 0.06

Data shown are mean ± s.e.mean.
n: number of experiments.
[a] Significantly different from control (CEC-untreated) ($P < 0.05$).
CEC: membranes were pretreated with 10 μM chloroethylclonidine (CEC) for 20 min and then washed.
The concentrations of [^{3}H]-prazosin used were 200 pM in CEC-untreated membranes and 1,000 pM in CEC-pretreated membranes, respectively.

CEC pretreatment in saturation experiments. A higher concentration of [^{3}H]-prazosin (1000 pM) was used to label the low affinity sites. Unlabelled prazosin also displaced the [^{3}H]-prazosin binding in a monophasic manner ($pK_i = 9.09 \pm 0.03$). HV723 displaced 1000 pM [^{3}H]-prazosin binding and the pK_i value was similar to that obtained in CEC-untreated membranes (Figure 2b, Table 1).

Displacement profiles for noradrenaline and methoxamine at prazosin-high and low affinity sites were also examined in CEC-untreated and pretreated membranes. These agonists inhibited the binding of [^{3}H]-prazosin. The pK_i value (5.84) for noradrenaline in CEC-untreated membranes was approximately 3 times higher than that (5.30) in CEC-pretreated membranes. Conversely, the affinity for methoxamine was only slightly higher in the CEC-pretreated membranes (Table 1).

Functional study

Noradrenaline and methoxamine produced a concentration-dependent contraction in rabbit isolated thoracic aorta. Prazosin antagonized the contractions induced by noradrenaline and produced a rightward shift of the concentration-response curves. The slope of the Schild plot for prazosin was significantly different from unity (slope = 0.723, 95% CL = 0.668–0.779), so the occurrence of two different affinity sites was suggested (Figure 3 and Table 2). However, the concentration-response curve for methoxamine was inhibited by prazosin in a simple competitive manner. WB4101 and HV723 also antagonized the contractions induced by both agonists with a Schild slope close to unity. Thus, single affinity constants for both antagonists were estimated (Table 2).

We have previously shown that CEC selectively masked the contractions mediated through high affinity sites for prazosin (Muramatsu *et al.*, 1990b). In the present study, pretreatment with CEC (10 μM) also shifted the concentration-response curve for noradrenaline to the right (pD_2; from 7.18 ± 0.04 to 6.88 ± 0.04, $n = 14$) without affecting the maximal contraction. On the other hand, the concentration-response curve for methoxamine was not significantly affected by CEC pretreatment (pD_2; 5.79 ± 0.04 and 5.75 ± 0.05 without and with CEC pretreatment, respectively, $n = 14$). All the antagonists tested blocked competitively the noradrenaline- and methoxamine-induced contractions in CEC-pretreated aorta (Table 2). The pK_B value for prazosin was similar to the value for low affinity site obtained in CEC-untreated aorta. The pK_B values for WB4101 and HV723 were not significantly different from the pK_B values in the CEC-untreated aorta.

To evaluate the affinity for noradrenaline and methoxamine in CEC-pretreated and untreated aortae, the effect of phenoxybenzamine was examined. Pretreatment with phenoxybenzamine (20 or 70 nM) produced an irreversible attenuation of contractile responses to noradrenaline or methoxamine. The calculated dissociation constant (pK_A value) for noradrenaline in CEC-untreated aorta was slightly higher than that in CEC-pretreated aorta. The pK_A values for methoxamine were not affected by CEC-pretreatment (Table 3).

Finally, effects of nifedipine were examined. Nifedipine (1 μM) slightly but significantly attenuated the contractile responses to noradrenaline or methoxamine both in CEC-untreated and pretreated aortae (Figure 4).

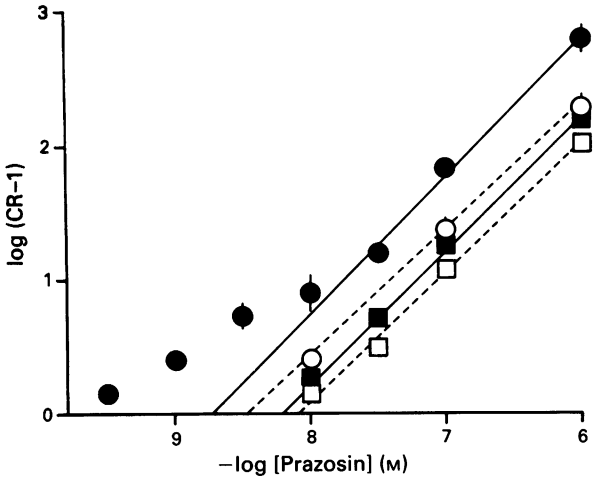


Figure 3 Schild plots for prazosin against contractile responses to noradrenaline (●, ○) or methoxamine (■, □). Filled symbols: chloroethylclonidine (CEC)-untreated aorta; open symbols: CEC-pretreated aorta.

Table 3 The dissociation constants (pK_A) of noradrenaline and methoxamine in rabbit thoracic aorta

	pK_A	
Treatment	*Noradrenaline*	*Methoxamine*
None	6.40 ± 0.11	4.48 ± 0.16
CEC	5.78 ± 0.10[a]	4.58 ± 0.13

Data shown are mean ± s.e.mean ($n = 8–10$).
CEC: 10 μM chloroethylclonidine.
The pK_A values were obtained by the method of Furchgott (1966) using 20 or 70 nM phenoxybenzamine.
[a] Significantly different from control (no treatment) ($P<0.05$).

Table 2 Antagonism by prazosin, WB4101 and HV723 of the contractile responses to noradrenaline and methoxamine in rabbit thoracic aorta

	pK_B (Slope, 95% CL)	
Antagonist	*No treatment*	*After CEC*
(I) Noradrenaline-induced contraction		
Prazosin	9.71 ± 0.08[a]	
WB4101	8.74 ± 0.15 (1.02, 0.85–1.19)	8.50 ± 0.09 (0.90, 0.79–1.01)
HV723	8.27 ± 0.05 (0.99, 0.92–1.06)	8.24 ± 0.07 (0.92, 0.83–1.02)
	8.49 ± 0.06 (0.95, 0.88–1.03)	8.33 ± 0.07 (0.92, 0.84–1.01)
(II) Methoxamine-induced contraction		
Prazosin	8.22 ± 0.04 (1.00, 0.93–1.06)	8.20 ± 0.03 (0.96, 0.91–1.01)
WB4101	8.49 ± 0.07 (0.98, 0.90–1.06)	8.51 ± 0.08 (0.98, 0.90–1.07)
HV723	8.16 ± 0.09 (1.07, 0.92–1.22)	8.36 ± 0.08 (0.94, 0.85–1.03)

[a] The pK_B value was estimated from the inhibitory effect of 300 pM prazosin. The other data were obtained from Schild plots.
CEC: 10 μM chloroethylclonidine.

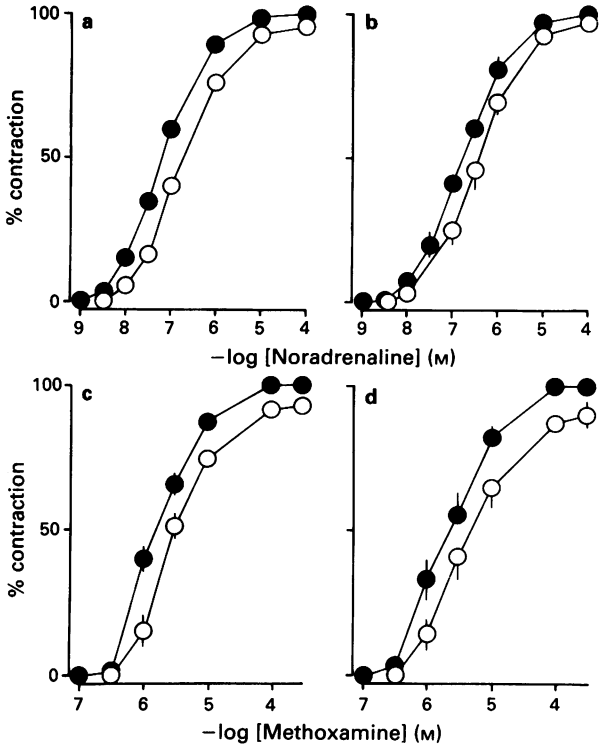


Figure 4 Effects of nifedipine on the concentration-response curves for noradrenaline (a, b) or methoxamine (c, d). (a, c) Chloroethylclonidine (CEC)-untreated aorta; (b, d): CEC-pretreated aorta. (●): control, (○): 1 μM nifedipine. The data shown are means ± s.e.mean of 5–6 experiments. All the contractile responses to noradrenaline or methoxamine were slightly but significantly ($P<0.05$) attenuated by nifedipine even though symbols overlap each other.

Discussion

The presence of two distinct α_1-adrenoceptors has been reported in the rabbit thoracic aorta (Babich *et al.*, 1987; Piascik *et al.*, 1988; Muramatsu *et al.*, 1990b). The present binding study reveals that α_1-adrenoceptors are clearly discriminated by prazosin; i.e., prazosin-high and low affinity sites. According to the α_{1H}, α_{1L}, α_{1N} subclassification (Muramatsu *et al.*, 1990a), both the sites appear to respectively correspond to α_{1H} and α_{1L} subtypes, as suggested previously (Muramatsu *et al.*, 1990b).

α_1-Adrenoceptors with high affinity for prazosin have been further subdivided into four subclasses (α_{1A}, α_{1B}, α_{1C} and α_{1D}) in binding and molecular biological studies (Han & Minneman, 1991; Lomasney *et al.*, 1991; Perez *et al.*, 1991; Hiramatsu *et al.*, 1992). We recently proposed the possibility that such subtypes may be included in the α_{1H} group designated in the α_{1H}, α_{1L}, α_{1N} subclassification (Muramatsu *et al.*, 1991; Oshita *et al.*, 1991; Ohmura *et al.*, 1992). Only the α_{1B} subtype among α_{1H} group shows low affinity for WB4101 and can be markedly inactivated by an alkylating agent, CEC. In this study, we determined which of the subtypes corresponds to the prazosin-high affinity site (α_{1H}) of rabbit thoracic aorta. The results obtained in binding experiments clearly indicate that the prazosin-high affinity site of rabbit thoracic aorta is a low affinity site for WB4101 and is markedly inactivated by CEC, corresponding to the features of an α_{1B}-adrenoceptor subtype. Expression of α_{1B}, but not α_{1A} and α_{1C} subtypes, in the rabbit thoracic aorta was also demonstrated by northern blot analysis (Schwinn *et al.*, 1991).

The prazosin-high and low affinity sites are also found from the antagonistic manner of prazosin against the contractile response to noradrenaline. However, WB4101 and HV723 cannot discriminate between the sites, resulting in a simple competition with a low affinity for each drug. Pretreatment with CEC abolished the inhibitory effect of low concentrations of prazosin but did not affect the inhibitory potency of WB4101 or HV723. These results support our previous view that noradrenaline-induced contraction is produced through two distinct α_1-adrenoceptor populations (presumably α_{1H} and α_{1L}) in the rabbit thoracic aorta (Muramatsu *et al.*, 1990b), and further suggest that the prazosin-high affinity site (α_{1H}) is characterized as an α_{1B} subtype.

In contrast to the response to noradrenaline, prazosin inhibited the contractile responses to methoxamine in a competitive manner and pretreatment with CEC failed to affect the response to methoxamine. Since similar and low pK_B values were estimated for prazosin, WB4101 or HV723 irrespective of CEC pretreatment, the contractile response to methoxamine appears to be produced through a single subtype (presumably α_{1L}). Alternatively, even though two different subtypes (α_{1B} and α_{1L}) are involved in the contractions induced by methoxamine, the α_{1L} subtype would play a more predominant role in the response to methoxamine than the α_{1B} subtype, because either prazosin at concentrations low enough to bind to the α_{1B} subtype or CEC to inactivate the α_{1B} subtype had no effect on the contractile response to methoxamine.

We compared the affinities of α_{1B} and α_{1L} subtypes for both agonists. Binding data clearly show that the α_{1B} subtype is slighly but significantly more sensitive to noradrenaline than the α_{1L} subtype, whereas methoxamine tends to reverse the selectivity slightly. In the functional experiments also, similar results were obtained for the dissociation constant (pK_A) for noradrenaline, but there was no significant difference in the values for methoxamine between CEC-untreated and pretreated aortae. This, together with the binding data mentioned above, suggests the predominant involvement of the α_{1L} adrenoceptor subtype in the contractile response to methoxamine compared with the response to noradrenaline.

The dissociation constants for α_1-adrenoceptor agonists and antagonists estimated in the functional experiments may be approximate values because, control concentration-response curves obtained in the absence of α_1-adrenoceptor antagonists might be caused through activation of two receptor subtypes, while the curves obtained after treatment with antagonist may be mainly produced through a single subtype. The contribution of each subtype to the total response may change depending on experimental conditions.

CEC was originally reported to produce a selective inactivation of the α_{1B} subtype (Minneman *et al.*, 1988). Although almost complete inactivation of the prazosin-high affinity site (α_{1B} subtype) was indeed produced by 10 μM CEC in the present study, [^{3}H]-prazosin binding to the low affinity site (α_{1L} subtype) was also in part inhibited. Such partial inactivation or inhibition of [^{3}H]-prazosin binding has been reported in other subtypes including α_{1A} and α_{1C} subtypes (Schwinn *et al.*, 1991; Oshita *et al.*, 1991; Ohmura *et al.*, 1992), indicating a relatively low selectivity of CEC to α_1-adrenoceptor subtypes.

Even under conditions where the α_{1L} subtype was in part inactivated by CEC, noradrenaline and methoxamine produced a maximum contraction in the rabbit thoracic aorta. This indicates that both drugs can act as full agonists for the α_{1L} subtype, and further suggests that there are spare receptors in the α_{1L} subtype-mediated contractions in the rabbit thoracic aorta. Higher density of α_{1L} than α_{1B} subtype and higher value of pD_2 than pK_A for noradrenaline or methoxamine would support this suggestion.

Han *et al.* (1987) have proposed that the α_{1A} receptor subtype selectively mediates the influx of extracellular Ca^{2+}, whereas the α_{1B} subtype controls the release of stored intracellular Ca^{2+} by an inositol phosphate mechanism. Following this original proposal, Suzuki *et al.* (1990) suggested that phenylephrine-induced contraction of the rabbit thoracic aorta may be mediated through α_{1A} and α_{1B} subtypes. However, mRNA for the α_{1A} subtype was not detected in the

rabbit aorta (Schwinn *et al.*, 1991) and WB4101-high affinity sites were not found in the present binding and functional studies. Furthermore, the contractile responses induced by noradrenaline or methoxamine in CEC-pretreated aorta were not abolished by nifedipine. Although nifedipine has been demonstrated to produce a non-selective inhibition of the contractile responses mediated through different α_1-adrenoceptor subtypes (Muramatsu *et al.*, 1990a; 1991; Ohmura *et al.*, 1992), the slight inhibition by nifedipine observed in the present study does not suggest the possible occurrence of the α_{1A} subtype in the rabbit thoracic aorta.

In conclusion, the present study shows the occurrence of two distinct α_1-adrenoceptor subtypes (prazosin-high and low affinity sites) in the rabbit thoracic aorta. Although the prazosin-high affinity site was previously designated as α_{1H} (Muramatsu *et al.*, 1990b), the pharmacological features obtained indicate that the site corresponds to the α_{1B} subtype (a member of α_{1H} group) according to the recently conciliated subclassification, while the prazosin-low affinity site is of the α_{1L} subtype which is clearly different from the other subtypes in its pharmacological features. It is likely that noradrenaline-induced contraction is mediated through both the α_{1B} and α_{1L} subtypes, whereas the response to methoxamine is predominantly caused through the α_{1L} subtype.

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References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BABICH, M., PEDIGO, N.W., BUTLER, B.T. & PIASIK, M.T. (1987). Heterogeneity of alpha$_1$ receptors associated with vascular smooth muscle: evidence from functional and ligand binding studies. *Life Sci.*, **41**, 663–673.

BRADFORD, 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.

CHENG, Y.-D. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

FLAVAHAN, N.A. & VANHOUTTE, P.M. (1986). α_1-Adrenoceptor subclassification in vascular smooth muscle. *Trends Pharmacol. Sci.*, **7**, 347–349.

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.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbuch der Experimentellen Pharmakologie*, Vol. 3, ed. Blaschko, H. & Muscholl, E. pp. 283–335. New York: Springer.

FURCHGOTT, R.F. (1981). The requirement for endothelial cells in the relaxation of arteries by acetylcholine and some vasodilators. *Trends Pharmacol. Sci.*, **2**, 173–176.

HAN, C., ABEL, P.W. & MINNEMAN, K.P. (1987). α_1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature*, **329**, 333–335.

HAN, C. & MINNEMAN, K.P. (1991). Interaction of subtype-selective antagonists with α_1-adrenergic receptor binding sites in rat tissues. *Mol. Pharmacol.*, **40**, 531–538.

HIRAMATSU, Y., MURAOKA, R., KIGOSHI, S. & MURAMATSU, I. (1992). 5-Methylurapidil may discriminate between α_1-adrenoceptors with a high affinity for WB4101 in rat lung. *Br. J. Pharmacol.*, **105**, 6–7.

LOMASNEY, J.W., COTECCHIA, S., LEFKOWITZ, R.J. & CARON, M.G. (1991). Molecular biology of α-adrenergic receptors: implications for receptor classification and for structure-function relationships. *Biochim. Biophys. Acta*, **1095**, 127–139.

MCPHERSON, G.A. (1985). Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. *Pharmacol. Methods*, **14**, 213–228.

MIGNOT, E., BOWERSOX, S.S., MADDALUNO, J., DEMENT, W. & CIARANELLO, R. (1989). Evidence for multiple [^{3}H]prazosin binding sites in canine brain membranes. *Brain Res.*, **486**, 56–66.

MINNEMAN, K.P. (1988). α_1-Adrenergic receptor subtypes, inositol-phosphate, and sources of cell Ca^{2+}. *Pharmacol. Rev.*, **40**, 87–119.

MINNEMAN, K.P., HAN, C. & ABEL, P.W. (1988). Comparison of α_1-adrenergic receptor subtypes distinguished by chlorethylclonidine and WB4101. *Mol. Pharmacol.*, **33**, 509–514.

MORROW, A.L. & CREESE, I. (1986). Characterization of α_1-adrenergic receptor subtypes in rat brain: A re-evaluation of [^{3}H] WB4101 and [^{3}H]prazosin binding. *Mol. Pharmacol.*, **29**, 321–330.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107**, 220–239.

MURAMATSU, I., OHMURA, T., KIGOSHI, S., HASHIMOTO, S. & OSHITA, M. (1990a). Pharmacological subclassification of α_1-adrenoceptors in vascular smooth muscle. *Br. J. Pharmacol.*, **99**, 197–201.

MURAMATSU, I., KIGOSHI, S. & OHMURA, T. (1991). Subtypes of α_1-adrenoceptors involved in noradrenaline-induced contractions of rat thoracic aorta and dog carotid artery. *Jpn. J. Pharmacol.*, **57**, 535–544.

MURAMATSU, I., KIGOSHI, S. & OSHITA, M. (1990b). Two distinct α_1-adrenoceptor subtypes involved in noradrenaline contraction of the rabbit thoracic aorta. *Br. J. Pharmacol.*, **101**, 662–666.

OHMURA, T., OSHITA, M., KIGOSHI, S. & MURAMATSU, I. (1992). Identification of α_1-adrenoceptor subtypes in the rat vas deferens: binding and functional studies. *Br. J. Pharmacol.*, **107**, 697–704.

OSHITA, M., KIGOSHI, S. & MURAMATSU, I. (1991). Three distinct binding sites for [^{3}H]-prazosin in the rat cerebral cortex. *Br. J. Pharmacol.*, **104**, 961–965.

PEREZ, D.M., PIASCIK, M.T. & GRAHAM, R.M. (1991). Solution-phase library screening for the identification of rare clones: isolation of an α_{1D}-adrenergic receptor cDNA. *Mol. Pharmacol.*, **40**, 876–883.

PIASCIK, M.T., KUSIAK, J.W., PITHA, J., BUTLER, B.T., LE, H.T. & BABICH, M. (1988). Alkylation of alpha-1 receptors with a chemically reactive analog of prazosin reveals low affinity sites for norepinephrine in rabbit aorta. *J. Pharmacol. Exp. Ther.*, **246**, 1001–1011.

SCHWINN, D.A., PAGE, S.O., MIDDLETON, J.P., LORENZ, W., LIGGETT, S.B., YAMAMOTO, K., LAPETINE, E.G., CARON, M.G., LEFKOWITZ, R.J. & COTECCHIA, S. (1991). The α_{1C}-adrenergic receptor: characterization of signal transduction pathways and mammalian tissue heterogeneity. *Mol. Pharmacol.*, **40**, 619–626.

SUZUKI, E., TSUJIMOTO, G., TAMURA, K. & HASHIMOTO, K. (1990). Two pharmacologically distinct α_1-adrenoceptor subtypes in the contraction of rabbit aorta: each subtype couples with a different Ca^{2+} signalling mechanism and plays a different physiological role. *Mol. Pharmacol.*, **38**, 725–736.

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Cellular mechanism of U78517F in the protection of porcine coronary artery endothelial cells from oxygen radical-induced damage

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1 The aim of this study was to clarify the role of lipid peroxidation in cellular injury as assessed by lactate dehydrogenase (LDH) release from cultured coronary artery endothelial cells of the pig. Cells exposed to H_2O_2 at concentrations of 0.1 to 20 mM or to a xanthine and xanthine oxidase (X/XO) reaction mixture released LDH into the medium. Significant release from X/XO-treated cells took place with a delay of 2 h.

2 Superoxide dismutase (SOD), catalase or dimethylthiourea attenuated the release of LDH from X/XO-treated cells. Similarly the putative inhibitor of lipid peroxidation, U78517F attenuated the release of LDH by X/XO with an IC_{50} of 0.08 μM.

3 H_2O_2 was continuously produced by the addition of X/XO to the medium alone. However, in the presence of endothelial cells, H_2O_2 was eliminated at 1 h. U78517F had no effect on either process.

4 The oxygen radical-induced release of LDH was associated with malondialdehyde (MDA) formation. U78517F inhibited the formation of MDA with an IC_{50} of 0.27 μM.

5 Reduction of the Ca^{2+} concentration in the incubation medium from 1.6 mM to 0.016 mM markedly attenuated the release of LDH from endothelial cells. Nifedipine (1 μM) did not attenuate the LDH release from the cells.

6 It is likely that porcine coronary artery endothelial cells can be thus injured by oxygen radicals presumably through hydroxyl radicals formed and consequent lipid peroxidation, and that the extracellular Ca^{2+} concentration plays an important role in the genesis of such endothelial cell damage.

Keywords: Lipid peroxidation; endothelial cells; cell injury; U78517F; oxygen radicals; Ca^{2+}; coronary artery

Introduction

Endothelial cells, which play a pivotal role in metabolism of peptide, biogenic amines, adenine nucleotides, eicosanoids, platelet activating factor as well as endothelium-derived relaxing factor (EDRF, nitric oxide) release, are both critical targets and sources of reactive oxygen species. A complex balance (Vercellotti *et al.*, 1988) between exogenous oxidants and endogenous antioxidants such as glutathione and catalase is likely to underlie the fate of reactive oxygen-exposed cells. There are several lines of evidence for endothelial cell injury induced by free radical reactions in interaction of leukocyte and endothelium (Sacks *et al.*, 1978; Weiss *et al.*, 1981; Martin, 1984). Prime targets for free radical reactions are the unsaturated bonds in membrane lipids. Consequent peroxidation causes a loss in membrane fluidity and receptor alignment and potentially cellular lysis (Machlin & Bendich, 1987).

The 21-aminosteroid compound (U74006F), an inhibitor of lipid peroxidation, has been shown to be effective in protecting brain tissue from irreversible cell damage resulting from iron-dependent lipid peroxidation after brain or spinal cord injury, or cerebral ischaemia (Hall, 1988; Hall & Yonkers, 1988; Hall *et al.*, 1988; 1989). U78517F (2-[[4(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl] methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol,dihydrochloride) has been developed as a second-generation series of compounds, possessing various amino functions of U74006F combined with the antioxidant ring portion of α-tocopherol. U78517F is approximately ten fold more potent than U74006F for inhibition of lipid peroxidation in rat brain homogenates (Hall *et al.*, 1990). It is not known, at present, whether inhibition of lipid peroxidation protects endothelial cells from reactive oxygen-induced injury as effectively as neuronal tissues.

In hepatocyte injury by toxins, a common functional consequence involves an increase in intracellular Ca^{2+} concentrations, most likely representing an influx of Ca^{2+} across the damaged plasma membrane and down a steep concentration gradient (Schanne *et al.*, 1979). Our previous study (Kimura *et al.*, 1992) has shown that exposure of coronary artery endothelial cells to hydrogen peroxide at a low concentration leads to sustained increase in intracellular Ca^{2+} concentration. However, it has not yet been elucidated whether the increased cytosolic Ca^{2+} concentration is directly linked to membrane lipid peroxidation in the processes of cell injury.

The present study was undertaken to clarify the role of lipid peroxidation in the cellular injury, expressed as a release of lactate dehydrogenase (LDH), which was induced by exposure of porcine coronary artery endothelial cells to xanthine and xanthine oxidase (X/XO). The link between lipid peroxidation and an extracellular Ca^{2+} source was also investigated.

Methods

Endothelial cell culture

Porcine hearts were isolated at a local slaughter house, and kept under sterile conditions for the short period of transportation (approximately 30 min). The coronary arterial segments of left circumflex, right circumflex and descending arteries were cleaned of surrounding adipose and connective tissue, and cut open longitudinally. The endothelial cells were

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harvested by scraping the luminal surface of the coronary artery with a razor blade gently under sterile conditions. The collected cells were suspended in Dulbecco's modified Eagle's (DME) medium supplemented with 10% foetal bovine serum, penicillin G (100 u ml^{-1}) and streptomycin (100 $\mu g\ ml^{-1}$), seeded into gelatin-coated 60 mm culture dish, and cultured under 95% humidified atmosphere containing 5% CO_2 at 37°C. After the first medium change, the concentration of penicillin G and streptomycin was reduced to 10 u ml^{-1} and 10 $\mu g\ ml^{-1}$, respectively. After the cells reached confluence, they were expanded by brief trypsinization with 0.05% trypsin in phosphate buffered saline containing 0.53 mM EDTA. Endothelial cells were seeded into 24 well plates and used in experiments when confluent.

Endothelial cell injury

Endothelial cells in culture monolayers were exposed to (1) H_2O_2 at concentrations varying from 0.1 to 20 mM in DME medium or (2) a reaction mixture containing 0.5 mM xanthine and 0.02 u ml^{-1} xanthine oxidase (X/XO) in DME medium. Since xanthine oxidase may have protease activity which could contribute to the X/XO-induced injury, cells were exposed to xanthine oxidase at concentrations of 0.02 and 0.1 u ml^{-1} in the absence and presence of 0.5 mM xanthine for 5 h. In separate studies, oxygen radical scavengers were added to the reaction mixture of X/XO. When the effect of extracellular Ca^{2+} on cell injury induced by the X/XO was examined, DME medium was replaced with Hanks' balanced salt solution as follows (mM): NaCl 137, KCl 5.4, Na_2HPO_4 0.34, KH_2PO_4 0.44, $MgSO_4$ 0.81, glucose 5.6, and $CaCl_2$ at three different concentrations of 0.016, 0.16 or 1.6 mM.

The cells were treated with 0.1% Triton X-100 for 15 min to measure the total lactate dehydrogenase (LDH); total LDH activity was 0.737 ± 0.039 u/10^6 cells (n = 12). Released LDH activity was measured by determining the first-order rate of NADH oxidation spectrophotometrically at 340 nm using pyruvate and NADH (Kornberg, 1955). The activity was expressed in units (1 unit oxidized 1 μmol of NADH per min).

Assay for H_2O_2 and malondialdehyde (MDA) formation

H_2O_2 generated in the external medium following treatment with X/XO was determined according to colorimetric procedures reported by Thurman *et al.* (1972). X/XO was added to the Hanks' balanced salt solution with and without endothelial cells. Aliquots of the reaction mixture were then put into a solution including 0.1 ml of 25% trichloroacetic acid (TCA), 0.2 ml of 10 mM ferrous ammonium sulphate and 0.1 ml of 2.5 M potassium thiocyanate. The spectrophotometric absorbance was determined at 490 nm. Known amounts of H_2O_2 was added to the medium to obtain the standard curve under the same conditions. Lipid peroxidation was estimated as MDA formation which forms a pink chromogen thiobarbituric acid (TBA) (Gutteridge, 1987). After treatment of the cells with the 0.25 ml reaction mixture for 5 h, 2.8% TCA in a volume of 0.25 ml was added. After a 5 min incubation period at room temperature, 0.4 ml of the reaction mixture was treated with 0.2 ml of 1% TBA in 0.05 N NaOH. The mixture was heated in a boiling water bath for 10 min, then cooled and centrifuged at 2500 r.p.m. The absorbance for MDA was determined at 532 nm against appropriate blanks and the MDA concentration was estimated based on the molar extinction coefficient of the MDA-TBA chromophore of 156,000 $M^{-1}\ cm^{-1}$ at 532 nm.

Materials and statistical analysis

Materials and drugs used were superoxide dismutase (SOD), catalase, butylated hydroxytoluene (BHT), deferoxamine, glutathione, oxypurinol, indomethacin, nifedipine, xanthine, xanthine oxidase grade I, Dulbecco's modified Eagle's medium, Hanks' salts without Mg^{2+} and Ca^{2+}, penicillin G and streptomycin; all from Sigma Chemical Co. (St. Louis, MO, U.S.A.). In addition, foetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.), hydrogen peroxide (Wako Pure Chem., Osaka, Japan), U78517F (Upjohn, Kalamazoo, MI, U.S.A.) were used for the study. Other reagents used were of the highest quality commercially available. Nifedipine in solution was shielded from light with aluminium foil, and during the 5 h incubation of the drug-containing medium was in a dark CO_2 incubator.

Results were expressed as mean ± s.e.mean or mean with 95% confidence limits in parentheses. Statistical analyses were made according to Dunnet's multiple range test or Student's unpaired t test.

Results

Damage to cells by oxidizing agents

Addition of H_2O_2 to the medium at a concentration of 0.1 mM or more increased LDH released from endothelial

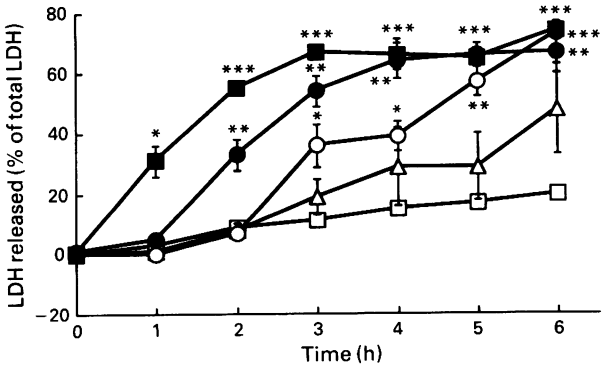


Figure 1 H_2O_2-induced release of lactate dehydrogenase (LDH) from endothelial cells. Confluent monolayers of cells were exposed to H_2O_2 at 0.1 to 20 mM: saline (□); H_2O_2 0.1 mM (△); 1 mM (○); 10 mM (●), and 20 mM (■). LDH released into medium was expressed as % of the total LDH assessed after treatment of cells with 0.1% Triton X-100; total LDH was 0.199 ± 0.013 u/well. Data points represent mean with s.e.mean of three experiments with duplicate wells within each experiment. Significantly different from control values (saline): $*P<0.05$; $**P<0.01$; $***P<0.001$.

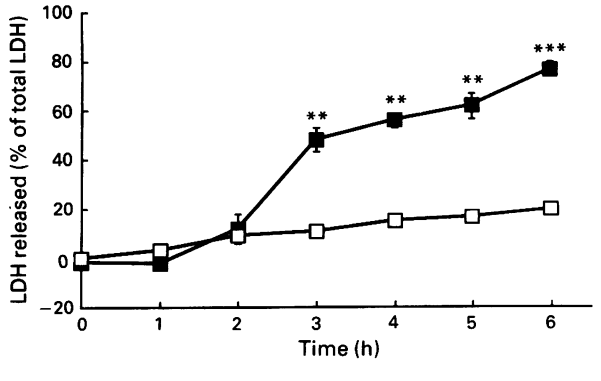


Figure 2 Slowly developed increase in lactate dehydrogenase (LDH) following exposure of endothelial cells to xanthine/xanthine oxidase (X/XO) reaction mixture (■). Confluent monolayers were incubated with 0.5 mM xanthine and 0.02 u ml^{-1} xanthine oxidase for 6 h. LDH released into medium was expressed as % of the total LDH assessed after treatment of cells with 0.1% Triton X-100; 0.199 ± 0.013 u/well. Data points represent means, with s.e.mean of three experiments with duplicate wells within each experiment. Significantly different from control (saline, □): $**P<0.01$; $***P<0.001$.

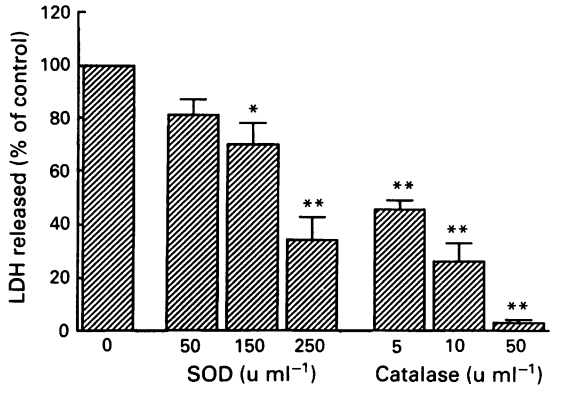


Figure 3 Effect of superoxide dismutase (SOD) and catalase on lactate dehydrogenase (LDH) release from endothelial cells by xanthine/xanthine oxidase (X/XO). Confluent monolayers were incubated with 0.5 mM xanthine and 0.02 u ml^{-1} xanthine oxidase in the absence or presence of scavengers for 5 h. X/XO-induced increase in LDH over the baseline value (without X/XO) was 0.072 ± 0.010 u/well. Columns represent means, with s.e.mean of three experiments with duplicate wells within each experiment. Significantly different from control of '0': $*P < 0.05$; $**P < 0.01$.

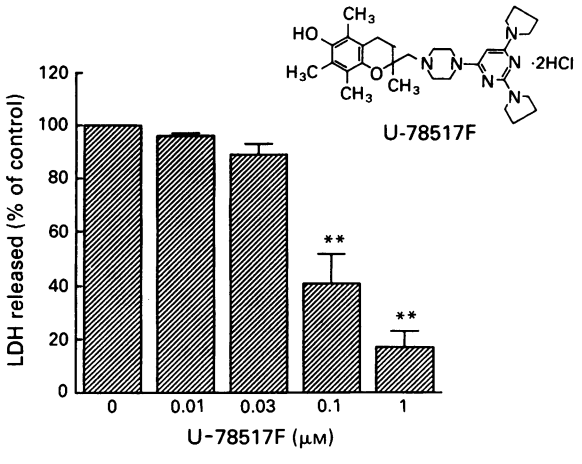


Figure 4 Concentration-dependent inhibition by U78517F of lactate dehydrogenase (LDH)-release following treatment with xanthine/xanthine oxidase (X/XO) reaction mixture. Confluent monolayers were incubated with 0.5 mM xanthine and 0.02 u ml^{-1} xanthine oxidase for 5 h. X/XO-induced increase in LDH over the baseline value (without X/XO) was 0.094 ± 0.012 u/well. Columns represent means with s.e.mean of five experiments with duplicate wells within each experiment. Significantly different from control of '0': $**P < 0.01$.

Table 1 IC_{50} values of U78517F and butylated hydroxytoluene for their inhibitory effects on xanthine and xanthine oxidase-induced release of lactate dehydrogenase (LDH)

Compound	n	*IC_{50}* (μM)
U78517F	5	0.08 (0.04–0.19)
Butylated hydroxytoluene	4	0.87 (0.29–2.6)

Cells were exposed to 0.5 mM xanthine and 0.02 u ml^{-1} xanthine oxidase for 5 h. Figures in parentheses are 95% confidence limits in the study with duplicate wells. LDH released in the absence of the compounds over the baseline value (without X/XO) was 0.090 ± 0.014 u/well for U78517F and 0.077 ± 0.005 u/well for butylated hydroxytoluene.

cells, compared to that of saline-treated cells, over a 6 h time period (Figure 1). Modest to moderate increases in LDH started after a delay of 2 h with 0.1 and 1.0 mM H_2O_2. Higher concentrations of H_2O_2 produced more rapid increase in LDH release. At 3 h, LDH release by H_2O_2 was concentration-dependent.

When endothelial cells were exposed to oxygen radicals generated by xanthine (0.5 mM) plus xanthine oxidase (0.02 u ml^{-1}) (X/XO), there was a significant release of LDH, compared to the saline group (Figure 2). No increase in LDH was seen until 2 h after initiating the X/XO reaction. When endothelial cells were incubated with xanthine oxidase alone, release of LDH 5 h later was slight and not concentration-dependent; 8% (0.011 ± 0.008 u/well) of total LDH, 7% (0.010 ± 0.005 u/well) and 6% (0.008 ± 0.003 u/well) for xanthine oxidase at 0, 0.02 and 0.1 u ml^{-1}, respectively ($n = 3$). Study of xanthine oxidase at concentrations lower than 0.02 u ml^{-1} were not conducted. Even when xanthine oxidase was increased from 0.02 to 0.1 u ml^{-1} together with 0.5 mM xanthine, no substantial further increase in LDH was induced: 58% (0.092 ± 0.005 u/well) of total LDH for 0.02 u ml^{-1} xanthine oxidase and 49% (0.078 ± 0.004 u/well) for 0.1 u ml^{-1} xanthine oxidase ($n = 3$). Therefore, LDH released by X/XO could not be attributed to the action of xanthine oxidase alone.

Effect of anti-oxidants/scavengers on LDH release

Antioxidants and scavengers were added to the cell medium along with the X/XO reaction mixture, and LDH release was determined after 5 h incubation. As shown in Figure 3, SOD at 150 and 250 u ml^{-1} reduced LDH release from cells exposed to X/XO. By contrast with the incomplete inhibition of LDH release in the presence of SOD, catalase inhibited the LDH-release in a concentration-dependent fashion, abolishing the release at a concentration of 50 u ml^{-1}. The LDH-release was not significantly reduced by treatment with dimethyl sulphoxide (50 mM) or mannitol (50 mM); 98 ± 7% ($n = 5$) and 96 ± 5% ($n = 3$) of the control with X/XO, respectively. On the other hand, dimethylthiourea (40 mM) reduced LDH release from the endothelial cells significantly; to 6 ± 2% of the control with X/XO ($n = 3$, $P < 0.01$). When endothelial cells were treated with U78517F at concentrations of 0.01 to 1.0 μM, X/XO-induced release of LDH was attenuated in a concentration-dependent fashion (Figure 4). The IC_{50} for U78517F against LDH-release was 0.08 μM (Table 1). Butylated hydroxytoluene (BHT, 0.3 to 3 μM) reduced the X/XO-induced release of LDH concentration-dependently with an IC_{50} of 0.87 μM. Treatment with nifedipine (1 μM) did not attenuate release of LDH in response to X/XO, but rather, exhibited a trend to augment the release (Table 2). There was no significant inhibition of the LDH-release by indomethacin (30 μM) or oxypurinol (100 μM).

Table 2 Effects of various compounds on xanthine and xanthine oxidase-induced release of lactate dehydrogenase (LDH) from endothelial cells

Compound	*Concentration* (μM)	n	*Inhibition* (%)
Glutathione	5000	2	82
Oxypurinol	100	3	30 ± 14
Indomethacin	30	5	5 ± 6
Nifedipine	1	5	−21 ± 14[1]

LDH released in the absence of the compounds over the baseline value (without X/XO) was 0.101 u/well for glutathione, 0.060 ± 0.005 u/well for oxypurinol, 0.066 ± 0.008 u/well for indomethacin and 0.063 ± 0.007 u/well for nifedipine. Data are expressed as mean ± s.e.mean of n experiments with duplicate wells within each experiment, and where [1] indicates release of LDH was augmented by the treatment.

Table 3 Effect of reduction in external Ca^{2+} concentration on lactate dehydrogenase (LDH)-release in response to reaction mixture of xanthine (0.5 mM) and xanthine oxidase (0.02 u ml^{-1}) for 5 h

Ca^{2+} concentration (mM)	*LDH released* with X/XO (%)	without X/XO (%)
1.6	41 ± 6	10 ± 2
0.16	24 ± 3*	9 ± 2
0.016	15 ± 1**	7 ± 2

Results are expressed as % of total LDH. Total LDH which was determined with Triton X-100-induced lysis was 0.170 ± 0.010 u/well. Confluent monolayers were incubated in Hanks' balanced salt solutions with three different Ca^{2+} concentrations. Data are expressed as mean ± s.e.mean of four experiments with duplicate wells within each experiment.
Significant difference from values at 1.6 mM Ca^{2+}: *$P < 0.05$; **$P < 0.01$.

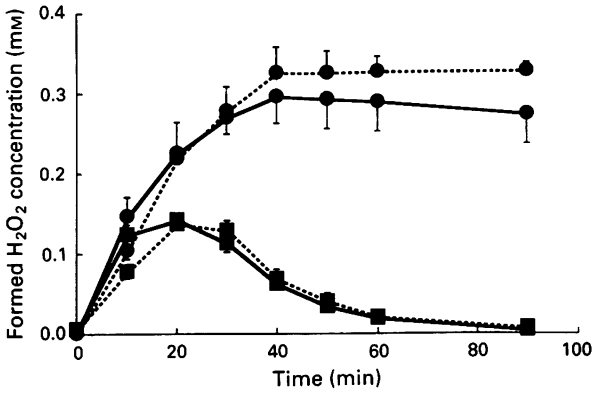


Figure 5 Effect of U78517F on H_2O_2 production following addition of xanthine/xanthine oxidase (X/XO) reaction mixture to the culture medium in the presence (■) or absence (●) of endothelial cells: saline, solid lines; U78517F, dotted lines. Data points represent means with s.e.mean of five experiments with duplicate wells within each experiment. There was no significant difference between saline- and U78517F-treated groups, except values at 10 min incubation with endothelial cells.

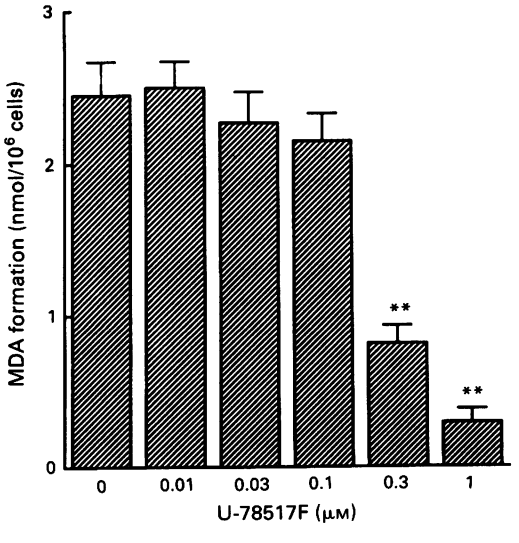


Figure 6 Concentration-dependent inhibition by U78517F of formation of malondialdehyde (MDA) in endothelial cells exposed to xanthine/xanthine oxidase (X/XO). Confluent monolayers were incubated with 0.5 mM xanthine and 0.02 u ml^{-1} xanthine oxidase for 5 h. Columns represent mean with s.e.mean of three experiments with triplicate wells within each experiment. Significantly different from control of '0': **$P < 0.01$.

Effect of extracellular Ca^{2+}

Endothelial cells were incubated with X/XO reaction mixture in Hanks' balanced salt solution medium containing a reduced Ca^{2+} concentration (0.16 mM and 0.016 mM). The release of LDH from endothelial cells following addition of X/XO was markedly attenuated, depending on the extent of the reduction in the external Ca^{2+} concentration (Table 3).

Mechanism of action of U78517F

In the absence of endothelial cells, H_2O_2 generated by X/XO was increased for 40 min and levelled off (Figure 5). In contrast, in the presence of endothelial cells, the X/XO-induced formation of H_2O_2 peaked at 20 min and then decreased to control levels by 60 min. U78517F did not alter the X/XO-induced formation of H_2O_2 in the medium or its degradation in either the presence or absence of endothelial cells.

When endothelial cells were exposed to X/XO for 5 h, MDA was formed, reaching a maximum concentration of 2.9 nM/10^6 cells (Figure 6). U78517F (0.1 to 1.0 μM) inhibited the formation of MDA with an IC_{50} of 0.27 μM (0.09–0.79 μM). BHT also markedly attenuated the formation of MDA in the cells exposed to X/XO with an IC_{50} of 0.92 μM (0.17–4.48 μM, $n = 3$).

Discussion

LDH was released into the extracellular medium gradually after treatment of coronary endothelial cells with X/XO. The time-dependent increase in LDH release was markedly inhibited by U78517F, with an IC_{50} of 0.08 μM. There are several agents reported to inhibit endothelial oxygen radical-induced injury including deferoxamine at 5 mM (Kvietys *et al.*, 1989), a ferric iron chelating agent, allopurinol at 200 μM (Ratych *et al.*, 1987), a xanthine oxidase inhibitor, SOD at 250 u ml^{-1} (Ratych *et al.*, 1987) and catalase at 200 u ml^{-1} (Ody & Junod, 1985). Although LDH is crude marker for cell damage in that it is generally indicative of severe cell membrane disruption and cell death (Whorton *et al.*, 1985), our results with this marker suggest a very potent cell-protecting activity of the lipid peroxidation inhibitor against cytotoxic oxygen radicals.

X/XO-induced release of LDH was inhibited by catalase in a concentration-dependent manner. Exogenous H_2O_2 which was applied to the cell, increased LDH release concentration-dependently at a concentration of 0.1 mM or more. In addition, formation of H_2O_2 by X/XO preceded release of LDH. From these findings, H_2O_2 may be responsible for the injury of cells exposed to X/XO. H_2O_2 acts directly on cells with consequent damage of cells (Sacks *et al.*, 1978; Weiss *et al.*, 1981; Harlan *et al.*, 1984). Link & Riley (1988) suggested in mammalian epithelial cells that H_2O_2, a stable product in the system investigated, is responsible for the death of cells after the exposure to X/XO system. As H_2O_2 can readily pass through cell membranes, it interacts with intracellular iron to form highly reactive oxidants such as the hydroxyl radical (Grisham & Granger, 1988). Dimethylthiourea, a hydroxyl radical scavenger with iron chelating action, protected endothelial cells from the oxygen radical-induced injury, while mannitol and dimethyl sulphoxide failed to protect them. The discrepancy in effects of the scavengers may derive from the inability of mannitol to pass through plasma membranes to reach the intracellular site of hydroxyl radical attack. Since generation of cytotoxic hydroxyl radicals within endothelial cells involves an iron catalyzed, site-specific reaction (Kvietys *et al.*, 1989), metal promoters of the reaction may not be suppressed by dimethyl sulphoxide at the specific site. Alternatively, it is conceivable that dimethylthiourea reacts with H_2O_2 and alkoxy radicals, since thiourea has nonspecific characteristics as a hydroxyl radical scavenger

(Cederbaum *et al.*, 1979; Winston *et al.*, 1983).

U78517F which has the antioxidant chromanol moiety of α-tocopherol, is potent in inhibiting MDA production in brain homogenates (Hall *et al.*, 1990). In the current study, endothelial cells were protected from the oxygen radical-induced injury by U78517F, being accompanied by a decrease in MDA formation at concentrations ranging from 0.1 to 1 μM. BHT, which also protected the cells from injury, serves as a chain breaker and acts as an electron donor to quench radical species (De Long, 1988). Thus, it is likely that lipid peroxidation which takes place as a consequence of free radical attack on unsaturated bonds in membrane lipids is crucial to oxygen radical-induced cell injury, and that the inhibitory effect of U78517F on the peroxidation results in minimizing a loss in membrane fluidity and cellular lysis. The cell protection by U78517F does not seem to be due to either decrease in H_2O_2 generated by X/XO or facilitated degradation of H_2O_2, because H_2O_2 generation was not altered by U78517F. Although there was a moderate difference in IC_{50} for U78517F between LDH release (0.08 μM) and MDA formation (0.27 μM), no difference was seen for BHT; 0.87 and 0.92 μM, respectively. Assuming that MDA formation reflects progress of lipid peroxidation in the cell, it is postulated that U78517F may act on another process(es) leading to the cell damage via mechanisms other than lipid peroxidation. For instance, U78517F may interact with intracellular iron, low molecular weight iron chelators and/or heme-associated iron (Kvietys *et al.*, 1989) which is responsible for hydroxyl radical formation and subsequent cytotoxicity.

Reduction in the external Ca^{2+} concentration markedly attenuated release of LDH from endothelial cells. In human endothelial cells, it has been shown that depletion of extracellular Ca^{2+} inhibits cytotoxicity induced by lymphokine-activated killer cells (Kotasek *et al.*, 1988). It is, therefore, conceivable that intracellular Ca^{2+} concentration is increased by the oxygen radical through facilitated Ca^{2+} transport across the membrane, e.g. increase in a leak channel of Ca^{2+} which is dependent on the electrochemical gradient for Ca^{2+}, and an increase in non-selective action channel as proposed for Ca^{2+} entry associated with EDRF release (Adams *et al.*, 1989). The hypothesis is consistent with recent findings (Kimura *et al.*, 1992) that there is gradual increase in resting (quiescent phase) intracellular Ca^{2+} concentration of cells exposed to 0.1 mM H_2O_2. Nifedipine at a concentration of 1 μM, which is sufficient to block voltage-dependent Ca^{2+} channels in vascular smooth muscle (Godfraind & Miller, 1983), did not protect the endothelial cells from the injury. The findings are compatible with a lack of the inhibitory effect on intracellular Ca^{2+} rise following exposure of cells to reactive oxygen (Kimura *et al.*, 1992). It is, thus, unlikely that voltage-dependent Ca^{2+} channel of coronary endothelial cell plays an important role in the genesis of oxidant-induced cell damage.

X/XO mixture generates superoxide anion which is likely to attack the cell. SOD inhibited the rise in LDH release following an addition of X/XO into the cell medium, suggesting involvement of superoxide anion in the cell injury. However, LDH release was attenuated by SOD and oxypurinol as well as catalase. Release of LDH took place in 2 h or later following radical exposure, but not in as early a period of exposure as presumed from production of the labile superoxide anion. Superoxide anion cannot cross biological membranes unless there is an anion channel for it (Halliwell & Gutteridge, 1986). As considered in cerebral arterioles as a target for free radical damage (Kontos, 1985), superoxide anion is produced during prostaglandin hydroperoxidase reactions following metabolism of arachidonic acid via activation of phospholipases and cyclo-oxygenase. Lack of an attenuating effect of indomethacin on LDH-release at the high concentration of 30 μM suggests little involvement of cyclo-oxygenase activation in this cell damage. Superoxide anion generated in the X/XO mixture may be a small driving force to cell injury compared to the subsequent intracellular attack by hydroxyl radicals. However, a potential role of superoxide anion in triggering cell injury could not be ruled out since no determination of labile superoxide anion was made in the present study.

An intact glutathione redox cycle (Harlan *et al.*, 1988) and catalase (Vercellotti *et al.*, 1988) are critical for the maintenance of endothelial cell integrity in the face of a H_2O_2 oxidant challenge. The glutathione redox cycle and/or intracellular activity of catalase and vitamin E may be altered by U78517F, since cell damage occurs when the ability of the cell to degrade exogenous oxidants with endogenous antioxidants is overwhelmed (Vercellotti *et al.*, 1988). For the quiescent period without LDH release, endogenous antioxidants within the cell may be consumed to balance oxygen radicals of extracellular origin. The presence of cultured endothelial cells in the medium, in fact, clearly decreased H_2O_2 generated by X/XO to control levels in 1 h. As detected at 5 h with formation of MDA, however, lipid peroxidation is no longer held in check, ultimately resulting in cell lysis.

In conclusion, porcine coronary artery endothelial cells are injured by oxygen radicals presumably through hydroxyl radicals formed and consequent lipid peroxidation. The extracellular Ca^{2+} concentration plays a critical role in the genesis of endothelial cell damage. Data support the conclusion that U78517F inhibits endothelial cell damage and lipid peroxidation, by a mechanism other than interfering with H_2O_2 production. However, further study is required to determine mechanism of action, e.g., assessment of glutathione levels in cells and effect of U78517F on oxidative state.

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References

ADAMS, D.J., BARAKETH, J., LASKEY, R. & VAN BREEMEN, C. (1989). Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J.*, **3**, 2389–2400.

CEDERBAUM, A.I., DICKER, E., RUBIN, E. & COHEN, G. (1979). Effects of thiourea on microsomal oxidation of alcohols & associated microsomal functions. *Biochemistry*, **18**, 1187–1191.

DE LONG, M.J. (1988). Induction by substituted phenols of enzymes that inactivate electrophilic compounds. In *CRC Handbook of Free Radicals and Antioxidants in Biomedicine*. ed. Miquel, J. *et al.*, vol. II, pp. 63–76. Boca Raton: CRC Press.

GODFRAIND, T. & MILLER, R.C. (1983). Specificity of action of Ca^{2+} entry blockers: a comparison of their actions in rat arteries and in human coronary arteries. *Circ. Res.*, **52** (suppl I), 81–91.

GRISHAM, M.B. & GRANGER, D.N. (1988). Neutrophil-mediated mucosal injury. Role of reactive oxygen metabolites. *Dig. Dis. Sci.*, **33**, 6S–15S.

GUTTERIDGE, J.M. (1987). Ferrous-salt-promoted damage to deoxyribose and benzoate. The increased effectiveness of hydroxyl-radical scavengers in the presence of EDTA. *Biochem. J.*, **243**, 709–714.

HALL, E.D. (1988). Effects of the 21-amino steroid U74006F on post-traumatic spinal cord ischemia in cats. *J. Neurosurg.*, **68**, 462–465.

HALL, E.D., BRAUGHLER, J.M., YONKERS, P.A., PAZARA, K.E., SMITH, S.L., SCHNEIDER, D.I., VAN DOORNIK, F.J. & JACOBSEN, E.J. (1989). U78517F, a second generation lazaroid with potent anti-oxidant and cerebroprotective activity in models of CNS injury and ischemia. *J. Neurotrauma*, **6**, 213.

HALL, E.D., PAZARA, K.E., BRAUGHLER, J.M., LINSEMAN, K.L. & JACOBSEN, E.J. (1990). Nonsteroidal lazaroid U78517F in models of focal and global ischemia. *Stroke*, **21** (suppl III), III-83–III-87.

HALL, E.D. & YONKERS, P.A. (1988). Attenuation of postischemic cerebral hypoperfusion by the 21-amino steroid U74006F. *Stroke*, **19**, 340–344.

HALL, E.D., YONKERS, P.A., McCALL, J.M. & BRAUGHLER, J.M. (1988). Effects of the 21-amino steroid U74006F on experimental head injury in mice. *J. Neurosurg.*, **68**, 456–461.

HALLIWELL, B. & GUTTERIDGE, J.M.C. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.*, **246**, 501–514.

HARLAN, J.M., LEVINE, J.D., CALLAHAN, K.S., SCHWARTS, B.R. & HARKER, L.A. (1984). Glutathione redox cycle protects cultured endothelial cells against lysis by extracellularly generated hydrogen peroxide. *J. Clin. Invest.*, **73**, 706–713.

KIMURA, M., MAEDA, K. & HAYASHI, S. (1992). Cytosolic calcium increase in coronary endothelial cells after H_2O_2 exposure, and the inhibitory effect of U78617F. *Br. J. Pharmacol.*, **107**, 488–493.

KONTOS, H.A. (1985). Oxygen radicals in cerebral vascular injury. *Cir. Res.*, **57**, 508–516.

KORNBERG, A. (1955). Lactic dehydrogenase of muscle. *Methods Enzymol.*, **1**, 441–443.

KOTASEK, D., VERCELLOTTI, G.M., OCHOA, A.C., BACH, F.H., WHITE, H.G. & JACOB, H.S. (1988). Mechanism of cultured endothelial injury induced by lymphokine-activated killer cells. *Cancer Res.*, **48**, 5528–5532.

KVIETYS, P.R., INAUEN, W., BACON, B.R. & GRISHAM, M.B. (1989). Xanthine oxidase-induced injury to endothelium: role of intracellular iron and hydroxyl radical. *Am. J. Physiol.*, **257**, H1640–H1646.

LINK, E.M. & RILEY, P.A. (1988). Role of hydrogen peroxide in the cytotoxicity of the xanthine/xanthine oxidase system. *Biochem. J.*, **249**, 391–399.

MACHLIN, L.J. & BENDICH, A. (1987). Free radical tissue damage: protective role of antioxidant nutrients. *FASEB J.*, **1**, 441–445.

MARTIN, W.J. (1984). Neutrophils kill pulmonary endothelial cells by a hydrogen-peroxide-dependent pathway: an in vitro model of neutrophil-mediated lung injury. *Am. Rev. Respir. Dis.*, **130**, 209–213.

ODY, C. & JUNOD, A.F. (1985). Effect of variable glutathine peroxidase activity on H_2O_2-related cytotoxicity in cultured aortic endothelial cells. *Proc. Soc. Exp. Biol. Med.*, **180**, 103–111.

RATYCH, R.E., CHUKNYISKA, R.S. & BULKLEY, G.B. (1987). The primary localization of free radical generation after anoxia/reoxygenation in isolated endothelial cells. *Surgery*, **102**, 122–131.

SACKS, T., MOLDOW, C.F., CRADDOCK, P.R., BOWERS, T.K. & JACOB, H.S. (1978). Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes: an in vitro model of immuno vascular damage. *J. Clin. Invest.*, **61**, 1161–1167.

SCHANNE, F.A.X., KANE, A.B., YOUNG, E.E. & FARBER, J.L. (1979). Calcium dependence of toxic cell death: a final common pathway. *Science*, **206**, 700–702.

THURMAN, R.G., LEY, H.G. & SCHOLZ, R. (1972). Hepatic microsomal ethanol oxidation hydrogen peroxide formation and the role of catalase. *Eur. J. Biochem.*, **25**, 420–430.

VERCELLOTTI, G.M., DOBSON, M., SCHORER, A.E. & MOLDOW, C. (1988). Endothelial cell heterogeneity: antioxidant profiles determine vulnerability to oxidant injury. *Proc. Soc. Exp. Biol. Med.*, **187**, 181–189.

WEISS, S.J., YOUNG, J., LoBUGLIO, A.F., SLIVKA, A. & NIMEK, N.F. (1981). Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. *J. Clin. Invest.*, **68**, 714–721.

WINSTON, G.W., HARVEY, W., BERL, L. & CEDERBAUM, A.I. (1983). The generation of hydroxyl and alkoxyl radicals from the interaction of ferrous bipyridyl with peroxides. *Biochem. J.*, **216**, 415–421.

WHORTON, A.R., MONTGOMERY, M.E. & KENT, R.S. (1985). Effect of hydrogen peroxide on prostaglandin production and cellular integrity in cultured porcine aortic endothelial cells. *J. Clin. Invest.*, **76**, 295–302.

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Virus-induced airway hyperresponsiveness in the guinea-pig: possible involvement of histamine and inflammatory cells

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1 Guinea-pig tracheal contractions by histamine and by the cholinoceptor agonist, arecoline, are significantly enhanced (30% and 20%, respectively), 96 h after intra-tracheal inoculation with Parainfluenza-3 (PI-3) virus.

2 The airway hyperresponsiveness in animals inoculated with virus coincides with a significant increase in the number of broncho-alveolar cells (82%), and in the albumin concentration (121%) in lung lavage fluid, relative to values obtained in guinea-pigs challenged with control solution.

3 The chemiluminescence production by isolated broncho-alveolar cells, obtained from virus-infected guinea-pigs 96 h after inoculation stimulated with PI-3 virus *in vitro*, is significantly reduced by 42% relative to broncho-alveolar cells obtained from animals inoculated with control solution. This diminution was not specific for stimulation by PI-3 virus since the chemiluminescence production was also significantly reduced by 30% in response to zymosan.

4 Pretreatment of the guinea-pigs with the anti-allergic drugs, oxatomide (2.5 mg kg^{-1}) or nedocromil (2.5 mg kg^{-1}), or the specific H_1-histamine receptor antagonist, levocabastine (0.25 mg kg^{-1}), administered intra-peritoneally twice a day for five successive days, inhibits the virus-induced airway hyperresponsiveness, suppresses the influx of broncho-alveolar cells and increase in albumin content, and corrects the reduced chemiluminescence production by broncho-alveolar cells in response to zymosan.

5 In contrast, the cyclo-oxygenase inhibitor, suprofen (5.0 mg kg^{-1}), the 5-HT_2 receptor antagonist, ketanserin (0.63 mg kg^{-1}), or the Ca^{2+} overload blocker, flunarizine (2.5 mg kg^{-1}) do not modify the above mentioned processes.

6 The platelet-activating factor receptor antagonist, WEB 2170 (10 mg kg^{-1}), reduces virus-induced airway hyperresponsiveness and influx of broncho-alveolar cells into the lungs but does not attenuate the increase of albumin in the bronchial lavage fluid.

7 Guinea-pigs nebulized with histamine, twice a day (30 min) during 4 successive days, do not demonstrate an increased airway responsiveness, but instead show tachyphylaxis in response to histamine *in vitro*. In addition, no influx of inflammatory cells is found in these animals.

8 These results suggest that histamine does not directly increase the responsiveness of the guinea-pig trachea; however, histamine may be involved in a cascade of events leading to airway hyperresponsiveness after a viral infection, a process that could be related to an influx and/or an activation of broncho-alveolar cells after PI-3 virus stimulation.

Keywords: Guinea-pig airways; Parainfluenza 3 virus; viral respiratory infections; tracheal responsiveness; inflammatory cell counts; albumin; chemiluminescence; oxatomide

Introduction

Children infected with parainfluenza or respiratory syncytial virus are prone to develop bronchiolitis, airway hyperresponsiveness, asthma, or abnormalities of pulmonary function (Minor *et al.*, 1974; Glezen *et al.*, 1982; Chanock & McIntosh, 1985; Busse, 1990). Approximately 40% of acute asthmatic attacks in children are associated with acute viral respiratory infections (McIntosh *et al.*, 1973). Moreover, acute viral respiratory infections may initiate development of bronchial hyperresponsiveness in normal as well as in asthmatic patients (Empey *et al.*, 1976; Laitinen & Kava, 1980). Such an airway hyperresponsiveness often develops within two to three days after manifestation of symptoms expressing viral infections, and can persist several weeks thereafter (Little *et al.*, 1978; Aquilina *et al.*, 1980).

Despite efforts, the mechanism underlying this virus-induced airway hyperresponsiveness and the possible role of the number and/or activity of inflammatory cells in the lungs remain to be clarified. Recently, we developed a model in guinea-pigs in which a long-lasting airway hyperresponsiveness was documented *in vitro* and *in vivo* after viral respiratory tract infection (Folkerts *et al.*, 1992a,b). The increased airway contraction of histamine (H_1) or muscarinic receptor stimulation, in such pathological conditions compared to control animals, was associated with an increased number of cells in the broncho-alveolar lavage fluid up to 16 days after intra-tracheal inoculation of Parainfluenza-3 (PI-3) virus, effects being most pronounced 4 days after infection (Folkerts *et al.*, 1992a,b). Furthermore, we found that PI-3 virus is a potent stimulus for broncho-alveolar lavage cells *in vitro*. Indeed, broncho-alveolar cells obtained from virus-infected animals, display a significantly reduced capacity to generate reactive oxygen metabolites upon additional PI-3 virus stimulation *in vitro*, reflecting stimulation and exhaustion *in vivo* (Folkerts *et al.*, 1992a,b).

The purpose of the present study was to elucidate further the importance of inflammatory cell number, type, and function as well as that of various allergic mediators in the airway hyperresponsiveness following viral infections. For this purpose virus-infected guinea-pigs were pretreated with a number of drugs that interfere with the synthesis and/or effect of various autocoids. To quantify such pharmacological modulation, we measured (a) tracheal responsiveness to histamine and the cholinoceptor agonist, arecoline

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in vitro, (b) the number and types of broncho-alveolar cells, (c) albumin concentration in lung lavage fluid, and (d) broncho-alveolar cells function/activity *in vitro* by means of chemiluminescence production after stimulation with PI-3 virus, or with zymosan.

Methods

Animals

Specified-pathogen-free guinea-pigs (400–500 g, male Dunkin Hartley, Harlan Olac Ltd, England) were housed in isolators under controlled conditions. Water and commercial chow were allowed *ad libitum*. The guinea-pigs were free of respiratory airway infections as assessed by the health monitoring quality control report by Harlan Percellus (England), and by histological examination.

Intra-tracheal inoculation

Suspensions of virus (1 ml; Tissue Culture Infective Dose $(TCID)_{50} = 10^{8.9}\ ml^{-1}$) were centrifuged at 100,000 *g* and resuspended in a 1/10 reduced volume (0.1 ml) of sterile pyrogen-free saline in order to minimize possible mechanical effects of the inoculation procedure. Growth medium was subjected to a similar procedure in order to serve as a control solution.

The animals were anaesthetized with ether and were placed in a supine position on a small table. The jaws were kept open by two elastic rubber rings and a needle with a bulbous tip was placed just behind the glottis. The rubber rings were removed and the animal was placed in an upright position. Thereafter, 0.1 ml of the inoculum was gently injected in the trachea. Such a procedure results in infection with PI-3 virus in all animals subjected to the procedure (Folkerts *et al.*, 1992a,b). Control guinea-pigs, housed in a separate isolator, were treated in the same way with 0.1 ml of a control solution.

Bovine Parainfluenza 3 virus (PI-3) was kindly provided by Duphar B.V. (Weesp, The Netherlands).

Experimental protocol

Guinea-pigs were pretreated with pharmacological agents intra-peritoneally (i.p.), twice a day (08 h 00 min and 17 h 30 min) for five successive days, starting on day 0 and ending on day 4. On day 1 the guinea-pigs were inoculated with the control solution or PI-3 virus at 10 h 00 min and received an additional dose of the pharmacological agent at 12 h 00 min. On day 5, the animals received no antagonist and were killed. Drugs were injected into the right or left side of the abdomen alternately.

Pharmacological agents

Drugs were dissolved in β-hydroxypropyl-cyclodextrine solution (20% w/v in diluted water). The sham group received this solvent ($1.0\ ml\ kg^{-1}$) in the same schedule as the drug-treated animals. The drugs used were: suprofen ($5.0\ mg\ kg^{-1}$), a cyclo-oxygenase inhibitor (De Clerck *et al.*, 1975); flunarizine ($2.5\ mg\ kg^{-1}$), a Ca^{2+} overload blocker (Todd & Benfield, 1989); ketanserin ($0.63\ mg\ kg^{-1}$), a 5-HT_2 receptor antagonist (De Clerck *et al.*, 1984); nedocromil ($2.5\ mg\ kg^{-1}$), anti-allergic compound (Auty, 1986); oxatomide ($2.5\ mg\ kg^{-1}$), an anti-allergic compound with H_1 histamine receptor blocking properties (Awouters *et al.*, 1979; De Clerck *et al.*, 1981); WEB 2170 (Bepafant; $10\ mg\ kg^{-1}$), a platelet-activating factor (PAF) receptor antagonist (Heuer *et al.*, 1990); levocabastine ($0.25\ mg\ kg^{-1}$), a specific H_1 histamine receptor antagonist (Van Wauwe, 1989).

Airway responsiveness in vitro

Tracheal responsiveness was measured as described by Folkerts *et al.* (1989). Briefly, guinea-pigs were killed with an overdose of pentobarbitone sodium (Nembutal, 30 mg $100\ g^{-1}$ body weight, i.p.). Trachea were dissected free of connective tissue and blood vessels and were mounted in a siliconized organ bath filled with Krebs-bicarbonate buffer (37°C, pH = 7.4) of the following composition (mM): NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 8.3, which was continuously gassed with a 5% CO_2 and 95% O_2 gas mixture. Each trachea was cut in a spiral, and divided into two parts of seven rings each. One end of a trachea was fixed in the organ bath, the other end was attached to an isotonic-transducer (Harvard Bioscience). Tracheal spirals were kept under a load of 0.8 g throughout the experiment. All tissues were washed three times at 15 min intervals, after which a stable tone was reached (within 45 min). The proximal or distal part of the tracheal spiral was used alternatively for the construction of histamine or arecoline cumulative concentration-response (C/R) curves to avoid possible differences between parts. Only one C/R curve was obtained on a tissue segment. Tracheal contractions were displayed on a two-channel pen recorder (Servogor, type 220).

Lung lavages

In order to perform lung lavages, several groups of animals were killed by the administration of $30\ mg\ 100\ g^{-1}$ body weight pentobarbitone sodium (i.p.). Tracheae were trimmed free of connective tissue and a small incision was made for insertion of a cannula into the trachea (Folkerts *et al.*, 1988). The lungs were filled with 12 ml NaCl-EDTA-buffer (0.15 M NaCl, 2.6 mM EDTA) *in situ* under a pressure of 30 cmH_2O. After gentle lung massage for 1 min, 7 ml fluid was withdrawn from the lungs for further processing to determine the albumin content (see albumin content). Such a procedure was repeated without notion of the amount of fluid recovered from the lungs, until 80 ml fluid was obtained from each animal. Cells were then sedimented by centrifugation at 400 *g* for 10 min at 4°C, and washed twice with Krebs-bicarbonate buffer. The cells were stained with Türk's solution and counted in a Bürker-Türk bright-line counting chamber.

All cell preparations were analysed morphologically after centrifugation on microscope slides. Air dried preparations were fixed and stained with Diff-Quik (Merz + Dade A.G., Düdingen, Switzerland). The cells were differentiated into alveolar macrophages, monocytes, lymphocytes, eosinophils and neutrophils by light microscopical observation. Viability was >95% as assessed by the trypan blue exclusion test.

Only plastics and siliconized glassware were used throughout the isolation procedure in order to minimize adherence of the cells to the walls of the tubes.

Albumin content

In the first 7 ml of the lung lavage fluid the content of albumin was measured. For that purpose tubes were centrifuged at 400 *g*, the cell-pellet was resuspended in Krebs and added to the respective cell-pool that was recovered from the 80 ml washing (see lung lavage). The supernatant was stored at −80°C. After thawing, fluids were again centrifuged at 1000 *g*, and in the resultant supernatant the albumin concentration was measured in a Cobas-Bio centrifugal analyzator (Hoffman-LaRoche/Co., Limited Company, Diagnostiga, Basel, Switzerland) with bromocresol green (Boehringer Mannheim GmbH, Mannheim, FRG) as reagent (Doumas *et al.*, 1971).

Table 1 Various parameters† derived from histamine concentration-response curves on isolated tracheal spirals from guinea-pigs pretreated with pharmacological probes and inoculated with control solution or PI-3 virus

Treatment	*Dose* (mg kg^{-1})	*Maximal contractions* (mm)	EC_{50} ($\times 10^{-6}$ M)	*Slope factor*	n
Sham	1.0 ml				
Control		3.5 ± 0.2	4.0 ± 1.0	1.12 ± 0.12	11
PI-3**		4.6 ± 0.3§§	4.4 ± 1.0	1.09 ± 0.06	10
Suprofen	5.0				
Control		3.0 ± 0.3	2.6 ± 0.4	0.87 ± 0.09	6
PI-3**		3.8 ± 0.2§	3.4 ± 0.3	0.93 ± 0.05	6
Oxatomide	2.5				
Control		3.2 ± 0.1	11.0 ± 2.1	1.92 ± 0.25	10
PI-3**		2.7 ± 0.2§	13.0 ± 3.0	2.02 ± 0.34	7
Nedocromil	2.5				
Control		3.7 ± 0.3	2.6 ± 0.9	0.87 ± 0.11	4
PI-3		3.7 ± 0.5	2.4 ± 0.6	1.02 ± 0.06	5
Flunarizine	2.5				
Control		2.1 ± 0.3	8.2 ± 2.2	0.89 ± 0.08	6
PI-3**		3.0 ± 0.1§	7.4 ± 2.9	0.71 ± 0.06	6
Ketanserin	0.63				
Control		3.4 ± 0.4	3.0 ± 0.8	0.89 ± 0.09	4
PI-3**		4.3 ± 0.3	2.5 ± 0.1	1.02 ± 0.03	4
WEB 2170 BS	10.0				
Control		3.2 ± 0.2	2.2 ± 0.3	0.96 ± 0.04	6
PI-3		3.5 ± 0.5	2.1 ± 0.4	0.95 ± 0.08	6
Levocabastine	0.25				
Control		3.0 ± 0.2	4.7 ± 0.7	0.79 ± 0.02	6
PI-3		3.2 ± 0.3	4.7 ± 0.6	0.80 ± 0.03	6

**$P<0.01$; the curves were significantly shifted upwards or downward as calculated by a two-way analysis of variance (ANOVA).
†The parameters were calculated by means of a computerized analysis of the individual curves based on the 4-parameter logistic equation. Results are expressed as means ± s.e.mean.
§$P<0.05$; §§$P<0.01$: significantly different from the respective control group, Student's t test (unpaired).
n = number of observations.

Table 2 Various parameters† derived from arecoline concentration-response curves on isolated tracheal spirals from guinea-pigs pretreated with pharmacological probes and inoculated with control solution or PI-3 virus

Treatment	*Dose* (mg kg^{-1})	*Maximal contractions* (mm)	EC_{50} ($\times 10^{-7}$ M)	*Slope factor*	n
Sham	1.0 ml				
Control		4.9 ± 0.3	7.2 ± 1.3	0.86 ± 0.05	13
PI-3**		5.9 ± 0.4§	5.8 ± 0.9	0.87 ± 0.05	12
Suprofen	5.0				
Control		4.1 ± 0.4	5.4 ± 0.8	0.82 ± 0.04	5
PI-3**		4.9 ± 0.4	6.3 ± 0.9	0.80 ± 0.02	5
Oxatomide	2.5				
Control		5.0 ± 0.3	7.5 ± 1.3	0.96 ± 0.06	11
PI-3		4.2 ± 0.4	8.5 ± 1.0	0.84 ± 0.05	11
Nedocromil	2.5				
Control		4.4 ± 0.6	4.9 ± 1.4	0.82 ± 0.02	5
PI-3		4.2 ± 0.3	4.1 ± 0.7	0.86 ± 0.04	5
Flunarizine	2.5				
Control		4.9 ± 0.4	12.0 ± 5.0	0.80 ± 0.04	6
PI-3		4.5 ± 0.2	8.0 ± 1.6	0.80 ± 0.02	5
Ketanserin	0.63				
Control		4.7 ± 0.6	4.9 ± 0.8	0.77 ± 0.04	4
PI-3**		5.5 ± 0.3	3.7 ± 0.3	0.81 ± 0.02	4
WEB 2170 BS	10.0				
Control		4.5 ± 0.3	4.6 ± 1.1	0.85 ± 0.04	6
PI-3*		5.1 ± 0.5	4.3 ± 0.6	0.83 ± 0.02	6
Levocabastine	0.25				
Control		4.5 ± 0.4	5.7 ± 0.7	0.78 ± 0.02	6
PI-3*		4.7 ± 0.2	3.8 ± 0.5	0.80 ± 0.03	6

*$P<0.05$; **$P<0.01$; the curves were significantly increased as calculated by a two-way analysis of variance (ANOVA).
†The parameters were calculated by means of a computerized analysis of the individual curves based on the 4-parameter logistic equation. Results are expressed as means ± s.e.mean.
§$P<0.05$; significantly different from the control group, Student's t test (unpaired).
n = number of observations.

Generation of chemiluminescence

The concentration of broncho-alveolar cells was adjusted to give 5×10^6 cells ml^{-1} and 200 µl of this suspension was used to measure chemiluminescence generation in a luminometer (type 1251 LKB Wallac, Turku, Finland) at 37°C in the presence of 0.5 mM lucigenin (50 µl). The virus suspension ($TCID_{50} = 10^{8.9}$ ml^{-1}) was centrifuged at 100,000 *g* and resuspended in Krebs-buffer (in 2 times less volume). Growth medium was treated in the same way and served as a control to determine basal chemiluminescence production. The end volume of every tube was adjusted to 0.5 ml with Krebs-buffer. The broncho-alveolar cells were stimulated with: (A) 200 µl control solution, (B) 200 µl PI-3 virus suspension and (C) 2.5 mg zymosan (preopsonized in 100% pooled guinea-pig serum). Chemiluminescence was measured every 60 s over a 40 min period; the peak value was used for data processing.

Histamine nebulization

In additional experiments, conscious guinea-pigs were exposed to a histamine aerosol for 30 min, twice a day, during 4 days. Histamine (2 mg ml^{-1}) was dissolved in sterile saline and nebulized (1 ml min^{-1}) with an electronic ultrasonic nebulizer (Medix, Mecomfa, Lisse, The Netherlands). Control animals were treated in the same way receiving an aerosol of sterile saline. Before the animals were placed in the perspex chamber (7.5 l), saline or histamine was aerosolized during 2 min in order to fill the system completely. In preliminary experiments it was found that 2 mg ml^{-1} histamine was the dose at which the guinea-pigs could stay in the chamber for 30 min, during 4 successive days, without developing severe bronchoconstriction.

On day 5, tracheal responsiveness to histamine and arecoline was measured, the total numbers of broncho-alveolar cells was determined, and the chemiluminescence production in response to zymosan was registrated.

Statistical analysis

Differences between groups after cumulative C/R curves with histamine and arecoline on isolated tissues were tested by two way analysis of variance (ANOVA). The ANOVA calculates the area under the curve of each single C/R curve. Thereafter, the mean of the AUC of the C/R curves within one group was calculated. Accordingly, the AUC of two different groups were compared with one another. In addition, the C/R curves for histamine and arecoline were analysed by means of a computerized curve fitting technique based on the 4-parameter logistic equation (De Lean *et al.*, 1978). The parameters defining the sigmoidal curve, i.e. the maximal response (in mm contraction), the EC_{50} value (the concentration that gives a half maximal response), and the slope factor were determined for each individual C/R curve. The respective parameters were subsequently averaged for the various experimental groups with respect to the maximal response, the EC_{50}, and slope factor of the C/R curves, and were statistically evaluated with Student's *t* test (unpaired). Differences in the total number or differential cell counts and chemiluminescence production were tested by Student's *t* test (unpaired). All *P* values <0.05 were considered to reflect a statistically significant difference (Siegel, 1956).

Chemicals

Oxatomide, flunarizine, levocabastine, suprofen and ketanserin were gifts from Janssen Research Foundation, Beerse, Belgium. Nedocromil was a gift from Fisons, Loughborough, Leicestershire. WEB 2170 BS was a gift from Boehringer Ingelheim KG, Ingelheim am Rhein, Germany. Nembutal, containing 60 mg ml^{-1} pentobarbitone sodium, was purchased from Abbott Laboratories (North Chicago, IL, U.S.A.). Histamine phosphate, was obtained from the Onder-

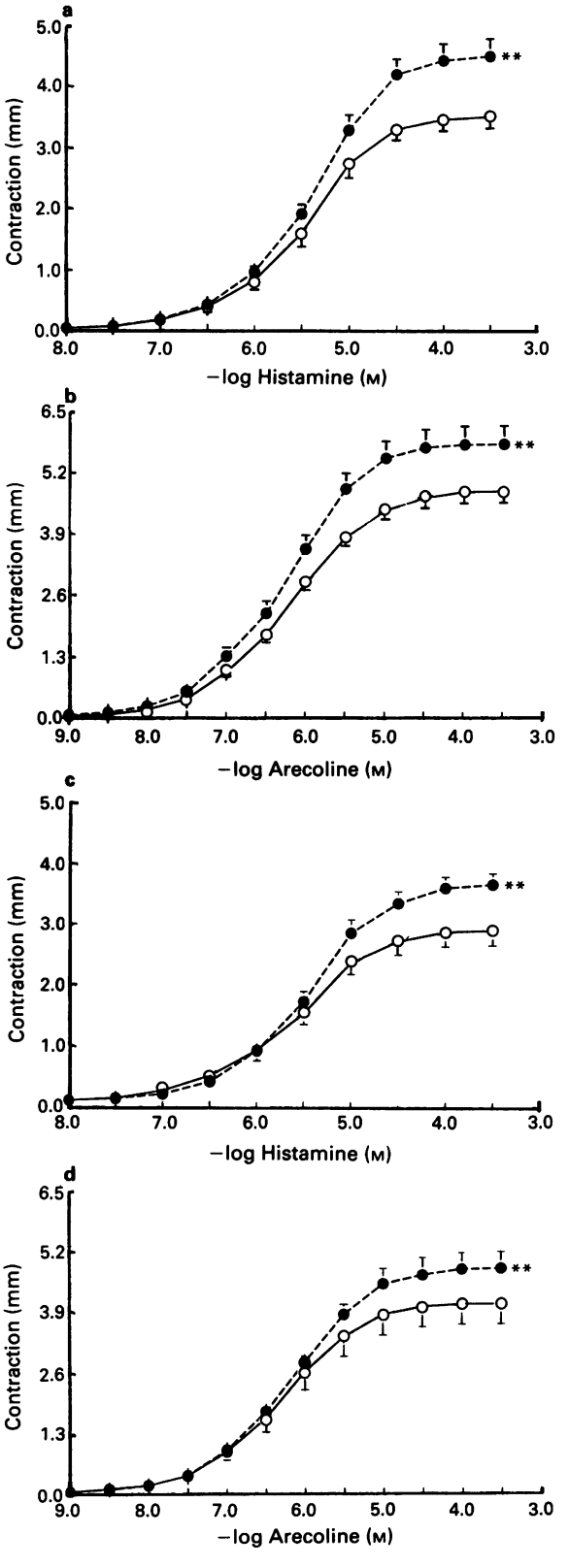


Figure 1 Concentration-response curve for histamine (a and c), and arecoline (b and d) on isolated tracheal spirals from guinea-pigs inoculated with control solution (○) or PI-3 virus (●). Pretreatment with solvent solution (1.0 ml kg^{-1} b.wt., a and b) or suprofen (5.0 mg kg^{-1}, c and d). The results are presented as the means ± s.e.mean. See Tables 1 and 2 for the parameters: maximal response, EC_{50} value, and slope factor of these curves and the number of experiments. The C/R curves for histamine and arecoline were significantly shifted upwards (**$P<0.01$, two way ANOVA) in the PI-3 virus groups when compared to the control group.

linge Pharmaceutische Groothandel (Utrecht, The Netherlands). Arecoline, lucigenin (*bis*-N-methylacridinium nitrate) and zymosan were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Results

Airway responsiveness in vitro

Concentration/response (C/R) curves for histamine and arecoline on tracheal spirals obtained from animals inoculated with PI-3 virus were significantly ($P<0.01$, ANOVA) shifted upwards as compared to controls (Figure 1a and b, respectively). Maximal contractions induced by histamine were significantly increased by approximately 30% ($P<0.01$, Table 1) and that by arecoline approximately 20% ($P<0.05$, Table 2) in the group inoculated with virus relative to those inoculated with control solution. The EC_{50} value of the arecoline C/R curve was slightly, but not significantly, lower (Table 2) in the virally-infected preparations.

Inhibition of cyclo-oxygenase activity by suprofen, which attenuates the production of all prostanoids derived from arachidonic acid during the time period of the viral-infection, did not affect the airway hyperresponsivess to histamine (Figure 1c, Table 1) and arecoline C/R (Figure 1d, Table 2). The C/R curves for both agonists were shifted upwards ($P<0.01$) in the virus-treated animals.

Treatment of the animals during the viral infection period with the anti-allergic compound oxatomide which has potent H_1-histamine receptor antagonist properties also, or with the mast cell stabilizer nedocromil inhibited the development of tracheal hyperresponsiveness in response to histamine and arecoline (Figure 2, Tables 1 and 2). Treatment with oxatomide reduced the sensitivity to histamine of tracheal preparations, as reflected by a 3 fold shift to the right of the C/R curve relative to that in the sham group ($P<0.01$, Table 1) in both the groups inoculated with control solution and that inoculated with virus. Interestingly, in the PI-3 virus group the histamine C/R curve was additionally shifted downwards ($P<0.05$, ANOVA) as compared with the control group (Figure 2a). The maximal contraction of the virus-inoculated guinea-pigs, after pretreatment with oxatomide, was reduced by more than 40% ($P<0.01$) as compared to the virus-infected sham group (Table 1). Similar to the results obtained with histamine, increased tracheal contractions to arecoline were also inhibited by pretreatment with oxatomide (Table 2; Figure 2b). Treatment with nedocromil inhibited the increase in tracheal contraction to histamine (Figure 2c, Table 1) as well as to arecoline (Figure 2d, Table 2).

Ca^{2+}-overload blockade by flunarazine during the development phase of the virally-induced airway hyperresponsiveness affected the histamine C/R curve in both groups. Maximal responses were decreased ($P<0.01$) and the EC_{50} values were non-significantly increased (Figure 3a, Table 1) as compared to the sham group (Figure 1a, Table 1). Nevertheless, such a pharmacological manipulation did not affect the virus-induced airway hyperresponsiveness. The histamine C/R curve was still shifted upwards in the PI-3 virus group (Figure 3a, $P<0.01$, ANOVA): the maximal contraction being increased by 40% ($P<0.05$, Table 1). However, Ca^{2+}-overload blockade with flunarizine abolished the development of functional hyperresponsiveness to cholinoceptor stimulation, with arecoline as agonist (Figure 3b, Table 2). Blockade of 5-HT_2 binding sites by ketanserin did not affect the virus-induced airway hyperresponsiveness to histamine (Figure 3c, Table 1, $P<0.01$ ANOVA) or arecoline (Figure 3d, Table 2, $P<0.01$ ANOVA) as well.

The PAF-receptor antagonist, WEB 2170, as well as the specific H_1-histamine receptor antagonist, levocabastine, suppressed the increase in maximal tracheal contraction to histamine (Figure 4a and c, Table 1) and to arecoline (Figure 4b and d, Table 2). While the histamine C/R curves on tracheae

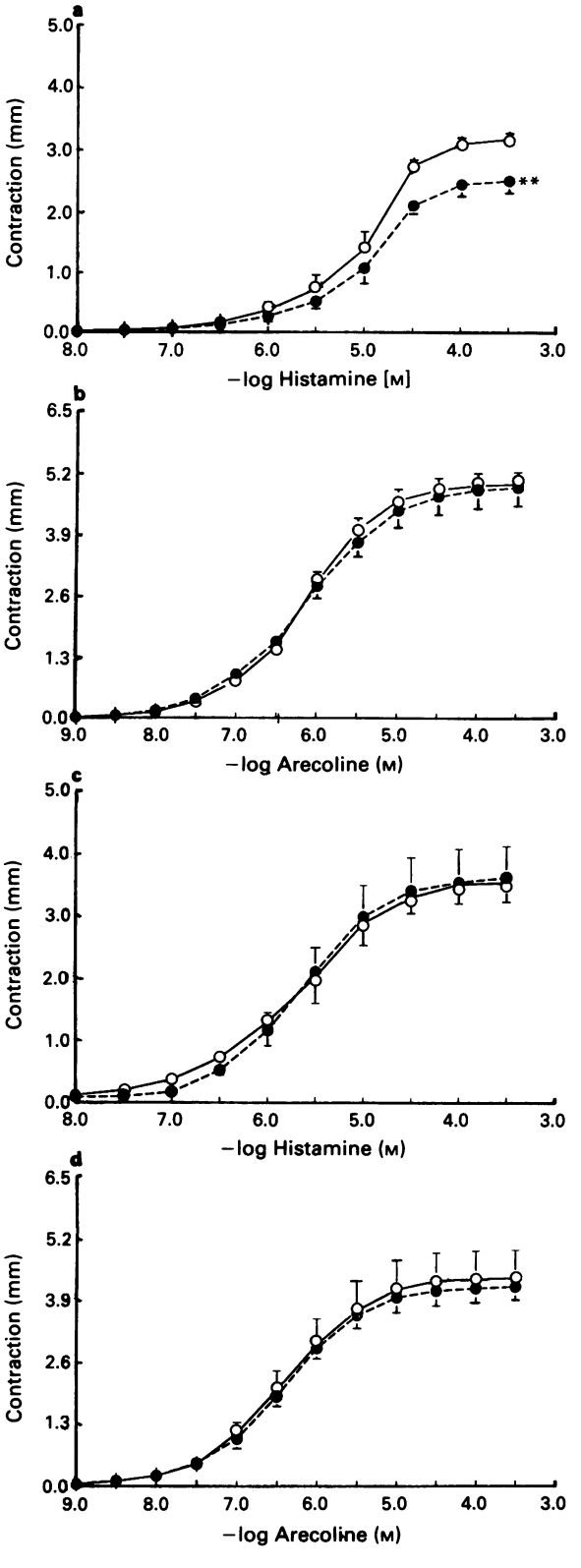


Figure 2 Concentration-response curves for histamine (a and c) and arecoline (b and d) on isolated tracheal spirals from guinea-pigs inoculated with control solution (○) or PI-3 virus (●). Pretreatment with oxatomide (2.5 mg kg^{-1} b.wt., a and b) or nedocromil (2.5 mg kg^{-1} b.wt., c and d). The results are presented as the means ± s.e.mean. See Tables 1 and 2 for the parameters: maximal response, EC_{50} value, and slope factor of these curves and the number of experiments. Oxatomide and nedocromil inhibited the increased tracheal contraction in response to histamine and arecoline in the PI-3 virus-treated groups.

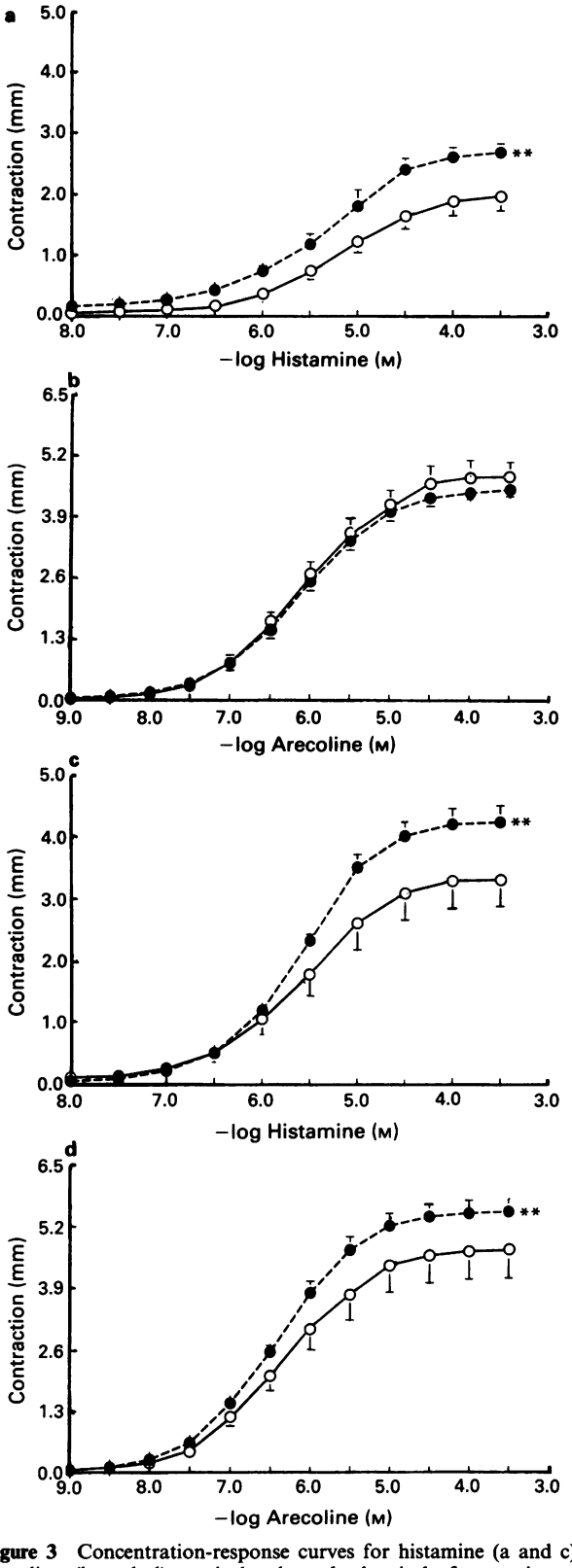


Figure 3 Concentration-response curves for histamine (a and c) or arecoline (b and d) on isolated tracheal spirals from guinea-pigs inoculated with control solution (○) or PI-3 virus (●). Pretreatment with flunarizine (2.5 mg kg^{-1} b.wt., a and b) or ketanserin (0.63 mg kg^{-1} b.wt., c and d). The results are presented as the means ± s.e.mean. See Tables 1 and 2 for the parameters: maximal response, EC_{50} value, and slope factor of these curves and the number of experiments. The drugs did not affect the virus-induced airway hyperresponsiveness to histamine. The C/R curves were significantly shifted upwards (**$P<0.01$, two way ANOVA) in the PI-3 virus groups when compared to the control group. Flunarizine (2.5 mg kg^{-1} b.wt.) suppressed the histamine C/R curve in both the group inoculated with control solution and that inoculated with PI-3 virus (a). Flunarizine inhibited the virus-induced tracheal hyperresponsiveness to arecoline (b).

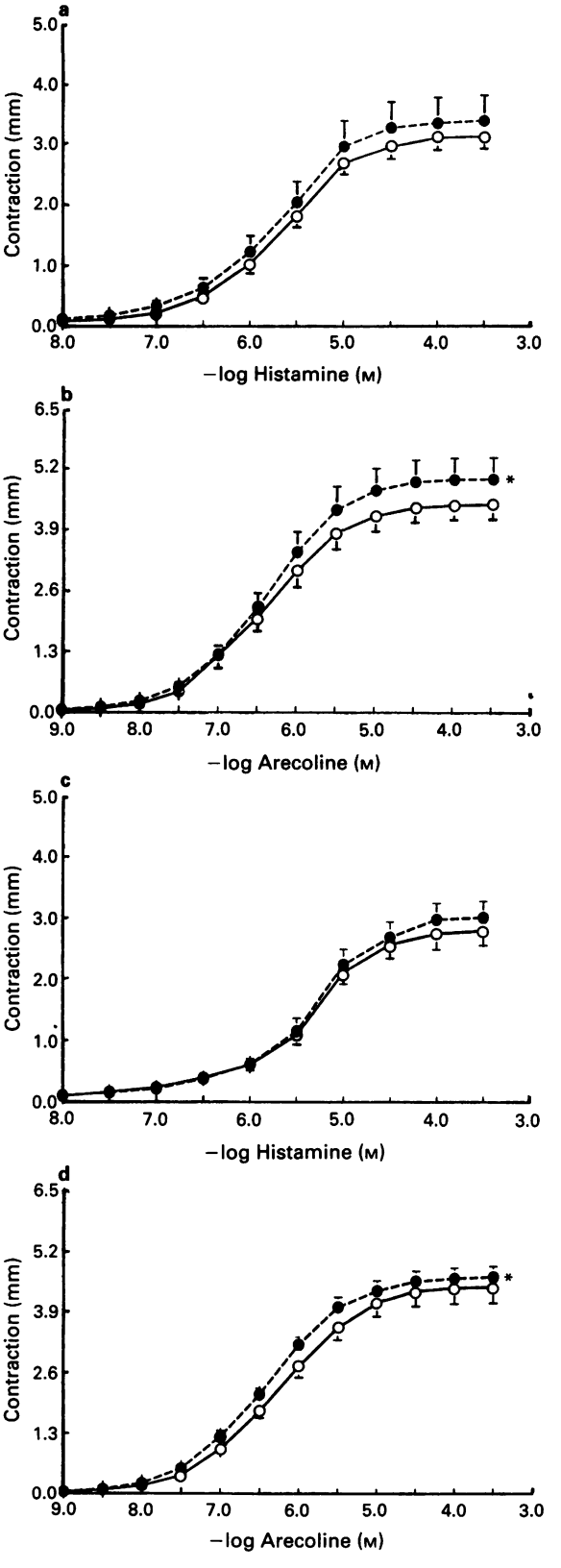


Figure 4 Concentration-response curves to histamine (a and c) and arecoline (b and d) on isolated tracheal spirals from guinea-pigs inoculated with control solution (○) or PI-3 virus (●). Pretreated with WEB 2170 (10 mg kg^{-1} b.wt., a and b) or levocabastine (0.25 mg kg^{-1} b.wt., c and d). The results are presented as the means ± s.e.mean. See Tables 1 and 2 for the parameters: maximal response, EC_{50} value, and slope factor of these curves and the number of experiments. WEB 2170 and levocabastine inhibited the increased airway contraction in response to histamine in the PI-3 virus-treated groups. The arecoline C/R curves were still slightly enhanced in the PI-3 virus group after pretreatment with WEB 2170 or levocabastine ($P<0.05$, two way ANOVA).

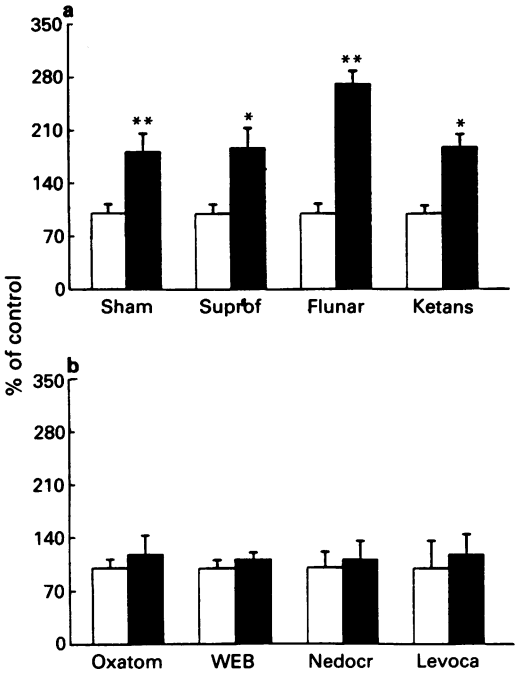


Figure 5 Broncho-alveolar cells in lung lavage fluid from guinea-pigs, inoculated with control solution (open columns) or PI-3 virus (solid columns), and pretreated with pharmacological probes (a). Solvent solution (1.0 ml kg^{-1}, b.wt.), suprofen (Suprof. 5.0 mg kg^{-1}, b.wt.), flunarizine (Flunar 2.5 mg kg^{-1}, b.wt.), ketanserin (Ketans, 0.63 mg kg^{-1}, b.wt.); (b) oxatomide (Oxatom, 2.5 mg kg^{-1}, b.wt.), WEB 2170 (10 mg kg^{-1}, b.wt.), nedocromil (Nedocr, 2.5 mg kg^{-1}, b.wt.), levocabastine (Levoca, 0.25 mg kg^{-1}, b.wt.). The data are expressed as percentage of the mean number of broncho-alveolar cells of each respective control group. The increase in the number of broncho-alveolar cells induced by PI-3 virus is inhibited by oxatomide, WEB 2170, nedocromil, and levocabastine. $*P<0.05$, $**P<0.01$ Student's t test (unpaired); $n = 5-8$. The relative numbers of the individual cell types are presented in Table 3.

isolated from animals inoculated with control solution or PI-3 virus and medicated with WEB 2170 or levocabastine did not differ significantly (Figure 4a and c), arecoline C/R curves in medicated animals remained slightly shifted upward ($P<0.05$, ANOVA). The EC_{50} value for both agonists did not differ between preparations obtained from virus-infected animals relative to those from guinea-pigs inoculated with control solution (Tables 1 and 2).

There were no differences in the slope factors of the histamine or arecoline C/R curves between tracheae obtained from animals inoculated with control solution or PI-3 virus, irrespective of the pharmacological probes used (Tables 1 and 2).

Number and types of broncho-alveolar cells

The absolute number of broncho-alveolar cells in the lung lavage fluid in the control animals was $14.5 \pm 1.8 \times 10^6$ cells. This number was almost doubled, to $26.3 \pm 3.5 \times 10^6$, in the virus-treated guinea-pigs. The increase in the number of broncho-alveolar cells was due to an increase in the absolute number of cell types: alveolar macrophages 1.5 fold. monocytes 3.6 fold, lymphocytes 20.0 fold, eosinophils 3.9 fold and neutrophils 3.6 fold.

Suprofen, flunarizine or ketanserin pretreatment did not modify the increase in the number of broncho-alveolar cells in the lung lavage fluid of guinea-pigs inoculated with PI-3 virus (Figure 5a). However, oxatomide, WEB 2170, nedocromil, and levocabastine which inhibited the virus-induced tracheal hyperresponsiveness, also inhibited the increase in the number of broncho-alveolar cells (Figure 5b).

The inhibition of the increase in *absolute* number of broncho-alveolar cells in the virus-treated animals by oxatomide, WEB 2170, nedocromil, and levocabastine was mainly due to a reduction in the *absolute* number of alveolar macrophages.

In the virally-infected group, the relative number of the alveolar macrophages was decreased ($P<0.01$), while that of all other cell types significantly increased. Changes induced in

Table 3 Mean distribution of broncho-alveolar cells as % of total in guinea-pigs pretreated with pharmacological probes and inoculated with control solution or PI-3 virus

Treatment	*Macrophages*	*Monocytes*	*Lymphocytes*	*Eosinophils*	*Neutrophils*
Sham					
Control	88.3 ± 2.10‡	0.63 ± 0.26	0.25 ± 0.16	7.54 ± 1.45	3.25 ± 1.15
PI-3	71.4 ± 1.81§§	1.71 ± 0.36§	3.71 ± 1.02§§	15.5 ± 0.16§§	7.71 ± 1.41§
Suprofen					
Control	89.7 ± 2.80	1.00 ± 0.45	0.16 ± 0.16	4.17 ± 0.75	5.52 ± 2.97
PI-3	74.3 ± 3.79§§	1.83 ± 0.54	1.33 ± 0.21§	17.3 ± 3.00§§	5.17 ± 1.08
Flunarizine					
Control	88.7 ± 2.50	0.33 ± 0.33	0.33 ± 0.21	4.67 ± 0.99	6.00 ± 1.98
PI-3	75.8 ± 4.15§	2.00 ± 0.63§	2.50 ± 0.56§§	10.7 ± 2.16§	9.31 ± 1.60
Ketanserin					
Control	87.6 ± 1.10	3.43 ± 1.22	0.60 ± 0.40	4.62 ± 0.98	4.62 ± 0.98
PI-3	74.2 ± 5.29§	4.58 ± 0.92	5.40 ± 2.44	9.78 ± 2.77	5.98 ± 3.22
Oxatomide					
Control	80.2 ± 3.51	2.36 ± 0.37	1.75 ± 0.62	8.30 ± 1.08	7.40 ± 2.96
PI-3	64.7 ± 4.77§	2.71 ± 0.50	6.06 ± 0.88§§	12.4 ± 2.21	14.3 ± 3.98
WEB 2170					
Control	83.4 ± 3.30	1.52 ± 0.57	0.00 ± 0.00	9.38 ± 0.63	6.50 ± 2.59
PI-3	74.3 ± 4.06	1.17 ± 0.31	3.33 ± 0.67§§	16.2 ± 2.17§	4.67 ± 1.71
Nedocromil					
Control	79.9 ± 2.30	1.42 ± 0.84	0.86 ± 0.40	10.1 ± 1.01	7.57 ± 1.65
PI-3	75.0 ± 5.00	1.17 ± 0.52	4.13 ± 2.08	16.3 ± 3.53	2.33 ± 0.49§
Levocabastine					
Control	87.0 ± 5.27	1.51 ± 0.56	0.83 ± 0.47	5.50 ± 2.70	5.17 ± 2.29
PI-3	76.0 ± 3.80	2.17 ± 0.70	3.00 ± 0.93	11.2 ± 3.26	7.50 ± 1.90

‡ Results are expressed as means ± s.e.mean.
§ $P<0.05$; §§$P<0.01$ as compared with the control group.
$n = 5-8$.

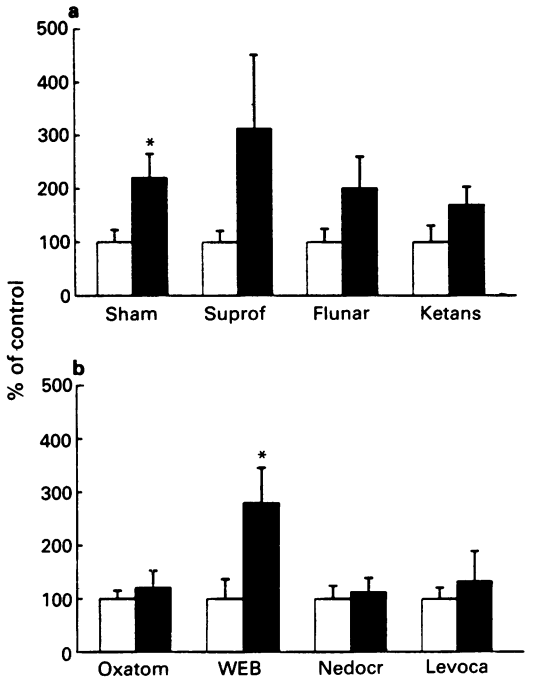


Figure 6 Albumin content in lung lavage fluid from guinea-pigs, inoculated with control solution (open columns) or PI-3 virus (solid columns), and pretreated with pharmacological probes. (a) Solvent solution (1.0 ml kg^{-1} b.wt.), suprofen (5.0 mg kg^{-1}, b.wt.), flunarizine (2.5 mg kg^{-1} b.wt.), ketanserin (0.63 mg kg^{-1} b.wt.); (b) oxatomide (2.5 mg kg^{-1}, b.wt.), WEB 2170 (10 mg kg^{-1}, b.wt.), nedocromil (2.5 mg kg^{-1}, b.wt.), levocabastine (0.25 mg kg^{-1}, b.wt.). Abbreviations as in Figure 5. Data (means ± s.e.mean) are expressed as percentage of the mean albumin concentration of each respective control group. The increase in albumin in lung lavage fluid induced by PI-3 virus is reduced by oxatomide, nedocromil, and levocabastine. $*P<0.05$, Student's t test (unpaired); $n = 5-8$.

the relative number of broncho-alveolar cells by pretreatment with the pharmacological probes were not directly associated with changes in airway reactivity (Table 3).

Albumin content in lung lavage fluid

In virally-infected animals, the albumin concentration in the broncho-alveolar lavage fluid was significantly higher (42.5 ± 8.5 μg ml^{-1}; $P<0.05$, $n = 8$) in comparison with that of guinea-pigs inoculated with control solution (19.2 ± 4.4 μg ml^{-1}; $n = 7$). After pretreatment with suprofen, ketanserin, or flunarizine, the albumin content was increased, although not significantly, in the PI-3 virus groups by 210%, 100% and 70%, as compared with the respective control groups (Figure 6a). PAF-receptor antagonism (WEB 2170) during the development phase of the virally-induced tracheal hyperresponsiveness did not modify the increase of albumin into the broncho-alveolar lavage fluid (Figure 6b, $P<0.05$). By contrast, treatment with either oxatomide, nedocromil or levocabastine abolished such an increased leakage of albumin (percentage increase: 20%, 13% and 33%, respectively; Figure 6b).

Chemiluminescence generation by broncho-alveolar cells

Basal chemiluminescence generation (i.e. stimulated with 200 μl control solution) by broncho-alveolar cells obtained from animals inoculated with control solution or PI-3 virus did not differ between the two groups, irrespective of the pretreatment. In the sham groups, the basal chemiluminescence production by broncho-alveolar cells obtained from animals inoculated with control solution was 148.9 ± 27.4 mV ($n = 9$) and with virus was 138.2 ± 24.1 ($n = 9$). H_1-receptor blockade (levocabastine) reduced the basal chemiluminescence production in both inoculated groups (control solution, 69.09 ± 9.20 mV; $n = 6$ vs virus, 88.70 ± 17.7 mV; $n = 6$). Such a decrease was also found after treatment with WEB 2170 (control solution, 93.20 ± 7.10 mV; $n = 6$ vs virus, 83.40 ± 8.50 mV; $n = 6$). The other drugs did not affect the basal chemiluminescence generation (data not shown). In the group inoculated with control solution, the addition of PI-3 virus to broncho-alveolar cells increased the chemiluminescence production to 1284.6 ± 130 mV; this increase was significantly less ($P<0.05$) in the animals infected with PI-3 virus (742.6 ± 164.4 mV). Suprofen, flunarizine and ketanserin did not abolish this diminution (Figure 7a). The chemiluminescence production after PI-3 virus stimulation was decreased in both groups after treatment with flunarizine, but the difference between the group inoculated with control solution and that with PI-3 virus remained (774.4 ± 125.0 mV vs 230.2 ± 43.8 mV, $P<0.05$). Pretreatment with oxatomide, WEB 2170, nedocromil and levocabastine did not modify this decrease either (Figure 7b). After stimulation of the broncho-alveolar cells obtained from virus-infected animals, with PI-3 the chemiluminescence was still, although not significantly, decreased as compared to the respective control groups.

The increase in chemiluminescence generation by broncho-alveolar cells after stimulation with zymosan was significantly diminished in the virally-infected group (control solution, 1176.7 ± 110.8 vs virus, 838.2 ± 46.1 mV, $P<0.05$, Figure 8a). Again, in the groups treated with flunarizine this stimulation

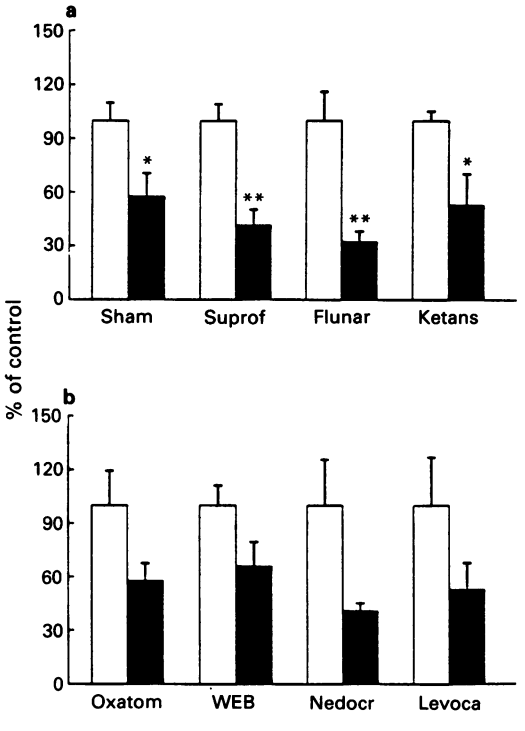


Figure 7 PI-3 virus-induced chemiluminescence generation by broncho-alveolar cells obtained from guinea-pigs inoculated with control solution (open columns) or PI-3 virus (solid columns), and pretreated with pharmacological probes. (a) Solvent solution (1.0 mg kg^{-1}, b.wt.), suprofen (5.0 mg kg^{-1}, b.wt.), flunarizine (2.5 mg kg^{-1}, b.wt.), or ketanserin (0.63 mg kg^{-1}, b.wt.). (b) Oxatomide (2.5 mg kg^{-1}, b.wt.), WEB 2170 (10 mg kg^{-1}, b.wt.), nedocromil (2.5 mg kg^{-1}, b.wt.), levocabastine (0.25 mg kg^{-1} b.wt.). Abbreviations as in Figure 5. The data are expressed as percentage of the mean peak chemiluminescence of each respective control group. The diminished chemiluminescence production by broncho-alveolar cells from animals treated with PI-3 virus was not affected by any pretreatment with drugs. $*P<0.05$; $**P<0.01$, Student's t test (unpaired); $n = 5-8$.

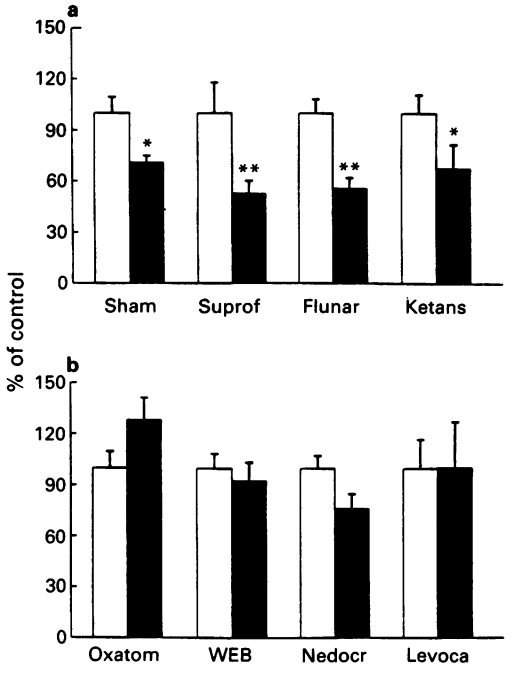


Figure 8 Zymosan (2.5 mg)-induced chemiluminescence generation by broncho-alveolar cells obtained from guinea-pigs inoculated with control solution (open columns) or PI-3 virus (solid columns), and pretreated with pharmacological probes. (a) Solvent solution (1.0 ml kg^{-1}, b.wt.), suprofen (5.0 mg kg^{-1}, b.wt.), flunarizine (2.5 mg kg^{-1}, b.wt.), or ketanserin (0.63 mg kg^{-1} b.wt.). (b) Oxatomide (2.5 mg kg^{-1}, b.wt.), WEB 2170 (10 mg kg^{-1}, b.wt.), nedocromil (2.5 mg kg^{-1}, b.wt.), levocabastine (0.25 mg kg^{-1}, b.wt.). Abbreviations as in Figure 5. The data are expressed as percentage of the mean peak chemiluminescence of every respective control group. The diminished chemiluminescence production by broncho-alveolar cells from animals treated with PI-3 virus was inhibited by oxatomide, WEB 2170, nedocromil, and levocabastine. $*P<0.05$; $**P<0.01$, Student's t test (unpaired); $n = 5-8$.

was less pronounced, but the difference remained (control solution, 764.8 ± 62.5 mV vs virus, 427.3 ± 47.2 mV, $P<0.05$). Suprofen and ketanserin did not affect the virus-induced reduction in chemiluminescence generation after stimulation with zymosan (Figure 8a). However, oxatomide, WEB 2170, levocabastine, and to a lesser extent nedocromil, attenuated the reduced chemiluminescence generation by broncho-alveolar cells obtained from virally-infected animals (Figure 8b).

Histamine nebulization

The histamine C/R curve on tissues obtained from histamine nebulized guinea-pigs ($n = 6$) was significantly ($P<0.01$, ANOVA) shifted downwards as compared with the tracheae obtained from saline-treated animals ($n = 6$, data not shown). The maximal response was decreased, although not significantly, by 20%. In contrast, the arecoline C/R did not differ between the saline and histamine nebulized group. In addition, the EC_{50} values and the slope factors did not differ significantly between both experimental groups after the histamine or arecoline C/R curves.

The total number of inflammatory cells isolated from the airways of animals nebulized with saline was $13.0 \pm 1.4 \times 10^6$ and with histamine $9.0 \pm 1.3 \times 10^6$ ($P>0.05$). The chemiluminescence production after stimulation with zymosan did not differ also between the saline-nebulized group (822.6 ± 159 mV) and the histamine-nebulized group (835.1 ± 47.0 mV).

Discussion

This study shows that virus-induced airway hyperresponsiveness in the guinea-pig is inhibited by drugs interfering with histamine release or with the binding of histamine to its receptor. Oxatomide and nedocromil have mast cell stabilizing effects (Awouters *et al.*, 1979; De Clerck *et al.*, 1981; Auty, 1986). In addition, oxatomide as well as levocabastine, blocks H_1-histamine receptor binding sites (Awouters *et al.*, 1979; De Clerck *et al.*, 1981; Van Wauwe, 1989). Histamine increases vascular permeability and has chemotactic activities (Clarck *et al.*, 1975; Hill, 1990). Indeed, oxatomide, nedocromil, and levocabastine inhibit the virus-induced increase in the number of inflammatory cells and the concentration of albumin in lung lavage fluid. In addition, these drugs inhibited the virus-induced decrease in chemiluminescence production after stimulation with zymosan. Interestingly, the drugs that did not modify the virus-induced tracheal hyperresponsiveness to histamine i.e. the cyclo-oxygenase inhibitor, suprofen, the 5-HT_2 receptor antagonist, ketanserin, and the Ca^{2+} overload blocker, flunarizine, did not modify the influx of inflammatory cells, the increase in albumin, or the decrease in chemiluminescence production after stimulation with zymosan. Flunarizine inhibited the histamine-induced contraction and shifted the C/R curve to the right; but the virus-induced airway hyperresponsiveness to histamine remained. The shape of the arecoline C/R curve was not changed as compared to the sham-treated group, but the virus-induced increased airway contraction was abolished. Histamine needs extracellular Ca^{2+} to elicit a contraction of the trachea, and arecoline increases the intracellular Ca^{2+} concentration via Ca^{2+} release from intracellular stores. This difference might offer a solution for the discrepant results.

The PAF antagonist, WEB 2170, was an exception to the rule in the way that this compound inhibited the increased airway responsiveness and the influx of inflammatory cells, and abolished the decrease in chemiluminescence production in response to zymosan, but did not affect the virus-induced increase of albumin in the lung lavage fluid. Therefore, an increase in albumin leakage may contribute, but is not primarily responsible for the airway hyperresponsiveness.

Basal chemiluminescence production of the broncho-alveolar cells did not differ between the groups inoculated with control solution and PI-3 virus in all pretreated animals. PI-3 virus was a very potent stimulus for broncho-alveolar cells as demonstrated by a marked increase in chemiluminescence production *in vitro*. The chemiluminescence production by broncho-alveolar cells isolated from virally-infected animals was reduced when stimulated with PI-3 virus. None of the pharmacological agents used could abolish this diminution. In contrast, the reduced chemiluminescence production found after stimulation with zymosan was inhibited by oxatomide, nedocromil, WEB 2170, and levocabastine; drugs that also inhibited the influx of inflammatory cells. It may be suggested that on the one hand the activity of broncho-alveolar cells is diminished in a non-specific way (for zymosan), and on the other hand the activity is diminished in a specific way (for PI-3 virus) since none of the drugs used, could inhibit the decreased chemiluminescence upon additional PI-3 virus stimulation. The possibility cannot be excluded that the increase in inflammatory cell numbers will result in a diminished activation of the cells, for instance by local changes in pH and/or release of reactive oxygen radicals and lysosomal enzymes. Therefore, the inhibition of the cell influx by oxatomide, nedocromil, WEB 2170, and levocabastine may prevent excessive release of mediator and enzyme release by inflammatory cells and subsequently inhibit the diminished activity of broncho-alveolar cells.

Many studies have suggested a role for inflammatory cells in the induction of airway hyperresponsiveness (Barnes *et al.*, 1988; Djukanovic *et al.*, 1990). Indeed, in the present study an association was found between the influx of inflammatory cells and the occurrence of airway hyperresponsiveness.

Drugs, such as oxatomide, nedocromil, WEB 2170, and levocabastine, that reduced the influx of cells, especially alveolar macrophages, also inhibited the increase in tracheal contractions. In our laboratory supporting evidence was found that inflammatory cells can modify airway responsiveness. Tracheae obtained from healthy non-treated guinea-pigs incubated *in vitro* with broncho-alveolar cells obtained from virus-infected animals, demonstrated an increased contraction in response to histamine and arecoline (Folkerts *et al.*, 1992c).

PAF and histamine can increase vascular permeability and can attract inflammatory cells into the respiratory tract (Clark *et al.*, 1975; Tate *et al.*, 1982; Lellouch-Tubiana *et al.*, 1988; O'Donnell & Barnett, 1987; Hill, 1990). PAF is one of the major products formed by alveolar macrophages. Histamine can be released by metachromatic cells after stimulation with: histamine releasing factors and interferons (Lett-Brown *et al.*, 1989), reactive oxygen radicals (Mannaioni & Masini, 1988) or PAF (Okuda *et al.*, 1988). These products are likely to be released during viral infections (Hall *et al.*, 1978). Recently we obtained evidence, that in the respiratory tract of virus-infected animals the mast cell morphology and number was changed and that the histamine concentration was doubled in the broncho-alveolar lavage fluid (Folkerts, 1993). Similar findings were published by others. Basal and stimulated histamine release from mast cells isolated from the airways of calves 7 days after inoculation with PI-3 virus, is significantly increased (Ogunbiyi *et al.*, 1988). Moreover, basophils isolated from guinea-pigs 4 days after PI-3 virus infection released more histamine after stimulation, than did basophils from control animals (Graziano *et al.*, 1989).

In patients infected with respiratory syncytial virus the release of histamine in nasopharyngeal secretions was detected significantly more often and in higher concentrations in patients with wheezing (Welliver *et al.*, 1981). In addition, in asthmatic patients positive correlations were found between the degree of bronchial hyperresponsiveness and (a) mast cell histamine release (Wardlaw *et al.*, 1988), (b) the number of mast cells in BAL fluid (Djukanovic *et al.*, 1990) and (c) the concentration of histamine in BAL fluid (Casale *et al.*, 1987). Interestingly, treatment of mild asthmatics with a specific non-sedating H_1-receptor blocker reduced asthmatic symptoms such as cough, wheeze, chest tightness, breathlessness, and inhibited the development of airway hyperresponsiveness (Rafferty, 1990). In contrast, however, nebulization of histamine for 4 successive days did not result in airway hyperresponsiveness, but instead, to histamine tachyphylaxis. Moreover, the number and activity of the broncho-alveolar cells was also not changed. These results support the idea that inflammatory cells/mediators are required in addition, to modify airway responses.

From literature data and the results presented in this study some suggestions can be made as to how viral respiratory infections cause airway hyperresponsiveness. Inflammatory cells seem to play an important role. Histamine and PAF, not only increase vascular permeability, but also attract inflammatory cells into the lungs. The expression of adhesion molecules on endothelial cells induced by histamine (Pober & Cotran, 1990) and on airway epithelial cells induced by viruses (Tosi *et al.*, 1992), probably potentiates the transport of inflammatory cells into the lumen of the airways. The inflammatory cells release an array of mediators that probably modify airway responses. The epithelial damage observed after a viral infection (Folkerts *et al.*, 1992a) may be caused by products released from these inflammatory cells and/or by the growth of viruses in epithelial cells. The loss of barrier function together with a decreased formation of an epithelium-derived relaxing factor(s) can also influence the contractility of the tracheae. Finally, epithelial damage exposes sensory nerve endings that can be stimulated by histamine and other mediators, leading to a release of neuropeptides (Barnes *et al.*, 1990; Maggi, 1991). Airway contractions in response to substance P and capsaicin are indeed increased in PI-3 virus-infected guinea-pigs (Saban *et al.*, 1987). Further, as shown by Jacoby *et al.* (1988), the enzymatic activity of enkephalinase is decreased and the reactivity to substance P is increased in ferret airway tissues incubated with influenza virus for 4 days. It cannot be excluded that an enhanced concentration of neuropeptides in the respiratory tract influences the contractility to histamine and arecoline.

It is concluded that histamine is probably involved in a cascade of events leading to airway hyperresponsiveness after a viral infection. It must be stressed that histamine is not solely responsible for the observed effects, and that inflammatory cells/mediators and epithelial damage are likely to be additional factors that contribute to the increased tracheal responsiveness.

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References

AQUILINA, A.T., HALL, W.J., DOUGLAS, R.G.J. & UTELL, M.J. (1980). Airway reactivity in subjects with viral upper respiratory tract infections: the effects of exercise and cold air. *Am. Rev. Resp. Dis.*, **122**, 3–10.

AUTY, R.M. (1986). The clinical development of a new agent for the treatment of airway inflammation, nedocromil sodium (Tilade). *Eur. J. Resp. Dis.*, **69** (suppl. 147), 120–131.

AWOUTERS, F., BORGERS, M. DE CLERCK, F. & NIEMEGEERS, C.J.E. (1979). Oxatomide: comparative experimental studies with other anti-allergic agents. *Tissue Reac.*, **1**, 95–102.

BARNES, P.J., BELVISI, M.G. & ROGERS, D.F. (1990). Modulation of neurogenic inflammation: novel approaches to inflammatory disease. *Trends Pharmacol. Sci.*, **11**, 185–189.

BARNES, P.J., CHUNG, K.F. & PAGE, C.P. (1988). Inflammatory mediators and asthma. *Pharmacol. Rev.*, **40**, 49–84.

BUSSE, W.W. (1990). Respiratory infections: their role in airway responsiveness and the pathogenesis of asthma. *J. Allergy Clin. Immunol.*, **85**, 671–683.

CASALE, T.B., WOOD, D., RICHERSON, H.B., TRAPP, S., METZGER, W.J., ZAVALA, D. & HUNNINGHAKE, G.W. (1987). Elevated bronchoalveolar lavage fluid histamine levels in allergic asthmatics are associated with methacholine bronchial hyperresponsiveness. *J. Clin. Invest.*, **79**, 1197–1203.

CHANOCK, R.M. & MCINTOSH, K. (1985). Parainfluenza viruses. In *Vitrology*. ed. Fields, B.N. pp. 1241–1253. New York: Raven Press.

CLARCK, R.A.F., GALLIN, J.I. & KAPLAN, A.P. (1975). The selective eosinophil chemotactic activity of histamine. *J. Exp. Med.*, **142**, 1462–1476.

DE CLERCK, F., VAN REEMPTS, J. & BORGERS, M. (1981). Comparative effects of oxatomide on the release of histamine from rat peritoneal mast cells. *Agents Actions*, **11**, 184–192.

DE CLERCK, F., VERMIJLEN, J. & RENEMAN, R. (1975). Effects of suprofen, an inhibitor of prostaglandin biosynthesis, on platelet function, plasma coagulation and fibrinolysis. In vitro experiments. *Arch. Int. Pharmacodyn. Ther.*, **216**, 262–279.

DE CLERK, F., XHONNEUX, B., LEYSEN, J. & JANSSEN, P.A.J. (1984). Evidence for functional 5-HT_2 receptor sites on human blood platelets. *Biochem. Pharmacol.*, **33**, 2807–2811.

DE LEAN, A., MUNSON, P.J. & RODBARD, R. (1978). Simultaneous analisis of families of sigmoidal curves: application of bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.*, **235**, E97–102.

DJUKANOVIC, R., ROCHE, W.R., WILSON, J.W., BEASLEY, C.R.W., TWENTYMAN, O.P., HOWARTH, P.H. & HOLGATE, S.T. (1990). Mucosal inflammation in asthma. *Am. Rev. Resp. Dis.*, **142,** 434–457.

DOUMAS, B.T., WATSON, W.A. & BIGGS, H.G. (1971). Albumin standards and the measurement of serum albumin with bromcresol green. *Clin. Chim. Acta*, **31,** 87–96.

EMPEY, D.W., LAUTINEN, L.A., JACOBS, L., GOLD, W.M. & NADEL, J.A. (1976). Mechanisms of bronchial hyperreactivity in normal subjects after upper respiratory tract infection. *Am. Rev. Resp. Dis.*, **113,** 131–139.

FOLKERTS, G., ENGELS, F. & NIJKAMP, F.P. (1989). Endotoxin-induced hyperreactivity of the guinea pig isolated trachea coincides with decreased prostaglandin E_2 production by the epithelial layer. *Br. J. Pharmacol.*, **96,** 388–394.

FOLKERTS, G., HENRICKS, P.A.J., SLOOTWEG, P.J. & NIJKAMP, F.P. (1988). Endotoxin-induced inflammation and injury of the guinea pig respiratory airways cause bronchial hyporeactivity. *Am. Rev. Resp. Dis.*, **137,** 1441–1448.

FOLKERTS, G., VERHEYEN, A. & NIJKAMP, F.P. (1992a). Viral infection in guinea pigs induces a sustained non-specific airway hyperresponsiveness and morphological changes of the respiratory tract. *Eur. J. Pharmacol. (section Environ. Toxicol. Pharmacol.)*, **228,** 121–130.

FOLKERTS, G., VERHEYEN, A.K.C.P., GEUENS, G.M.A., FOLKERTS, H.F. & NIJKAMP, F.P. (1993). Virus-induced changes in airway responsiveness, morphology and histamine levels in guinea-pigs. *Am. Rev. Resp. Dis.* (in press).

FOLKERTS, G., VAN ESCH, B., JANSSEN, M. & NIJKAMP, F.P. (1992b). Virus-induced airway hyperresponsiveness in guinea pigs in vivo: study of broncho-alveolar cell number and activity. *Eur. J. Pharmacol. (section Environ. Toxicol. Pharmacol.)*, **228,** 219–227.

FOLKERTS, G., VERHEYEN, A., JANSSEN, M. & NIJKAMP, F.P. (1992c). Virus-induced airway hyperresponsiveness in the guinea pig can be transferred by bronchoalveolar cells. *J. Allergy Clin. Immunol.*, **90,** 364–372.

GLEZEN, W.P., LODA, F.A. & DENNY, F.W. (1982). The parainfluenza viruses. In *Viral Infections of Humans. Epidemiology and Control.* ed. Evans, E.A. pp. 441–454. New York: Plenum.

GRAZIANO, F.M., TILTON, R., HIRTH, T., SEGALOFF, D., MULLINS, T., DICK, E., BUCKNER, C.K. & BUSSE, W.W. (1989). The effects of Parainfluenza 3 infection on guinea pig basophil and lung mast cell histamine release. *Am. Rev. Resp. Dis.*, **139,** 715–720.

HALL, C.B., DOUGLAS, R.G.J., SIMONS, R.L. & GEIMAN, J.M. (1978). Interferon production in children with respiratory syncytial influenza, and parainfluenza virus infections. *J. Pediatr.*, **93,** 28–32.

HEUER, R.O., CASALS-STENZEL, J., MUACEVIC, G. & WEBER, K.H. (1990). Pharmacologic activity of Bepafant (WEB 2170), a new and selective hetrazepinoic antagonist of platelet activating factor. *J. Pharmacol. Exp. Ther.*, **255,** 962–968.

HILL, S.J. (1990). Distribution, properties, and functional characteristics of three classes of histamine receptors. *Pharmacol. Rev.*, **42,** 46–83.

JACOBY, D.B., TAMAOKI, J., BORSON, D.B. & NADEL, J.A. (1988). Influenza infection causes airway hyperresponsiveness by decreased enkephalinase. *J. Appl. Physiol.*, **64,** 2653–2658.

LAITINEN, L.A. & KAVA, T. (1980). Bronchial reactivity following uncomplicated Influenza A infections in healthy subjects and in asthmatic patients. *Eur. J. Resp. Dis.*, **10,** 51–58.

LELLOUCH-TUBIANA, A., LEFORT, J., SIMON, M.T., PFISTER, A. & VERGAFTIG, B.B. (1988). Eosinophil recruitment into guinea pig lungs after PAF-acether and allergen administration. *Am. Rev. Resp. Dis.*, **137,** 984–954.

LETT-BROWN, M.A., ALAM, R. & GRANT. J.A. (1989). Regulation of basophil and mast cell activation by cytokines. In *Cytokines Regulating the Allergic Response.* ed. Sorg, C. pp. 90–99, Basel: S. Karger.

LITTLE, J.W., HALL, W.J., DOUGLAS, R.G.J., MUDHOLKAR, G.S., SPEERS, D.M. & PATEL, K. (1978). Airway hyperreactivity and peripheral airway dysfunction in Influenza A infection. *Am. Rev. Resp. Dis.*, **118,** 295–303.

MAGGI, C.A. (1991). The pharmacology of the efferent function of sensory nerves. *J. Auton. Pharmacol.*, **11,** 173–208.

MANNAIONI, P.F. & MASINI, E. (1988). The release of histamine by free radicals. *Free Radical Biol. Med.*, **5,** 177–197.

MCINTOSH, K., ELLIS, E., HOFFMAN, L., LYBASS, T., ELLER, J. & FULGINITI, V. (1973). The association of viral and bacterial respiratory infections with exacerbations of wheezing in young asthmatic children. *J. Pediatr.*, **82,** 578–590.

MINOR, T.E., DICK, E.C., DEMEO, A.N., OUELLETTE, J.J., COHEN, M. & REED, C.E. (1974). Viruses as precipitants of asthmatic attacks in children. *J. Am. Med. Ass.*, **227,** 292–298.

O'DONNELL, S.R. & BARNETT, C.J.K. (1987). Microvascular leakage to Platelet-Activating Factor in guinea pig trachea and bronchi. *Eur. J. Pharmacol.*, **138,** 385–396.

OGUNBIYI, P.O., BLACK, W.D. & EYRE, P. (1988). Parainfluenza-3 virus-induced enhancement of histamine release from calf lung mast cell: effect of levamisole. *J. Vet. Pharmacol. Ther.*, **2,** 338–344.

OKUDA, Y., TSUYUGUCHI, I. & YAMATODANI, A. (1988). Histamine release from human leukocytes by Platelet-Activating Factor. *Int. Arch. Appl. Immunol.*, **85,** 341–345.

POBER, J.S. & COTRAN, R.S. (1990). The role of endothelial cells in inflammation. *Transplantation*, **50,** 537–544.

RAFFERTY, P. (1990). Antihistamines in the treatment of clinical asthma. *J. Allergy Clin. Immunol.*, **86,** 647–650.

SABAN, R., DICK, E.C., FISHLEDER, R.I. & BUCKNER, C.K. (1987). Enhancement by parainfluenza 3 infection of the contractile response to substance P and capsaicin in airway smooth muscle of the guinea pig. *Am. Rev. Resp. Dis.*, **139,** 586–591.

SIEGEL, S. (1956). *Non-parametric Statistics for the Behavioural Sciences.* Tokio: McGraw-Hill.

TATE, R.M., VANBENTHUYSEN, K.M., SHASBY, D.M., MCMURTHY, I.F. & REPINE, J.E. (1982). Oxygen-radical-mediated permeability edema and vasoconstriction in isolated perfused rabbit lungs. *Am. Rev. Resp. Dis.*, **126,** 802–806.

TODD, P.A. & BENFIELD, P. (1989). Flunarizine: a reappraisal of its pharmacological properties and therapeutic use in neurological disorders. *Drug*, **38,** 481–499.

TOSI, M.F., STARK, J.M., HAMEDANI, A., SMITH, C.W., GRUENERT, D.C. & HUANG, Y.T. (1992). Intercellular adhesion molecule-1 (ICAM-1)-dependent and ICAM-1-independent adhesive interactions between polymorphonuclear leukocytes and human airway epithelial cells infected with parainfluenza virus type 2. *J. Immunol.*, **149,** 3345–3349.

VAN WAUWE, J.P. (1989). Animal pharmacology of levocabastine: a new type of H_1-antihistamine well suited for topical application. In *Rhinoconjunctivitis-New Perspectives in Topical Application.* ed. Mygind, N. & Naclerio, R.M. Toronto: Hografe & Huber.

WARDLAW, A.J., DUNNETTE, S., GLEICH, G.J., COLLINS, J.V. & KAY, A.B. (1988). Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am. Rev. Resp. Dis.*, **137,** 62–69.

WELLIVER, R.C., WONG, D.T., SUN, M., MIDDELTON, E.J., VAUGHAN, R.S. & OGRA, P.L. (1981). The development of respiratory syncytial virus-specific IgE and the release of histamine in nasopharyngeal secretions after infection. *N. Engl. J. Med.*, **305,** 841–846.

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Ap_4A and ADP-β-S binding to P_2 purinoceptors present on rat brain synaptic terminals

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1 Diadenosine tetraphosphate (Ap_4A) a dinucleotide stored and released from rat brain synaptic terminals presents two types of affinity binding sites in synaptosomes. When [^{3}H]-Ap_4A was used for binding studies a K_d value of 0.10 ± 0.014 nM and a B_{max} value of 16.6 ± 1.2 fmol mg^{-1} protein were obtained for the high affinity binding site from the Scatchard analysis. The second binding site, obtained by displacement studies, showed a K_i value of 0.57 ± 0.09 μM.

2 Displacement of [^{3}H]-Ap_4A by non-labelled Ap_4A and P_2-purinoceptor ligands showed a displacement order of Ap_4A > adenosine 5'-O-(2-thiodiphosphate) (ADP-β-S) > 5'-adenylyl-imidodiphosphate (AMP-PNP) > α,β-methylene adenosine 5'-triphosphate (α,β-MeATP) in both sites revealed by the K_i values of 0.017 nM, 0.030 nM, 0.058 nM and 0.147 nM respectively for the high affinity binding site and values of 0.57 μM, 0.87 μM, 2.20 μM and 4.28 μM respectively for the second binding site.

3 Studies of the P_2-purinoceptors present in synaptosomes were also performed with [^{35}S]-ADP-β-S. This radioligand showed two binding sites the first with K_d and B_{max} values of 0.11 ± 0.022 nM and 3.9 ± 2.1 fmol mg^{-1} of protein respectively for the high affinity binding site obtained from the Scatchard plot. The second binding site showed a K_i of 0.018 ± 0.0035 μM obtained from displacement curves.

4 Competition studies with diadenosine polyphosphates of [^{35}S]-ADP-β-S binding showed a displacement order of $Ap_4A > Ap_5A > Ap_6A$ in the high affinity binding site and K_i values of 0.023 nM, 0.081 nM and 5.72 nM respectively. The second binding site potency order was $Ap_5A > Ap_4A > Ap_6A$, with K_i values of 0.28 μM, 0.53 μM and 5.32 μM respectively.

5 Displacement studies of [^{35}S]-ADP-β-S with P_2-purinoceptor agonists showed the following potency pattern: ADP-β-S > AMP-PNP > α,β-MeATP with K_i values of 0.021 nM, 0.029 nM 0.215 nM respectively in the high affinity binding site. 2-Methylthio-adenosine 5'-triphosphate (2MeSATP) was unable to displace [^{35}S]-ADP-β-S in this binding site. The second binding site showed a profile of ADP-β-S > α,β-MeATP > AMP-PNP > 2MeSATP and K_i values of 0.018 μM, 0.212 μM, 0.481 μM and 18.04 μM respectively.

6 These studies suggest the presence of a new P_2-purinoceptor in rat brain synaptosomes with high affinity for diadenosine polyphosphates which we tentatively designate as P_{2d}.

Keywords: Adenosine-(5')-tetraphospho-(5')-adenosine (Ap_4A); diadenosine polyphosphates; P_{2d}-purinoceptors; synaptosome

Introduction

Since the idea that purinergic nerves exist was first conceived, intensive studies of the actions of adenosine and ATP, through plasma membrane receptors, have been carried out (Burnstock, 1972; Gordon, 1986; Williams, 1987). Separate receptors for adenosine and ATP were soon recognized and classified as P_1- and P_2-purinoceptors respectively (Burnstock, 1978). Subsequently, four different classes of P_2-purinoceptors have been proposed. P_{2x} and P_{2y} were the first to be found, based on the divergent effects of ATP and analogues, on vascular and visceral smooth muscles (Burnstock & Kennedy, 1985), P_{2t}-purinoceptors, the main natural agonist of which is ADP, are involved in platelet aggregation. P_{2z}-purinoceptors are linked to mast cell stimulation and ATP^{4-} is the active ligand (Cockcroft & Gomperts, 1980).

In spite of the broad studies in the field of P_2-purinoceptors, no potent, selective and reversible antagonists have yet been identified, but even in their absence the existence of other P_2-purinoceptors seems probable. For example, recently a receptor with different agonist order from that already described, has been reported (O'Connor *et al.*, 1991).

The presence of diadenosine polyphosphates as natural constituents of secretory granules, increases the diversity of secreted purinergic compounds and their possibilities of action as extracellular signals. The discovery of Ap_4A and Ap_5A, and more recently Ap_6A, in chromaffin cells increases the possibility of their role as neural transmitters and modulators (Rodriguez del Castillo *et al.*, 1988; Pintor *et al.*, 1992c). In this neural model, the release of these dinucleotides after carbachol stimulation allowed their quantification in the extracellular media at concentrations in the μM range (Pintor *et al.*, 1991a). It has also been possible to characterize receptors for Ap_xA with the highest affinities described for a nucleotide ($K_d \approx 10^{-11}$ M) (Pintor *et al.*, 1991b). Ap_4A and Ap_5A acting through P_{2y}-receptors are able to inhibit the catecholamine secretion induced by nicotine (Castro *et al.*, 1990). These purinoceptors subserve increases in the intracellular levels of Ca^{2+} when they are stimulated by diadenosine polyphosphates (Castro *et al.*, 1992).

This is not the only model in which diadenosine polyphosphates show biological actions. Ap_3A and Ap_4A, stored in dense secretory granules, inhibit platelet aggregation (Cusack & Hourani, 1982; Flodgaard & Klenow, 1982; Lüthje & Ogilvie, 1983; Zamecnick *et al.*, 1992). Macrophage membranes, which possess P_{2z}-purinoceptors, are susceptible to diadenosine pentaphosphate, inducing the typical pore formation on these membranes (Steinberg *et al.*, 1987). In other organs, such as the liver, diadenosine polyphosphates are able to displace ATP-γS from P_2-purinoceptors present on liver membranes (Keppens *et al.*, 1989). Noteworthy are the actions of Ap_4A and Ap_5A on vas deferens, which has similar effects to the potent P_{2x} agonist, α,β-MeATP (MacKenzie *et al.*, 1988). Ap_6A has also been found to act on the urinary bladder via P_{2x}-purinoceptors (Hoyle *et al.*, 1989). Studies

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have been carried out in sensory neurones where the effects of different nucleotides and diadenosine polyphosphates, have been reported. These compounds activate a receptor which opens cation channels with low ionic selectivity and strong inward-going rectification (Krishtal *et al.*, 1988).

The presence of diadenosine polyphosphates in the synaptosomes of rat brain, and their release by depolarizing agents, emphasized the importance of these substances as possible neurotransmitters in the central nervous system and invites the search for their physiological actions mediated by membrane receptors (Pintor *et al.*, 1992a).

The present paper deals with the existence of specific receptors for Ap_xA in brain synaptic terminals, and also the classification of these purinoceptors by use of different ATP analogues, in order to establish similarity to or difference from the P_2-purinoceptors previously described.

Methods

Synaptosomal preparation

Synaptosomes were prepared from middle cerebral cortices of male Wistar rats cervically dislocated and decapitated as described by Pintor *et al.* (1992a). Synaptosomes in 1 or 10 mg pellets, were resuspended into 1 ml incubation medium (composition mM: NaCl 122, KCl 3.1, KH_2PO_4 0.4, $NaHCO_3$ 5, $MgSO_4$ 1.2, glucose 10 and TES buffer 20, pH 7.4).

High performance liquid chromatography (h.p.l.c.) procedures

The h.p.l.c. equipment consisted of a Spectra Physics SP 8800 ternary pump, a Spectra 100 variable wavelength detector, a Reodyne injector and a Chrom Jet integrator. The column was an RP-18 (22 cm length, 0.46 mm diameter) from Brownlee (Applied Biosystems). The conditions of the mobile phase were as follows: KH_2PO_4 10 mM, tetrabutylammonium 2 mM, 10% acetonitrile, pH 7.5.

Concentrations of 1 μM diadenosine polyphosphates and ATP analogues were assayed in 1 ml of incubation media with 1 mg of synaptosomes ml^{-1}. Samples of 25 μl were taken for h.p.l.c. analysis.

Binding studies with [^{3}H]-Ap_4A

[^{3}H]-Ap_4A (6.3 Ci $mmol^{-1}$) binding assays to rat brain synaptosomes were performed with 0.6 mg of protein synaptosomal preparations in 1 ml of the incubation medium (described under 'synaptosomal preparation') and graded concentrations of [^{3}H]-Ap_4A, ranging from 0.025 nM to 2.5 nM. In order to obtain the saturation isotherms, the incubation conditions were 45 min at 25°C temperature. Binding was stopped by filtering through Whatman GF/C filters and washing with 10 ml of cold incubation medium containing 10 μM cold Ap_4A. The filters were then put into scintillation vials, with 5 ml of Beckman Ready Safe scintillation cocktail. Finally the vials were counted in a Beckman LS 3801.

In order to quantify the non-specific binding, the same procedure was carried out in the presence of 10 μM cold Ap_4A.

Displacement studies were performed with 0.6 mg of synaptosomes in a final volume of 1 ml incubation medium and in the presence of a concentration of 0.25 nM [^{3}H]-Ap_4A. P_2-purinoceptor agonists as well as Ap_4A were assayed in concentrations ranging from 10^{-12} to 10^{-3} M.

Binding studies with [^{35}S]-ADP-β-S

[^{35}S]-ADP-β-S (1242 Ci $mmol^{-1}$) binding assays to synaptic terminals were studied with 50 μg of synaptosomes in 1 ml of the incubation medium, as previously described, and in the presence of graded concentrations of [^{35}S]-ADP-β-S ranging from 0.025 nM to 2.5 nM. The incubation conditions were 45 min at 25°C. When necessary the binding experiments were performed at 4°C with 4 h incubation time. Binding was stopped by addition of 10 ml of cold incubation medium with 10 μM cold ADP-β-S after filtering through Whatman GF/C filters following the same protocol as described for [^{3}H]-Ap_4A.

Non-specific binding was evaluated by incubation in the presence of 10 μM ADP-β-S.

Competition binding assays were initiated with 50 μg of synaptosomes in 1 ml of incubation medium containing [^{35}S]-ADP-β-S 0.1 nM. Diadenosine polyphosphates and P_2-purinoceptor agonists were assayed in a concentration ranging from 10^{-12} to 10^{-3} M. 2MeSATP was assayed at 4°C for 4 h to avoid possible ectonucleotidease activities.

Computer analysis

Data from saturation and displacement experiments were processed, covering a range from 10^{-12} to 10^{-3} M Ap_4A and ADP-β-S. Binding curves were fitting directly to raw c.p.m. data, to avoid biasing during intermediate calculations, with the aid of the programmes EQUIL, kindly provided by Dr. R.F. Goldstein, Computer Center, Univ. Illinois of Chicago (Goldstein & Leung, 1990) and LIGAND (Munson & Rodbard, 1980). The equilibrium parameters were determined by the equations described by Casadó *et al.* (1990) to avoid the interference of non-specific binding by the inclusion of the corresponding unknown parameter in the equations. All the results obtained are means ± s.d.

Materials

[^{3}H]-Ap_4A (6.3 Ci $mmol^{-1}$) was purchased from Amersham (United Kingdom). [^{35}S]-ADP-β-S (1242 Ci $mmol^{-1}$) was from New England Nuclear (United Kingdom). All nucleotides and dinucleotides were obtained from Boehringer (Manheim, Germany) except Ap_6A which was purchased from Sigma (St. Louis, U.S.A.) and 2MeSATP which was purchased from RBI (U.S.A.). Other reagents were analytical grade purchased from Merck (Darmstadt, Germany).

Results

H.p.l.c. analysis

ATP analogues as well as diadenosine polyphosphates were analysed by h.p.l.c. as described in Methods, in order to verify their structure during the incubation period. Neither diadenosine polyphosphates nor ATP analogues were affected during the incubation period. As shown in Figure 1, Ap_4A and ADP-β-S were stable during the whole incubation time; the same procedure was followed with the other nucleotides and dinucleotides. 2MeSATP was incubated for 4 h at 4°C to avoid possible ectonucleotidase activity. This time is sufficient to reach the equilibrium for binding studies. H.p.l.c. analysis confirmed its stability during the incubation period (not shown). These results allowed us to develop the following binding experiments with the assurance of the stability of all nucleotides.

[^{3}H]-Ap_4A binding to rat brain synaptic terminals

[^{3}H]-Ap_4A data from six experiments were transformed and represented as a Scatchard plot (Figure 2). The results were analysed by the method for binding parameters estimation of Casadó *et al.* (1990). The results of computer studies showed the presence of a high affinity binding site in the nM range, with a K_d value of 0.10 ± 0.014 nM and a B_{max} value of 16.6 ± 1.2 fmol mg^{-1} of protein. The curvilinear profile in the

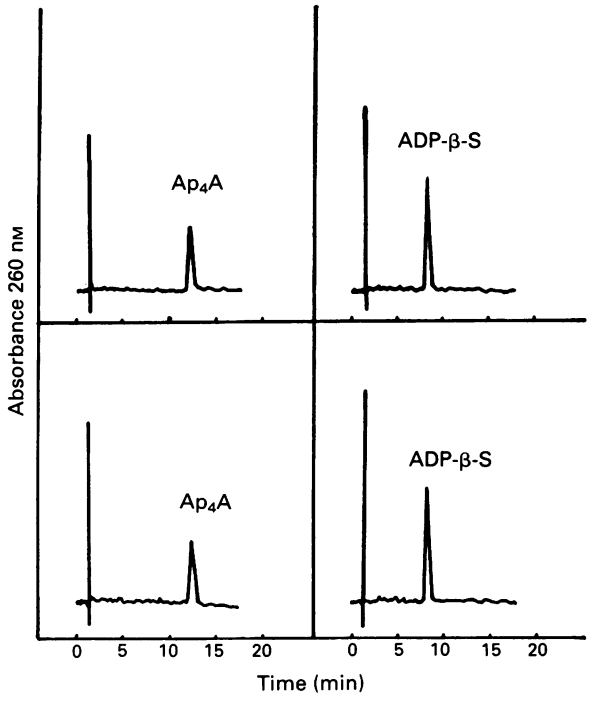


Figure 1 High performance liquid chromatography (h.p.l.c.) of Ap_4A and ADP-β-S stability during the binding study: (a) h.p.l.c. elution profile of 25 pmol Ap_4A after incubation with 1 mg synaptosomes ml^{-1} for 45 min under the conditions described in Methods; (b) h.p.l.c. elution profile of 25 pmol of standard Ap_4A; (c) elution profile of 25 pmol ADP-β-S after incubation for 45 min with 1 mg synaptosomes ml^{-1} under the conditions described in Methods; (d) elution profile of 25 pmol standard ADP-β-S. For abbreviations in this and subsequent legends, see text.

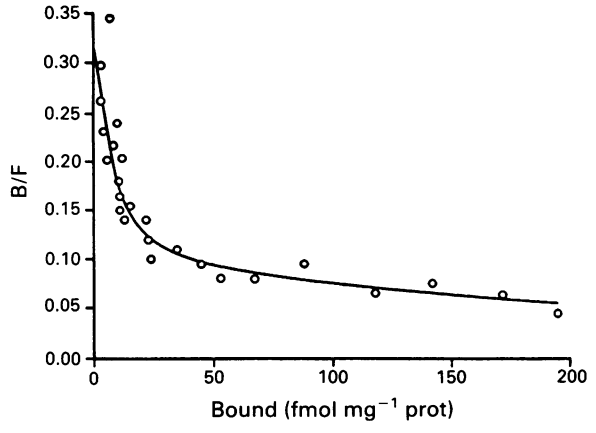


Figure 2 Scatchard analysis of equilibrium of [^{3}H]-Ap_4A binding to rat brain synaptosomes. Binding was studied with 0.6 mg synaptosomes ml^{-1} as described in Methods. K_d and B_{max} values were obtained by the method of Casadó *et al.* (1990). Results are means of six experiments in duplicate.

experimental concentration-range employed, is a consequence of an increasing non-specific binding, which is a difficult experimental value to obtain and in this way, it was included as an unknown parameter in the K_d determination equations (Casadó *et al.*, 1990).

[^{3}H]-Ap_4A competition studies with Ap_4A and P_2 agonists

Indirect binding assays were carried out with non-labelled Ap_4A and different P_2 agonists in order to characterize the pharmacological profile of the sites present in the synaptosomes.

In Figure 3 the displacement curves for Ap_4A, ADP-β-S, α,β-MeSATP and AMP-PNP are shown (results of four experiments in duplicate). The percentage displacement in the first binding site was approximately 15% of the total for the four compounds assayed. Data from saturation and displacement studies were processed together and fitted with the help of the computer programmes EQUIL and LIGAND (Goldstein & Leung, 1990; Munson & Rodbard, 1980) to obtain the K_i values. The displacement binding parameters (K_i) for the first and second steps are summarized in Table 1. The displacement of [^{3}H]-Ap_4A by non-labelled Ap_4A shows two different binding sites. The first was in the nM range with a K_i in the order of the K_d obtained in the Scatchard plot. The second was in the μM range and it was not detectable by Scatchard analysis in the experimental radioligand concentration.

The displacement order for the first binding site was $Ap_4A > ADP\text{-}\beta\text{-}S > AMP\text{-}PNP > \alpha,\beta\text{-}MeATP$. The second binding site showed a potency order of $Ap_4A > ADP\text{-}\beta\text{-}S > AMP\text{-}PNP > \alpha,\beta\text{-}MeATP$. The Hill numbers ($n_H$) for these compounds (less than 1) suggested the presence of more than one binding site.

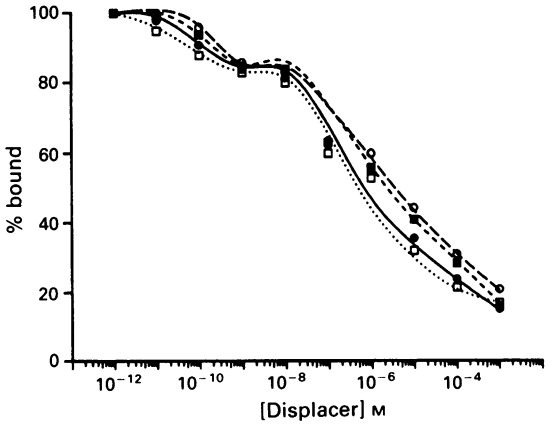


Figure 3 Displacement studies of radiolabelled diadenosine tetraphosphate ([^{3}H]-Ap_4A) by cold Ap_4A and P_2-receptor agonists. Synaptosomes in a concentration of 0.6 mg ml^{-1} were incubated in the presence of Ap_4A (□), ADP-β-S (●), AMP-PNP (■) and α,β-MeATP (○). All the experiments represented the mean of four experiments in duplicate. The K_i values are summarized in Table 1.

Table 1 K_i values for the [^{3}H]-diadenosine tetraphosphate ([^{3}H]-Ap_4A) binding displacement by diadenosine tetraphosphate and P_2 agonists

	[^{3}H]-Ap_4A		
Compound	K_{i_1} (nM)	K_{i_2} (μM)	n_H
Ap_4A	0.017 ± 0.004	0.57 ± 0.09	0.26
ADP-β-S	0.030 ± 0.006	0.87 ± 0.12	0.23
AMP-PNP	0.058 ± 0.006	2.20 ± 0.83	0.25
α,β-MeATP	0.147 ± 0.031	4.28 ± 1.03	0.24

Binding parameters for [^{3}H]-Ap_4A: K_d: 0.010 ± 0.014 nM; B_{max}: 16.6 ± 1.2 fmol mg^{-1} protein.
K_i values for Ap_4A and P_2 purinoceptor agonists correspond to Figure 2. The K_i values were calculated by the programme EQUIL (Goldstein & Leung, 1990) which adjusted data to non-linear weighted regressions. K_{i_1} and K_{i_2} correspond to the very high affinity and the high affinity binding sites respectively.
For abbreviations, see text.

Characaterization of the synaptic terminal binding sites with [35S]-ADP-β-S

[35S]-ADP-β-S, one of the most potent P_{2Y}-agonists, helped us to identify the binding sites present in synaptosomes. When the saturation studies were performed ($n = 6$), the Scatchard plot profile observed was very similar to that obtained with [3H]-Ap_4A (Figure 4). In the concentration-range used to obtain the equilibrium isotherms a curvilinear plot was obtained as a consequence of an increasing of non-specific binding. To avoid this interference, data were fitted to the equations described by Casadó *et al.* (1990). The results of these studies showed the presence of one binding site with a K_d value of 0.11 ± 0.022 nM, and a B_{max} of 3.9 ± 2.1 fmol mg^{-1} of protein.

[35S]-ADP-β-S displacement studies with diadenosine polyphosphates

The ability of Ap_4A, Ap_5A and Ap_6A to displace [35S]-ADP-β-S, was assayed with a concentration value in the order of its K_d value. In Figure 5 the behaviour of these compounds in displacing [35S]-ADP-β-S is shown (results of 4 experiments in duplicate). The K_i values for these dinucleotides in both binding sites, calculated with the help of the computer programmes, are summarized in Table 2. Ap_4A was the best competitor at the high affinity binding site but not in the second. On the other hand, Ap_5A was more active in displacing at the second site than any other dinucleotide. Ap_6A was the less potent compound at both sites.

[35S]-ADP-β-S competition studies with P_2 agonists

Looking for a pharmacological profile of the binding sites present in rat brain synaptic terminals, we performed competition studies of [35S]-ADP-β-S with ADP-β-S, α,β-MeATP, AMP-PNP and 2MeSATP (results of 4 experiments in duplicate). As shown in Figure 6, the P_2 pattern displacement was ADP-β-S > AMP-PNP > α,β-MeATP. It is necessary to emphasize that 2MeSATP was not able to displace [35S]-ADP-β-S from the high affinity binding site. The displacement order for the second binding site was: ADP-β-S > α,β-MeATP > AMP-PNP > 2MeSATP. This agonists order was different from any known classification for P_2 purinoceptors.

The K_i values for the four compounds and for both sites are summarized in Table 2. All the compounds appear to bind to more than one site, except for 2MeSATP with a Hill number close to 1.

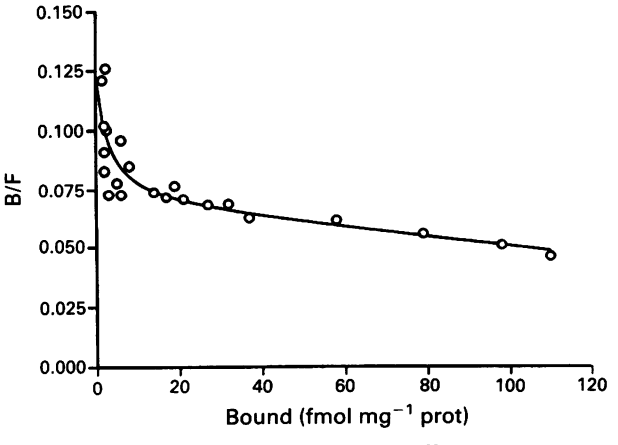


Figure 4 Scatchard analysis of equilibrium of [35S]-ADP-β-S binding to rat brain synaptosomes. Binding was studied with 50 μg synaptosomes ml^{-1} as described in Methods. K_d and B_{max} values were obtained by the method of Casadó *et al.* (1990). Results are means of six experiments in duplicate.

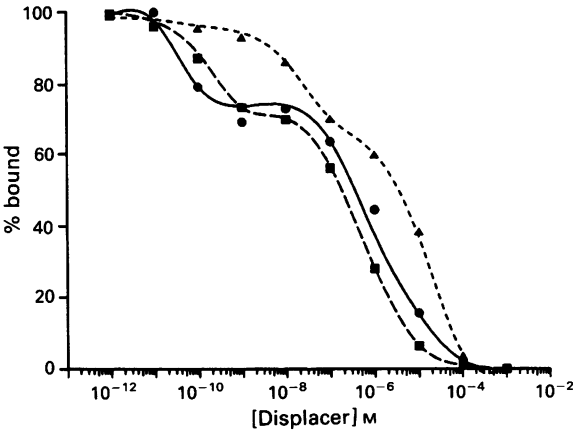


Figure 5 Inhibition of site specific binding of [35S]-ADP-β-S to rat brain synaptosomes. Synaptosomes were incubated in a concentration of 50 μg ml^{-1} in the presence of [35S]-ADP-β-S as described in Methods in the presence of each Ap_4A (●), Ap_5A (■) and Ap_6A (▲). Results are plotted as a percentage of site specific binding of [35S]-ADP-β-S in the absence of inhibitors. Results are means of four experiments in duplicate.

Table 2 K_i values for the [35S]-adenosine 5'-O-(2 thiodiphosphate ([35S]-ADP-β-S) binding displacement by diadenosine polyphosphates and P_2 receptor agonists

	[35S]-ADP-β-S		
Compound	K_{i_1} (nM)	K_{i_2} (μM)	n_H
Ap_4A	0.023 ± 0.007	0.53 ± 0.012	0.30
Ap_5A	0.081 ± 0.012	0.28 ± 0.027	0.36
Ap_6A	5.72 ± 0.85	5.32 ± 0.97	0.41
ADP-β-S	0.021 ± 0.007	0.018 ± 0.003	0.33
AMP-PNP	0.029 ± 0.008	0.481 ± 0.092	0.43
α,β-MeATP	0.215 ± 0.035	0.212 ± 0.046	0.62
2MeSATP	–	18.04 ± 3.72	0.86

Binding parameters for [35S]-ADP-β-S: K_d: 0.11 ± 0.022 nM; B_{max}: 3.9 ± 2.1 fmol mg^{-1} protein.
K_i values for diadenosine polyphosphates and P_2 purinoceptor agonists correspond to Figures 5 and 6. The K_i values were calculated by the computer programme EQUIL (Goldstein & Leung, 1990) which adjusted data to non-linear wieghted regressions. K_{i_1} and K_{i_2} correspond to the very high affinity and the high affinity binding sites respectively.
For abbreviations, see text.

As described in Methods, 2MeSATP experiments were performed at 4°C. To verify that temperature did not alter the binding features, i.e. as a consequence of lipid-phase transitions, binding experiments with [35S]-ADP-β-S were also performed at 4°C. Under these conditions the K_d and B_{max} values were respectively 0.083 ± 0.0015 nM and 3.0 ± 1.2 fmol mg^{-1} protein, being the ratio K_d/B_{max} 27600. The quotient K_d/B_{max} at 25°C was 28205 (see Table 2). These data suggest that the behaviour of 2MeSATP is not due to a problem with the temperature.

Discussion

In the present experimental work we show that diadenosine polyphosphates, which are stored and released from rat brain synaptosomes, can bind to P_2 purinoceptors present in these synaptic terminals. Two radioligands, [3H]-Ap_4A and [35S]-ADP-β-S were used to perform the binding studies.

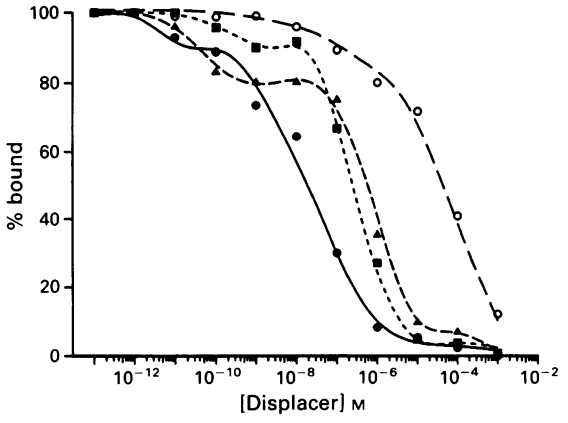


Figure 6 Competition studies of [^{35}S]-ADP-β-S binding by P_2 purinoceptor agonists. Synaptosomes were incubated in the presence of [^{35}S]-ADP-β-S as described in Methods. Synaptosomes in a concentration of 50 μg ml^{-1} were incubated in the presence of ADP-β-S (●), AMP-PNP (▲), α,β-MeATP (■) and 2MeSATP (○). All the experiments represent the mean of four experiments in duplicate.

The binding experiments carried out with [^{3}H]-Ap_4A showed the presence of two binding sites in middle brain synaptic terminals after data analysis. In chromaffin cells where [^{3}H]-Ap_4A binding studies have been done, two binding sites have also been characterized (Pintor *et al.*, 1991b). The high affinity binding site dissociation constant in synaptic terminals (K_d = 0.10 nM) was in the same order as that found in chromaffin cells (K_d = 0.080 nM). The second binding site, obtained from displacement studies, in the synaptosomes had a K_i value close to that described with respect to that of chromaffin cells (K_i = 0.13 μM).

Displacement studies of [^{3}H]-Ap_4A by Ap_4A as well as the ATP analogues showed, at the high affinity binding site, a similar pharmacological profile in rat brain synaptic terminals to that in chromaffin cells: Ap_4A > ADP-β-S > AMP-PNP > α,βMeATP (Figure 3). Furthermore, the inhibition constants obtained from the displacement curves were similar for each compound in both systems. The second binding site also showed a similar displacement profile to that described in chromaffin cells: Ap_4A > ADP-β-S > AMP-PNP > α,β-MeATP. Nevertheless, all the K_i values in middle brain synaptosomes were approximately five times higher than those described in cultured chromaffin cells.

[^{35}S]-ADP-β-S is a good radioligand to characterize P_{2y}-purinoceptors (Cooper *et al.*, 1989). Since diadenosine polyphosphates bind to a putative P_{2y}-purinoceptor in chromaffin cells, it was decided to study further the purinoceptors present in rat brain synaptosomes with this labelled ligand.

Binding studies performed with [^{35}S]-ADP-β-S also showed two binding sites on rat brain synaptosomes. The K_d value for the high affinity binding site was similar to that obtained with [^{3}H]-Ap_4A, both in the nanomolar range. The second binding site, calculated from the displacement studies, showed a K_i value in the same range as described in the literature (Williams, 1987; Cooper *et al.*, 1989; Keppens *et al.*, 1989).

The competition studies carried out with diadenosine polyphosphates, using [^{35}S]-ADP-β-S as radioligand, showed an affinity profile Ap_4A > Ap_5A > Ap_6A for the high affinity binding site. In chromaffin cells at the high affinity binding site the three polyphosphates displace the [^{3}H]-Ap_4A binding with the same potency (Pintor *et al.*, 1991b). In this case there is a clear difference in the displacement potency order which is not surprising with different neural models and different animal species. It is generally assumed that a typical P_{2y} profile shows the following order: 2MeSATP > ADP-β-S > AMP-PNP > α,β-MeATP, and a P_{2x}-purinoceptor profile an order of α,β-MeATP > AMP-PNP > ADP-β-S > 2MeSATP (Burnstock, 1991). When the pharmacological analysis using synthetic analogues is performed, synaptosomal binding sites do not seem to belong either to the P_{2y}, or to the P_{2x}. As 2MeSATP is not the most potent displacer, it is difficult to classify the high affinity binding site as a P_{2y}, in spite of the order of the other ligands (Figure 6). The results obtained with ATP synthetic analogues for the second binding site was different from any P_2 classification. At this site the most potent synthetic displacer was ADP-β-S followed by α,β-MeATP and AMP-PNP. The least potent agonist was 2MeSATP which was two orders of magnitude lower in potency than the other ligands (Figure 6).

The relevance of the P_2-purinoceptors with respect to the high affinity for diadenosine polyphosphates is due to the presence of these compounds and their calcium-dependent release from rat brain synaptic terminals (Pintor *et al.*, 1992a). These dinucleotides have recently been described in catecholaminergic neurones (Pintor *et al.*, 1992c) and in pure cholinergic vesicles from *Torpedo* electric organ (Pintor *et al.*, 1992b). Thus, the presence of P_2-purinoceptors, with high affinity for diadenosine polyphosphates, suggests the possibility of important physiological roles of these compounds in the central nervous system, as reported by Stone & Perkins (1981). They demonstrated that ATP, Ap_3A, and Ap_5A were able to depress the firing of spontaneously active cortical neurones. In cortex slices preloaded with [^{3}H]-adenosine, dinucleoside polyphosphates enhanced the spontaneous efflux of the labelled nucleoside. It is possible that those actions are mediated by the P_2-purinoceptors described in the present study. To support this possibility Tschöpl *et al.* (1992), working with *locus ceruleus* neurones, have demonstrated the presence of a P_2-purinoceptor with an agonist order α,β-MeATP > 2MeSATP, which is in accordance with our second binding site. Although these authors tried to classify the purinoceptor as a P_{2x}, the typical desensitization carried out by α,β-MeATP did not occur. The EC_{50} values obtained in these neurones, in the micromolar range, are similar to the K_i values described in the present work (Table 2).

Actions of ATP and diadenosine polyphosphates have been described in the peripheral nervous system and its innervated tissues. Rat nodose ganglia possess purinoceptors which bind ATP and Ap_xA (Krishtal *et al.*, 1988). These binding sites are coupled to a cation channel which is open to allow low specific inward currents.

In vas deferens and urinary bladder, diadenosine polyphosphates acting through P_{2x}-purinoceptors present the same features as α,β-MeATP (Hoyle, 1990).

Synaptosomes join a heterogeneous population of synaptic terminals. They can exhibit different ecto-enzymes and can store different neurotransmitters in their synaptic vesicles (Richardson *et al.*, 1987). In a pure catecholaminergic model, the chromaffin cell, P_2-purinoceptors with high affinity for diadenosine polyphosphates have been described (Pintor *et al.*, 1991b). In the cholinergic model of *Torpedo* synaptosomes the presence of P_2-purinoceptors has been demonstrated which also bind Ap_xA compounds with K_d values very similar to those described in the present work (Pintor & Miras-Portugal, 1993). The possible existence of these purinoceptors in other neurones, such as GABAergic or glutamatergic, ought to be taken into consideration. With our experimental model, it is not possible to know whether P_2-purinoceptors are present or not in all synaptic terminals. Moreover, Inoue *et al.* (1992), have described the modulation of glutamate release by ATP in cultured hippocampal neurones. These findings support the hypothesis of a wide P_2-purinoceptor distribution.

Our findings demonstrate that diadenosine polyphosphates can bind to synaptosomal P_2 purinoceptors. The pharmacological profiles with respect to the Ap_4A and ADP-β-S displacement studies do not agree with any specific P_2-purinoceptor subtype. These observations may relate to those made by Hoyle (1990), who demonstrated different phar-

macological properties of diadenosine polyphosphates in purinergically innervated tissues, suggesting the possible existence of a purinoceptor highly specific for adenine dinucleotides for which we suggest the designation, P_{2d}.

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References

BURNSTOCK, G. (1972). Purinergic nerves. *Pharmacol. Rev.*, **24**, 509–581.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptors. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*. ed. Staub, R.W. & Bolis, L. pp. 107–118. New York, NY: Raven Press.

BURNSTOCK, G. (1991). Distribution and roles of purinoceptors subtypes. *Nucleos. Nucleot.*, **10**, 917–930.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2 purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CASADO, V., MARTI, T., FRANCO, R., LLUIS, C., MALLOL, J. & CANELA, E.I. (1990). A method for binding parameters estimation of A_1 adenosine receptor subtype: a practical approach. *Anal. Biochem.*, **184**, 117–123.

CASTRO, E., PINTOR, J. & MIRAS-PORTUGAL, M.T. (1992). Ca^{2+} stores mobilization by diadenosine tetraphosphate, Ap_4A, through a putative P_{2y} purinoceptor in adrenal chromaffin cells. *Br. J. Pharmacol.*, **106**, 833–837.

CASTRO, E., TORRES, M., MIRAS-PORTUGAL, M.T. & GONZALEZ, M.P. (1990). Effect of diadenosine polyphosphates on catecholamine secretion from isolated chromaffin cells. *Br. J. Pharmacol.*, **100**, 360–364.

COCKCROFT, S. & GOMPERTS, B.A. (1980). The ATP^{4-} receptor of rat mast cells. *Biochem. J.*, **188**, 789–798.

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.*, **264**, 6202–6206.

CUSACK, N. & HOURANI, S.M.O. (1982). Adenosine 5'-diphosphate antagonists and human platelets: no evidence that aggregation and inhibition of stimulated adenylate cyclase are mediated by different receptors. *Br. J. Pharmacol.*, **76**, 221–227.

FLODGAARD, H. & KLENOW, H. (1982). Abundant amounts of diadenosine 5',5'''-p^1,p^4-tetraphosphate are present and releasable, but metabolically inactive in human platelets. *Biochem. J.*, **208**, 737–742.

GOLDSTEIN, R.F. & LEUNG, E. (1990). EQUIL: simulation and data analysis of binding reactions with arbitrary chemical models. *Anal. Biochem.*, **190**, 220–232.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

HOYLE, C.H.V. (1990). Pharmacological activity of adenine dinucleotides in the periphery. Possible receptor classes and transmitter function. *Gen. Pharmacol.*, **21**, 827–831.

HOYLE, C.H.V., CHAPPLE, C. & BURNSTOCK, G. (1989). Isolated human bladder: evidence for an adenine dinucleotide acting on P_{2x}-purinoceptors and for purinergic transmission. *Eur. J. Pharmacol.*, **174**, 115–118.

INOUE, K., NAKAZAWA, K., FUJIMORI, K., WATANO, T. & TAKANAKA, A. (1992). Extracellular adenosine 5'-triphosphate-evoked glutamate release in cultured hippocampal neurons. *Neurosci. Lett.*, **134**, 215–218.

KEPENS, S., VANDEKERCKHOVE, A. & DE WULF, H. (1989). Characterization of purinoceptors present on human liver plasma membranes. *FEBS Lett.*, **248**, 137–140.

KRISHTAL, O.A., MARCHENKO, S.M., OBUKHOV, A.G. & VOLKOVA, T.M. (1988). Receptors for ATP in rat sensory neurones: the structure-function relationship for ligands. *Br. J. Pharmacol.*, **95**, 1057–1062.

LUTHJE, J. & OGILVIE, A. (1983). Presence of diadenosine 5',5'''-p^1,p^4-triphosphate (Ap_3A) in human platelets. *Biochem. Biophys. Res. Commun.*, **115**, 253–260.

MACKENZIE, I., KIRKPATRIC, K.A. & BURNSTOCK, G. (1988). Comparative study of the actions of Ap_5A and α,β-CH_2-ATP on nonadrenergic, noncholinergic neurogenic excitation in the guinea-pig vas deferens. *Br. J. Pharmacol.*, **94**, 699–706.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107**, 220–239.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonists studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

PINTOR, J., DIAZ-REY, M.A., TORRES, M. & MIRAS-PORTUGAL, M.T. (1992a). Presence of diadenosine polyphosphates – Ap_4A and Ap_5A – in rat brain synaptic terminals. Ca^{2+} dependent release evoked by 4-aminopyridine and veratridine. *Neurosci. Lett.*, **136**, 141–144.

PINTOR, J., KOWALEWSKI, H.J., TORRES, M., MIRAS-PORTUGAL, M.T. & ZIMMERMANN, H. (1992b). Synaptic vesicle storage of diadenosine polyphosphates in the Torpedo electric organ. *Neurosci. Res. Commun.*, **10**, 9–14.

PINTOR, J. & MIRAS-PORTUGAL, M.T. (1993). Diadenosine polyphosphates – Ap_xA – as new neurotransmitters. *Drug Develop. Res.*, (in press).

PINTOR, J., ROTLLAN, P., TORRES, M. & MIRAS-PORTUGAL, M.T. (1992c). Characterization and quantification of diadenosine hexaphosphate in chromaffin cells: granular storage and secretagogue-induced release. *Anal. Biochem.*, **200**, 296–300.

PINTOR, J., TORRES, M. & MIRAS-PORTUGAL, M.T. (1991a). Carbachol induced release of diadenosine polyphosphates – Ap_4A and Ap_5A – from perfused bovine adrenal medulla and isolated chromaffin cells. *Life Sci.*, **48**, 2317–2324.

PINTOR, J., TORRES, M., CASTRO, E. & MIRAS-PORTUGAL, M.T. (1991b). Characterization of diadenosine tetraphosphate (Ap_4A) binding sites in cultured chromaffin cells: evidence for a P_{2y} site. *Br. J. Pharmacol.*, **103**, 1980–1984.

RICHARDSON, P.J., BROWN, S.J., BAILYES, E.M. & LUZIO, J.P. (1987). Ectoenzymes control adenosine modulation of immuno-isolated cholinergic synapses. *Nature*, **327**, 232–234.

RODRIGUEZ DEL CASTILLO, A., TORRES, M., DELICADO, E.G. & MIRAS-PORTUGAL, M.T. (1988). Subcellular distribution studies of diadenosine polyphosphates – Ap_4A and Ap_5A – in bovine adrenal medulla: presence in chromaffin granules. *J. Neurochem.*, **51**, 1696–1703.

STEINBERG, T.H., NEWMAN, A.S., SWANSON, J.A. & SILVERSTEIN, S.C. (1987). ATP^{4-} permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J. Biol. Chem.*, **262**, 8884–8888.

STONE, T.W. & PERKINS, M.N. (1981). Adenine dinucleotide effect on rat cortical neurones. *Brain Res.*, **229**, 241–245.

TSCHOPL, M., HARMS, L., NORENBERG, W. & ILLES, P. (1992). Excitatory effects of adenosine 5'-triphosphate on rat locus ceruleus neurones. *Eur. J. Pharmacol.*, **213**, 71–77.

WILLIAMS, M. (1987). Purine receptors in mammalian tissues: pharmacology and functional significance. *Annu. Rev. Pharmacol. Toxicol.*, **27**, 315–345.

ZAMECNIK, P.C., KIM, B., GAO, M.J., TAYLOR, G. & BLACKBURN, M.G. (1992). Analogues of diadenosine 5',5'''-p^1,p^4-tetraphosphate (Ap_4A) as potential anti-platelet-aggregation agents. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 2370–2373.

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Inhibition by recombinant SLPI and half-SLPI (Asn^{55}-Ala^{107}) of elastase and cathepsin G activities: consequence for neutrophil-platelet cooperation

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1 The capacity of recombinant human secretory leukocyte proteinase inhibitor (SLPI) to inhibit human leukocyte elastase (HLE) and cathespin G (Cat G) was investigated and compared with a recombinant truncated form (carboxyl-terminal domain, Asn^{55}-Ala^{107}) called 1/2 SLPI.

2 Both compounds were efficient when tested against enzymatic activities of purified HLE and Cat G indicating that the HLE- and Cat G-inhibitory sites were preserved in the truncated form. SLPI and 1/2 SLPI also affected platelet activation induced by 0.2 μM Cat G ($IC_{50} = 112 \pm 13$ nM for SLPI and 280 ± 12 nM for 1/2 SLPI).

3 The effects of SLPI and 1/2 SLPI were then tested against polymorphonuclear neutrophil (PMN)-mediated platelet activation, a cell-to-cell interaction mediated by HLE and Cat G released from PMN. In this experimental system, addition of SLPI or 1/2 SLPI before N-formyl-Met-Leu-Phe (fMLP) led to the inhibition of the resulting platelet activation. As was the case for Cat G enzymatic activity and Cat G-induced platelet activation, SLPI was more efficient than 1/2 SLPI ($IC_{50} = 676 \pm 69$ nM *vs* 1121 ± 150 nM).

4 The ratio of the IC_{50} against PMN-mediated platelet activation compared to purified Cat G-mediated platelet activation was 6.03 for SLPI and 4.32 for 1/2 SLPI. This difference may be due to the smaller size of the truncated form which could allow this molecule to diffuse more easily between PMN and platelets.

5 In conclusion, 1/2 SLPI could be a promising candidate in the treatment of pathological states linked to inflammation in which participation of HLE and Cat G has been evoked.

Keywords: Leukocytes; platelets; cathepsin G; elastase; secretory leukocyte proteinase inhibitor (SLPI); 1/2 SLPI

Introduction

Elastase (HLE) and cathesin G (Cat G) are two major neutral serine proteinases present in human polymorphonuclear neutrophils (PMN) (Henson & Johnston, 1987). While studies on the role of proteinases in pulmonary diseases have focused on HLE (Senior *et al.*, 1977; Janoff *et al.*, 1977; Snider *et al.*, 1984), the possible participation of Cat G has also to be considered. Indeed, although Cat G is weakly elastinolytic, it enhances the activity of HLE (Boudier *et al.*, 1981) and degrades cartilage matrix proteoglycans (Janusz & Doherty, 1991). Another recently described interesting property of this serine proteinase is its role as a mediator between PMN and platelets. Thus, when PMN are stimulated by N-formyl-Met-Leu-Phe (fMLP) or C5a, they release an array of substances among which is Cat G, responsible in turn for the activation of surrounding platelets (Chignard *et al.*, 1986; Selak *et al.*, 1988; Del Maschio *et al.*, 1990; Ferrer-Lopez *et al.*, 1990; Renesto *et al.*, 1990) via an interaction with a specific receptor (Selak & Smith, 1990). In fact, HLE which fails to activate platelets by itself (Renesto *et al.*, 1990), also plays a role in this system by potentiating the platelet activating activity of Cat G (Renesto & Chignard, unpublished). The physiological relevance of such a cell-to-cell cooperation is not yet defined. However, PMN and platelets have been described as potential contributors in some pulmonary diseases and more particularly in the adult respiratory distress syndrome (ARDS) (Warshawski *et al.*, 1986; Heffner *et al.*, 1987). Thus, a communication between these two cell populations, and consequently Cat G and HLE may play an important role in such an inflammatory state.

An imbalance between the PMN proteinase HLE and its plasma inhibitor, α_1-proteinase inhibitor, is a possible mechanism at the origin of destructive lung diseases (Cohen, 1983; Janoff, 1985). The same reasoning possibly holds true for Cat G because α_1-proteinase inhibitor is also a physiological inhibitor of this proteinase (Beatty *et al.*, 1980). Thus, administration of exogenous anti-proteinases has been proposed as a therapeutic weapon to arrest or delay tissue destruction (Crystal *et al.*, 1989). Another promising candidate for the treatment of diseases in which HLE and Cat G could be involved is the secretory leukocyte proteinase inhibitor (SLPI) secreted by clara and goblet cells within the respiratory tract (Mooren *et al.*, 1983; Puchelle *et al.*, 1985). Indeed, this molecule exhibits a high affinity for HLE and Cat G (Thompson & Olhsson, 1986; Boudier & Bieth, 1989). A recombinant form of SLPI has been constructed by genetic engineering, together with a recombinant truncated form (Masuda *et al.*, 1992), here called 1/2 SLPI and corresponding to the C-terminal domain (Asn^{55}-Ala^{107}) of the full length molecule. In this paper, we first compared their respective capacities for inhibiting HLE and Cat G enzymatic activities. Then, we tested their inhibitory potencies in the PMN-mediated platelet activation system. The aim was to compare two molecules of different mass (mol.wt 11.7 and 5 kDa for the full length and truncated forms, respectively) under biological conditions in which cell-to-cell contact exists and where HLE and Cat G are both involved.

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Methods

Production of recombinant SLPI and truncated SLPI

The two recombinant forms of SLPI were produced by Teijin Limited, Tokyo, Japan. Briefly, for the truncated form, DNA fragments encoding the carboxy-terminal domain (Asn^{55}-Ala^{107}) of SLPI were chemically synthesized using favourable codons in *E. coli*. Human growth hormone gene was fused to the (Asn^{55}-Ala^{107}) SLPI gene via a DNA sequence encoding Leu-Val-Pro-Arg cleavable by thrombin. The expression vector was constructed and introduced into *E. coli* HB 101. Transformed cells were cultured followed by collection of inclusion bodies, thrombin treatment, S-S refolding and purification by chromatography (Masuda *et al.*, 1992).

Purification of cathepsin G and human leukocyte elastase

Cat G and HLE were obtained from human neutrophils by the method of Baugh & Travis (1976) modified by Martodam *et al.* (1979). Briefly, cells were washed thrice and erythrocytes were removed by hypotonic shock. Granules containing Cat G and also HLE, were extracted by breaking PMN with a Potter-Elvejhem glass homogenizer. Nuclei and unbroken cells were sedimented at 1,000*g* for 15 min at 4°C and re-extracted twice. Granules were sedimented at 30,000 *g* for 30 min and were then broken with an Ultra-Turrax homogenizer followed by centrifugation at 1,000 *g* for 15 min. The Ultra-Turrax procedure was repeated twice. Supernatants were pooled and centrifuged at 30,000 *g* for 35 min at 4°C. The resulting supernatant, containing haemoglobin, myeloperoxidase, HLE and Cat G, was applied to an aprotinin-sepharose column. HLE and Cat G were both desorbed from the affinity chromatography column by elution with 0.05 M sodium acetate, 1 M NaCl, pH 5.5. After concentration and dialysis, the mixture was applied onto a CM-Trisacryl M column and the elution was carried out with a first buffer (0.05 M sodium acetate, 0.45 M NaCl, pH 5.5) followed by a second one with higher ionic strength (0.05 M sodium acetate, 1 M NaCl, pH 5.5), in order to elute HLE and Cat G, respectively. The elution profile was followed by spectrophotometric measurement at 280 nm. The first peak corresponded to HLE, and the second to Cat G. Fractions of each peak were pooled separately, and after concentration and dialysis, aliquots were stored at − 80°C.

The enzymatic activity of Cat G and HLE were determined spectrophotometrically according to Nakajima *et al.* (1979) using N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and N-succinyl-Ala-Ala-Ala-*p*-nitroanilide as specific substrates, respectively. For the determination of their active site concentration, a fixed concentration of the enzymes was reacted with increasing amounts of active site titrated α_1-proteinase inhibitor in 495 µl of 0.1 M Tris/HCl buffer, pH 8, containing 0.1% bovine serum albumin (BSA) in order to minimize adsorption of the enzymes on the test tube surface. After 1 min incubation at 37°C, residual enzyme activities were measured by addition of 5 µl of a 100 mM solution of the specific synthetic substrate in N-methylpyrrolidone. Hydrolysis of the substrate (1 mM final concentration) was monitored every 20 s during 5 min by following the release of *p*-nitroaniline at 410 nm. Proteinase active site concentrations were evaluated by extrapolation of the obtained inhibition curve. The molarities indicated in this article refer to concentrations of active enzymes (100% specific activity). The purified batches of HLE and Cat G which were devoid of contamination by Cat G and HLE, respectively (as determined by enzymatic activity) migrated on PAGE (Phast Gel Gradient 10-15, Phast System Separation, Pharmacia, Sweden) with an apparent molecular mass of 28 kDa.

Preparation of human purified neutrophils

Cells were obtained from blood of human volunteers collected in SAG-M solution for preservation of red blood cells (composition, mM: NaCl 187.6, dextrose 71, adenine 1950, mannitol 10.7, pH 5.1). Briefly, blood was mixed with dextran 3% in saline (2:1), and 30 min later the supernatant was centrifuged (350 *g*; 45 min) through a Ficoll-Paque gradient. The resulting pellet was resuspended in a lysis solution (composition, mM: NH_4Cl 155, $KHCO_3$ 2.96, EDTA $(Na)_2$ 3.72) and gently agitated for 5 min in order to remove remaining erythrocytes. The cell suspension was then centrifuged (350 *g*; 10 min) and PMN were washed twice in HBSS. The last pellet was resuspended in a volume of HBSS such that the cell concentration was 10^7 PMN ml^{-1}.

Cell viability determined by the blue Trypan dye exclusion method was 97 ± 2% and purity, determined with Türk's solution, was 95 ± 3% ($n = 9$).

Preparation of human washed platelets

Blood was collected as for PMN preparation. Platelet rich plasma obtained by a first centrifugation (180 *g*; 20 min) was incubated at 37°C for 30 min with [^{14}C]-5-HT at 5×10^{-2} µCi ml^{-1} (platelets were kept at this temperature throughout the preparation). Loaded platelet suspension was then centrifuged (1,600 *g*; 10 min) and the pellet was resuspended in modified Tyrode buffer (composition, mM: NaCl 137.0, KCl 2.68, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, $CaCl_2$ 2.0, $MgCl_2$ 1.0, glucose 5.5, HEPES 5.0, BSA 0.35%, pH 7.4) supplemented with heparin (50 iu ml^{-1}) and prostacyclin (10^{-8} M). Cells were washed a second time (1,600 *g*; 10 min) with the same mixture without heparin and the final pellet was resuspended in a volume of Tyrode buffer such that the final platelet concentration was 4×10^8 cells ml^{-1}.

Measurement of platelet aggregation and 5-hydroxytryptamine release

Experiments were performed using a Dual Aggro-meter (Chrono-Log-Corp., Hevertown, Pennsylvania, U.S.A.). Platelets (250 µl) and PMN (250 µl) were placed in siliconized glass test tubes with fibrinogen and cytochalasin B (0.7 mg ml^{-1} and 5 µg ml^{-1} final concentrations, respectively) under stirring (900 r.p.m.) at 37°C ($CaCl_2$ and $MgCl_2$ concentrations were adjusted to 1.0 and 1.3 mM, respectively before each experiment). Five minutes later, cells were stimulated with fMLP in the presence of various concentrations of SLPI or 1/2 SLPI (added 1 min before fMLP) and the resulting aggregation was evaluated by measuring changes in light transmission. The reaction was stopped at $t = 3$ min by addition of a stopping solution (composition, mM: EDTA 38.5, NaCl 68.88 and formaldehyde 2.05%) followed by a centrifugation (14,000 *g*; 2 min). Release of 5-HT was measured by scintillation counting from 400 µl of corresponding supernatants mixed with ACS II (Counter 1212 Rackbeta, LKB, Wallac, Stockholm, Sweden).

When platelets were studied alone, PMN were replaced by an equal volume of HBSS. Cells were preincubated for 1 min at 37°C in presence of fibrinogen and various concentrations of inhibitors before stimulation by purified Cat G. Samples were then processed as described above.

Aggregation was expressed as the percentage change in light transmission and 5-HT release as a percentage of the total [^{14}C]-5-HT content of platelets.

Enzymatic activity determination

Enzymatic activities of proteinases were determined spectrophotometrically following hydrolysis of their specific synthetic substrates. Experimental conditions corresponded to the protocol for which platelets were used as 'substrate'. Thus, increasing concentrations of SLPI or 1/2 SLPI and substrate (1 mM final concentration) were incubated for 1 min at 37°C in Tyrode/HBSS buffer (1:1) and the enzymatic reaction was initiated by addition of purified HLE or Cat G (200 nM). Hydrolysis of substrates was monitored

as decribed above. Results were expressed as the percentage of enzymatic activity present in controls without inhibitor.

β-Glucuronidase release determination

PMN (5×10^6 cells ml^{-1}) in HBSS/Tyrode (1:1) were incubated for 4 min with cytochalasin B (5 μg ml^{-1}) at 37°C. Various concentrations of SLPI or 1/2 SLPI were added, and 1 min later cells were challenged with 5×10^{-7} M fMLP. Reactions were stopped at $t = 5$ min by placing test tubes at 0°C in ice. After centrifugation (2,500 g; 15 min; 4°C), supernatants containing the released enzyme were stored at − 20°C and pellets were resuspended in 0.2% Triton X-100 and incubated overnight at 4°C in order to lyse PMN and to extract the remaining enzyme. Samples were then centrifuged and the corresponding supernatants were stored at − 20°C. The amount of β-glucuronidase was determined spectrophotometrically with 4-nitrophenyl-β-D-glucopyranosiduronic acid as substrate. Results were expressed as percentage of release compared to the total enzyme content.

Materials

Blood was obtained from the Centre National de Transfusion Sanguine, Paris, France. Ficoll-Paque was purchased from Pharmacia, Uppsala, Sweden. Aqueous Counting Scintillant (ACS II) and [^{14}C]-5-hydroxytryptamine (5-HT) were from Amersham International, Amersham, Buckinghamshire. Heparin was from Choay, Paris, France. FMLP, cytochalasin B, dextran, N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, N-succinyl-Ala-Ala-Ala-*p*-nitroanilide, prostacyclin, aprotinin, Tris and HEPES were obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A. CM-Trisacryl M was from IBF Biotechnics, Villeneuve-la-Garenne, France and HBSS without calcium, magnesium and phenol red from Gibco, Paisley, Scotland. 4-β-D-glucopyranosiduronic acid was from Merck, Darmstadt, Germany. BSA was purchased from Euromedex, Strasbourg, France. Human fibrinogen (Grade L) from Kabi, Stockholm, Sweden, was treated with diisopropyl fluorophosphate in order to remove the activity of coagulant contaminants. Active site-titrated α_1-proteinase inhibitor was kindly donated by Drs C. Boudier and J.G. Bieth (INSERM U237, Strasbourg, France).

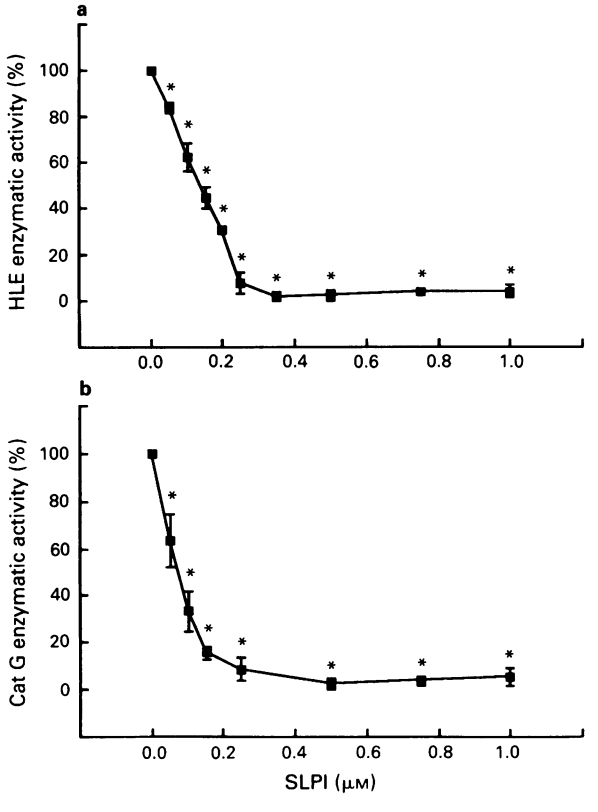


Figure 1 Inhibition by SLPI of human leukocyte elastase (HLE) and cathepsin G (Cat G) enzymatic activities. Increasing concentrations of SLPI were incubated for 1 min at 37°C in Tyrode/HBSS and in presence of 1 mM of substrate before addition of (a) 0.2 μM HLE or (b) Cat G. Enzymatic activities were measured by following hydrolysis of their specific substrate. Results are expressed as percentage of activity compared with control without inhibitor. Each point is the mean ± s.d. of 3 distinct experiments.

Statistical analysis

Results are expressed as mean ± s.d. of at least 3 distinct experiments. The data were analysed by Student's t test for unpaired samples to determine whether differences were statistically significant. In figures, asterisks indicate significance of differences corresponding to $P < 0.05$.

Results

Inhibition of the enzymatic activity of human leukocyte elastase and cathepsin G

We first evaluated the capacity of SLPI to inhibit the enzymatic activities of purified HLE and Cat G by incubating increasing concentrations with 0.2 μM of each serine proteinase. Results presented in Figure 1 show that HLE (1a) and Cat G (1b) were significantly inhibited in a concentration-dependent manner by SLPI above 0.05 μM ($P < 0.05$). Under our experimental conditions, SLPI was more efficient against Cat G than against HLE, the concentrations necessary to induce 50% inhibition being 77 ± 14 nM and 137 ± 3 nM ($n = 3$), respectively (Table 1). We then tested the capability of the truncated form of SLPI to affect these enzymatic activities using the same procedure (Figure 2). An inhibitory activity of SLPI against both proteinases was observed in the truncated form. Indeed, while 1/2 SLPI was less active than SLPI, concentrations necessary to obtain 50% inhibition were 130 ± 12 nM for Cat G ($n = 5$) and 305 ± 66 nM for HLE ($n = 4$) (Table 1).

Effects of SLPI and 1/2 SLPI on platelet aggregation

Since both molecules exhibited the property of inhibiting the enzymatic activity of Cat G, we investigated their effects against platelet activation induced by a submaximal concen-

Table 1 Comparison of the inhibitory effects of secretory leukocyte proteinase inhibitor (SLPI) and 1/2 SLPI in different experimental conditions in terms of IC_{50}

		SLPI (μM)	*1/2 SLPI* (μM)
1	HLE + substrate	0.137 ± 0.003	0.305 ± 0.066
2	Cat G + substrate	0.077 ± 0.014	0.130 ± 0.012
3	Cat G + platelets	0.112 ± 0.013	0.280 ± 0.120
4	fMLP + PMN + platelets	0.676 ± 0.069	1.121 ± 0.150
5	Ratio 4/3	6.03	4.32

1 and 2: Inhibitors were tested against the capability of the enzymes to hydrolyze their specific synthetic substrates (see original data in Figures 1 and 2). 3 and 4: Platelet activation triggered by Cat G (3) or activated PMN (4) were evaluated by measuring 5-HT release (see original data in Figures 3 to 6). The IC_{50} values (concentrations inhibiting 50% of the control responses) were interpolated from each single experiment. Data are mean ± s.d. of 3 to 7 distinct experiments.
For abbreviations, see text.

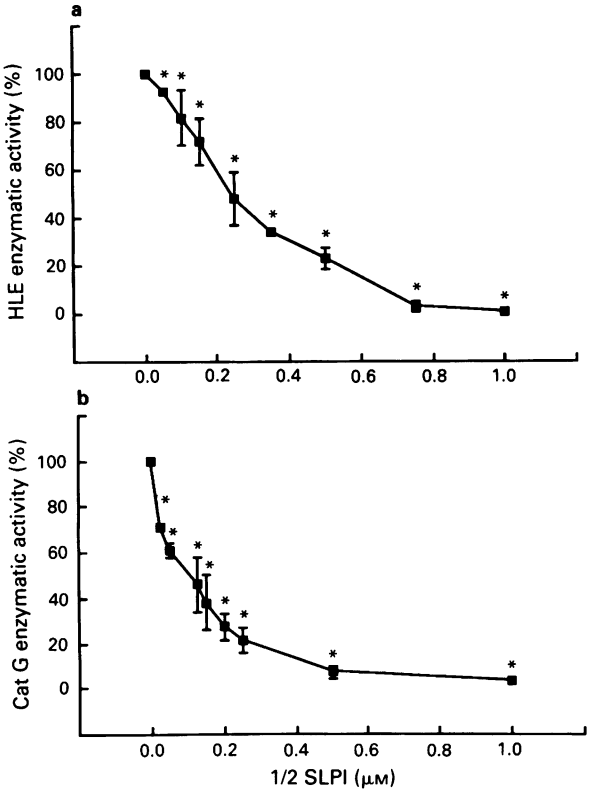


Figure 2 Inhibition by 1/2 SLPI of human leukocyte elastase (HLE) and cathepsin G (Cat G) enzymatic activities. For details see legend to Figure 1, SLPI being replaced by 1/2 SLPI.

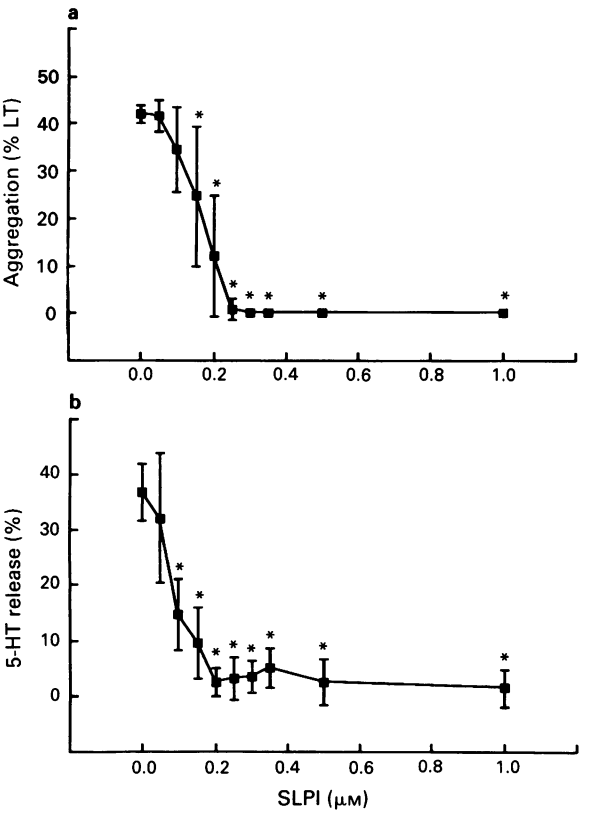


Figure 3 Inhibition by SLPI of platelet activation induced by purified cathepsin G (Cat G). Platelets (2×10^8 cells ml^{-1}) were incubated for 1 min at 37°C under stirring with increasing concentrations of SLPI before their stimulation by 0.2 μM of purified Cat G. Resulting aggregations (a) were measured as percentage change in light transmission (LT) and 5-hydroxytryptamine (5-HT) release (b) as a percentage of the total 5-HT content. Each point is the mean ± s.d. of 5 to 7 distinct experiments.

tration of this serine proteinase (0.2 μM). SLPI effectively inhibited platelet aggregation (Figure 3a) and 5-HT release (Figure 3b), the active concentrations ranging from 0.15–1 μM and from 0.1–1 μM, respectively. As observed for the enzymatic activity, the same effect was obtained with 1/2 SLPI which induced a significant inhibition of platelet activation above 0.2 μM for aggregation (Figure 4a) and 0.1 μM for 5-HT release (Figure 4b). This inhibition was specific because 2 μM SLPI as well as 2 μM 1/2 SLPI failed to affect platelet aggregation induced by 0.5 μM of the endoperoxide analogue U-46619 (49.1 ± 9.8% for control *vs* 51.1 ± 8.4% with SLPI and 47.3 ± 2.1% with 1/2 SLPI; $n = 3$) or by 2 μM of platelet-activating factor (22.6 ± 13.1% for control *vs* 26.6 ± 14.5% with SLPI and 22.8 ± 7.1% with 1/2 SLPI; $n = 3$).

Inhibition by SLPI and 1/2 SLPI of the PMN-mediated platelet activation

Having demonstrated the capacity of SLPI and 1/2 SLPI to inhibit enzymatic and biological activities of Cat G, we tested their effects in an experimental model in which PMN and platelets were mixed. In this system, stimulation of PMN with fMLP results in a strong platelet activation, the responsible mediators being Cat G and HLE (Renesto & Chignard, unpublished). As shown in Figure 5, addition of increasing concentrations of SLPI in the test tube 1 min before the challenge with 0.5 μM fMLP, resulted in a concentration-dependent inhibition of platelet activation. The smallest concentrations able significantly to inhibit platelet aggregation (Figure 5a) and 5-HT release (Figure 5b) were 1 and 0.75 μM, respectively ($P < 0.05$). Interestingly, in this series of experiments, the same profile of inhibition was obtained with 1/2 SLPI which significantly inhibited platelet aggregation above 1 μM (Figure 6a) and 5-HT release above 0.75 μM (Figure 6b). These effects were the consequence of the interaction between proteinases and inhibitors and were not due to an indirect effect on PMN. Indeed, the release of β-glucuronidase from fMLP-activated PMN was identical irrespective of whether cells were pretreated with 2 μM of these anti-proteinases (40.1 ± 2.8% for control *vs* 44.1 ± 0.4% with SLPI and 39.8 ± 3.8% with 1/2 SLPI; $n = 3$).

Discussion

In this paper we have confirmed that SLPI is a potent inhibitor of HLE and Cat G enzymatic activities. Indeed, it has been reported that the association rate constants (K_{on}) for HLE and Cat G were 1.06 ± 10^5 and 2.48 ± 10^4 M^{-1} s^{-1}, respectively (Smith & Johnson, 1985) and that the dissociation constants (K_d) of the SLPI-HLE complex and the SLPI-Cat G complex were 0.2 ± 1 nM and 5 ± 2 nM, respectively (Thompson & Olhsson, 1986). We found that recombinant SLPI inhibited HLE and Cat G enzymatic activities (both enzymes used at 0.2 μM) with an IC_{50} of 137 ± 3 nM ($n = 3$) and 77 ± 14 nM ($n = 3$), respectively ($P < 0.05$). This reverse order of potency may be due to a phenomenon recently observed by Boudier & Bieth (1992). They demonstrated that SLPI is capable of inhibiting Cat G with a enzyme:inhibitor molar ratio of 1:1 or 2:1, depending on the use of low (nM range) or high (mM range) concentrations of the enzyme, respectively. They reported that previous enzyme titrations performed with low concentrations such as those mentioned above could not unambiguously detect the 2:1 binding. By contrast we have used 0.2 μM of Cat G, an intermediate concentration, which may have favoured the formation of ternary complexes along with binary complexes. The presence

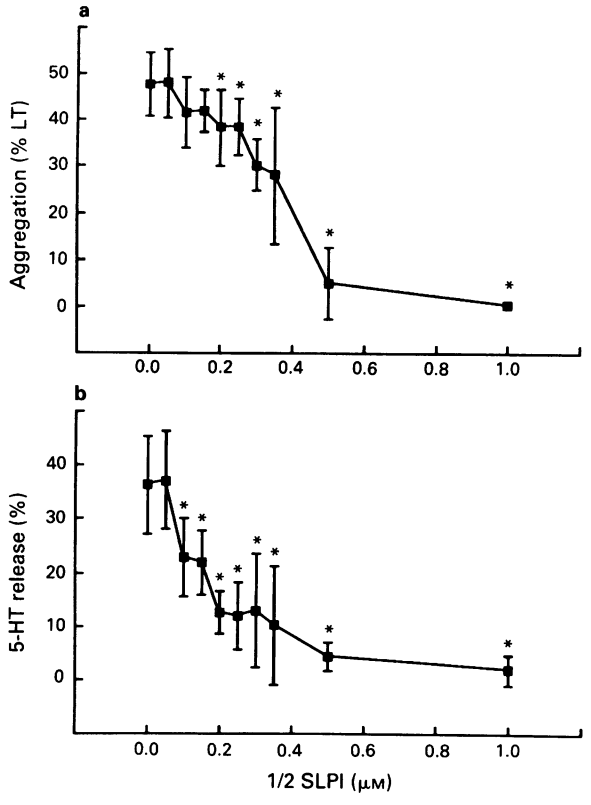


Figure 4 Inhibition by 1/2 SLPI of platelet activation induced by purified cathepsin G (Cat G). For details see legend to Figure 3, SLPI being replaced by 1/2 SLPI.

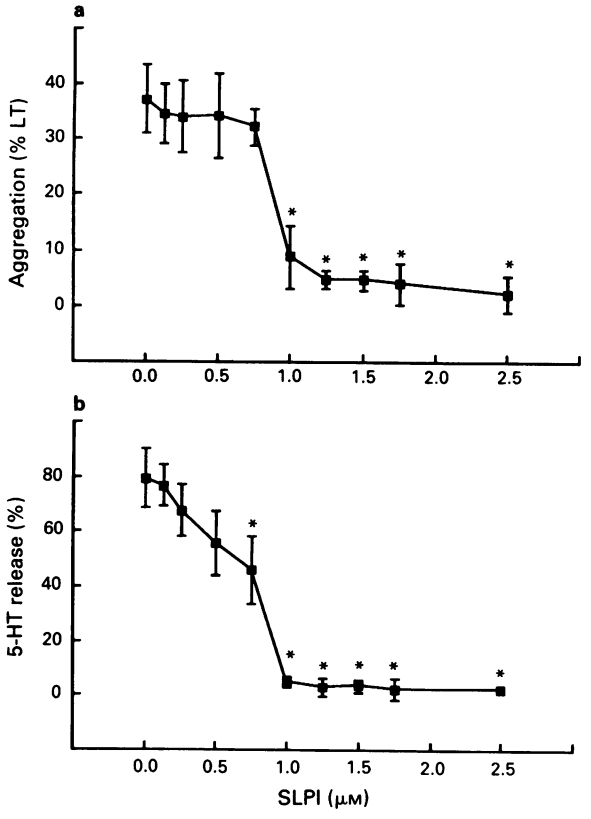


Figure 5 Inhibition by SLPI of platelet activation induced by N-formyl-Met-Leu-Phe (fMLP)-stimulated polymorphonuclear neutrophils (PMN). PMN (5×10^6 cells ml^{-1}) and platelets (2×10^8 cells ml^{-1}) were incubated together for 4 min at 37°C with stirring. Increasing concentrations of SLPI were then added and 1 min later cells were stimulated with 0.5 μM fMLP. Parameters of platelet activation, i.e. aggregation and 5-HT release are expressed as in Figure 3. Each point is the mean ± s.d. of 5 distinct experiments.

of these ternary complexes could then explain the relatively higher potency of SLPI towards Cat G compared to HLE because for the latter the molar ratio is always 1:1, independent of the concentration of the enzyme.

Interestingly, we observed that the truncated form, Asn^{55}-Ala^{107}, shared the same profiles of activity as the full length molecule, although being less potent with IC_{50} values of 305 ± 66 nM ($n = 4$) and 130 ± 12 nM ($n = 5$) for HLE and Cat G, respectively. Here again, Cat G enzymatic activity was more susceptible to inhibition than HLE enzymatic activity ($P < 0.05$). This inhibition of both proteinases is in agreement with the fact that the carboxyl-terminal domain of SLPI contains the HLE and Cat G inhibitory site (Thompson & Olhsson, 1986; Eisenberg *et al.*, 1990) and proves that the site was still operative in the truncated recombinant form, although to a lesser extent.

Due to these properties, SLPI and 1/2 SLPI also inhibited the biological activity of purified Cat G, i.e. its capacity to activate human platelets, the minimal concentration necessary to induce a significant inhibition and the IC_{50} being a little higher for 1/2 SLPI than for SLPI. These results are comparable to those obtained with other proteinase inhibitors, such as α_1-antichymotrypsin (Ferrer-Lopez *et al.*, 1990) and eglin C (Renesto *et al.*,1990). Indeed, α_1-antichymotrypsin and eglin C which present a high degree of specificity for Cat G with an association rate constant (K_{on}) of $5.1 \times 10^7\ M^{-1}s^{-1}$ and $2 \times 10^6\ M^{-1}s^{-1}$, respectively (Beatty *et al.*, 1980), were able to inhibit enzymatic and biological activities of the purified serine proteinase. In fact, the concentrations of SLPI and 1/2 SLPI necessary to inhibit platelet activation in terms of 5-HT release induced by 0.2 μM of Cat G, although close, were not strictly comparable to those efficient against the enzymatic activity. Thus, the IC_{50} (in nM) were 77 ± 14 ($n = 3$) and 112 ± 13 ($n = 5$) for SLPI and 130 ± 12 ($n = 5$) and 280 ± 120 ($n = 7$) for 1/2 SLPI for enzymatic and biological activities, respectively. It can be hypothesized that, when platelets are present, the inhibitors become bound to cell membranes and thus the amount available to interact with Cat G is lowered.

Finally, and as expected, SLPI and 1/2 SLPI inhibited PMN-induced platelet activation. This preventive effect was related to an inhibition of Cat G and HLE, the mediators responsible for this cell-to-cell cooperation (Renesto & Chignard, unpublished). However, we verified the specificity of the reaction. Thus, neither PMN nor platelets were directly affected, as assessed by measurements of β-glucuronidase release and platelet activation induced by other platelet agonists evaluated in presence of these inhibitors. Efficient concentrations in this experimental model were higher than those active against platelets used alone and activated by purified Cat G. As observed in Table 1, a six fold higher concentration of SLPI was needed to inhibit by 50% the control responses. A similar shift has been reported for eglin C (Renesto *et al.*, 1990). To explain this result, several hypotheses can be put forward: (i) the concentration of Cat G released from fMLP-activated PMN is not 200 nM but 240 nM (Renesto & Chignard, unpublished); (ii) not only Cat G, but also HLE which is released from activated PMN, interferes with SLPI and 1/2 SLPI as observed in the present study; (iii) SLPI and 1/2 SLPI could be partially inactivated by oxidative substances released from PMN (Smith *et al.*, 1987; Gervaix *et al.*, 1992); (iv) cell-to-cell contact could prevent the access of inhibitors to endogenously released proteinases.

Considering the last hypothesis (partial exclusion of inhibitors), an interesting observation can be made. In the PMN-platelet cooperation system, the concentrations of

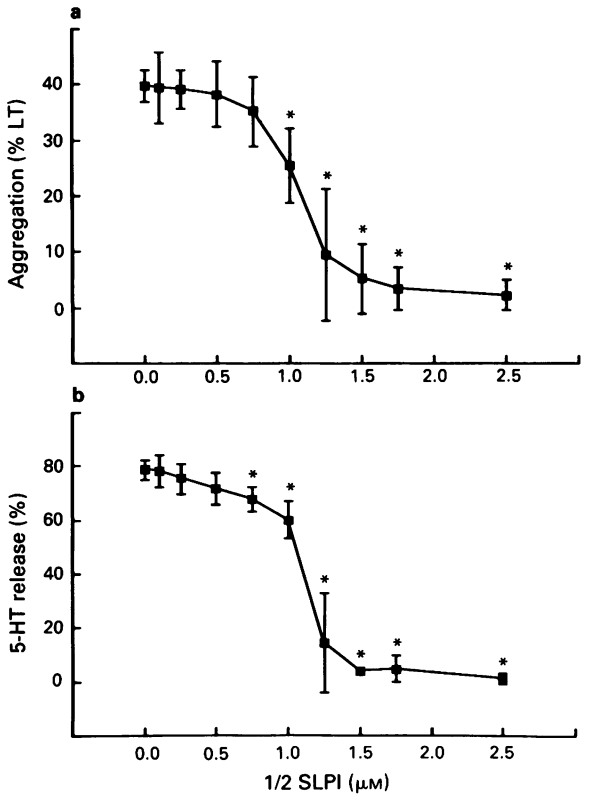


Figure 6 Inhibition by 1/2 SLPI of platelet activation induced by N-formyl-Met-Leu-Phe (fMLP)-stimulated polymorphonucleur neutrophils (PMN). For details see legend to Figure 5, SLPI being replaced by 1/2 SLPI.

SLPI and 1/2 SLPI necessary to induce a 50% inhibition of 5-HT release were different. However, when compared to concentrations inducing 50% inhibition of the purified Cat G-induced platelet 5-HT release, they were 6.0 times higher for SLPI but only 4.3 times higher for 1/2 SLPI (the difference was even more striking when considering aggregation, i.e. 6.3 compared to 2.6). These results could be explained by the fact that the low molecular weight of 1/2 SLPI (5 kDa) allows this molecule to diffuse more easily than the full length SLPI (11.7 kDa) between PMN and platelets, this property counterbalancing its lower potency for the two proteinases involved. This hypothesis is sustained by observations made in other experimental systems. For example PMN proteinases degrade fibronectin, elastin and fibrinogen, even in the presence of physiological concentrations of plasmatic inhibitors, particularly α_1-proteinase inhibitor (51 kDa) (Weiss & Regiani, 1984; Weitz *et al.*, 1987). More interestingly, stimulated PMN rapidly solubilized a subjacent fibronectin substrate even in the presence of plasma, but were unable to do so when plasma was supplemented with SLPI (Rice & Weiss, 1990). It was concluded that such a small molecular weight inhibitor is able to intervene in situations where natural defences fail. Consequently, 1/2 SLPI, with its even lower molecular weight of 5 kDa could be better for practical therapeutic use than the full length SLPI.

Diseases resulting from a proteinase-proteinase inhibitor imbalance and which may be treated by proteinase inhibitors include pulmonary emphysema (Janoff, 1985), chronic bronchitis (Eriksson, 1978), periodontitis (Olhsson *et al.*, 1973), rheumatoid arthritis (Saklatvala & Barret, 1980), cystic fibrosis (Suter *et al.*, 1986) and ARDS (Merrit *et al.*, 1983). Serum-derived α_1-proteinase inhibitor was first proposed for replacement therapy in pulmonary emphysema, but its limited availability and its human origin (fear of viral contamination) are major drawbacks. The recombinant form overcame these disadvantages while bringing some others, e.g. lack of carbohydrate side chain causing a reduced biological half-life (Hubbard *et al.*, 1989). Eglin C, a small proteinase inhibitor (8.1 kDa) was proposed as a potential therapeutic agent for the treatment of disease states associated with inflammation (Braun & Schnebli, 1986). However, its allergenic potential in man due to its animal origin, is an impediment for its development. Our preliminary results show that 1/2 SLPI could be a promising new candidate for the treatment of destructive lung diseases. In initial studies, it has been shown that SLPI was effective *in vivo*. Thus, an instillation of SLPI into the trachea of hamster resulted in a significant protection against lung destruction and the development of bronchial epithelial cell metaplasia (Snider, 1989), these results being corroborated with observations made by Lucey *et al.* (1990). It has also been reported that aerosolization of SLPI in sheep enhances the anti-HLE capacity of the lung, acting at least on pulmonary epithelial surface and possibly also in the pulmonary interstitium (Volgelmeier *et al.*, 1990).

In summary, we have demonstrated that biochemical and biological inhibitory activities of SLPI against HLE and Cat G were present in its truncated form. While 1/2 SLPI was less efficient than the native form against synthetic substrates, it appeared that the inhibitory activity of both compounds were close in the PMN-platelet cooperation system. We can put forward the facility of this low molecular weight compound to intervene at the cell-to-cell contact. Thus, if 1/2 SLPI diffused more easily than SLPI between PMN and the targets of these proteinases, it could be a promising candidate for the treatment of pathologies in which HLE and Cat G are involved.

References

BAUGH, R.J. & TRAVIS, J. (1976). Human leukocyte granule elastase: rapid isolation and characterization. *Biochemistry*, **15**, 836–841.

BEATTY, K., BIETH, J.G. & TRAVIS, J. (1980). Kinetics of association of serine proteinases with native and oxidized α-1-proteinase inhibitor and α-1-antichymotrypsin. *J. Biol. Chem.*, **255**, 3931–3934.

BOUDIER, C. & BIETH, J.G. (1989). Mucus proteinase inhibitor. A fast acting inhibitor of leukocyte elastase. *Biochem. Biophys. Acta*, **995**, 36–41.

BOUDIER, C. & BIETH, J.G. (1992). The proteinase: mucus proteinase inhibitor binding stoichiometry. *J. Biol. Chem.*, **267**, 4370–4375.

BOUDIER, C., HOLLE, C. & BIETH, J.G. (1981). Stimulation of the elastinolytic activity of leukocyte elastase by leukocyte cathepsin G. *J. Biol. Chem.*, **256**, 10256–10258.

BRAUN, N.J. & SCHNEBLI, H.P. (1986). A brief review of the biochemistry and pharmacology of eglin C, an elastase inhibitor. *Eur. J. Respir. Dis.*, **69**, 541–547.

CHIGNARD, M., SELAK, M.A. & SMITH, J.B. (1986). Direct evidence for the existence of a neutrophil-derived platelet activator (neutrophilin). *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 8609–8613.

COHEN, A.B. (1983). Supplement: proteases and antiproteases in the lung. *Am. Rev. Respir. Dis.*, **127** (part 2), S2–S58.

CRYSTAL, R.G., BRANTLY, M.L., HUBBARD, R.C., CURIEL, D.T., STATES, D.J. & HOLMES, M.D. (1989). The alpha$_1$-antitrypsin gene and its mutation. Clinical consequences and strategies for therapy. *Chest*, **95**, 196–208.

DEL MASCHIO, A., EVANGELISTA, V., RAJTAR, G., MIN CHEN, Z., CERLETTI, C. & DE GAETANO, G. (1990). Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. *Am. J. Physiol.*, **258**, H870–H879.

EISENBERG, S.P., HALE, K.K., HEIMDAL, P. & THOMPSON, R.C. (1990). Location of the protease-inhibitory region of secretory leukocyte protease inhibitor. *J. Biol. Chem.*, **265**, 7976–7981.

ERIKSSON, S. (1978). Proteases and protease inhibitors in chronic obstructive lung disease. *Acta Med. Scand.*, **203**, 449–455.

FERRER-LOPEZ, P., RENESTO, P., SCHATTNER, M., BASSOT, S., LAURENT, P. & CHIGNARD, M. (1990). Activation of human platelets by C5a-stimulated neutrophils. A role for cathepsin G. *Am. J. Physiol.*, **258**, C1100–C1107.

GERVAIX, A., THOMPSON, R., BIETH, J.G., NICK, H.-P. & SUTER, S. (1992). Secretory leucocyte proteinase inhibitor: inhibitor of fibronectin degradation by neutrophil elastase. *Eur. Respir. J.*, **5**, 566–575.

HEFFNER, J.E., SAHN, S.A. & REPINE, J.E. (1987). The role of platelets in the adult respiratory distress syndrome. Culprits or bystanders? *Am. Rev. Respir. Dis.*, **135**, 482–492.

HENSON, P.M. & JOHNSON Jr, R.B. (1987). Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. *J. Clin. Invest.*, **79**, 669–674.

HUBBARD, R.C., MCELVANEY, N.G., SELLERS, S.E., HEALY, J.T., CZERSKI, D.B. & CRYSTAL, R.G. (1989). Recombinant DNA-produced α1-antitrypsin administered by aerosol augments lower respiratory tract antineutrophil elastase defenses in individuals with α_1-antitrypsin deficiency. *J. Clin. Invest.*, **84**, 1349–1354.

JANOFF, A. (1985). Elastases and emphysema. Current assessment of the protease-antiproteases hypothesis. *Am. Rev. Respir. Dis.*, **132**, 417–433.

JANOFF, A., SLOAN, B., WEINBAUM, G., DAMIANO, V., SANHAUS, R.A., ELIAS, J. & KIMBEL, P. (1977). Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *Am. Rev. Respir. Dis.*, **115**, 461–478.

JANUSZ, M.J. & DOHERTY, N.S. (1991). Degradation of cartilage matrix proteoglycan by human neutrophils involves both elastase and cathepsin G. *J. Biol. Chem.*, **146**, 3922–3928.

LUCEY, E.C., STONE, P.J., CICCOLELLA, D.E., BREUR, R., CHRISTENSEN, T.G., THOMPSON, R.C. & SNIDER, G.L. (1990). Recombinant human secretory leukocyte-protease inhibitor: in vitro properties, and amelioration of human neutrophil elastase-induced emphysema and secretory cell metaplasia in the hamster. *J. Lab. Clin. Med.*, **115**, 224–232.

MARTODAM, R.R., BAUGH, R.J., TWUMASI, D.Y. & LIENER, I.E. (1979). A rapid procedure for the large scale purification of elastase and cathepsin G from human sputum. *Prep. Biochem.*, **9**, 15–31.

MASUDA, K., IMAIZUMI, A., KAMIMURA, T., WATANABE, K., SUGIYAMA, T., SUZUKI, Y. & OHTSUKA, E. (1992). Expression of the C-terminal domain of secretory leukoprotease inhibitor in *E coli* as a thrombin-cleavable fusion protein. *Am. Rev. Resp. Dis.*, **145**, A201.

MERRIT, T.A., COCHRANE, C.G., HOLCOMB, K., BOHL, B., HALLMAN, D., STRAYER, D., EDWARDS, D.K. & GLUCK, L. (1983). Elastase and α1-PI proteinase inhibitor activity in tracheal aspirates during respiratory distress syndrome. *J. Clin. Invest.*, **72**, 656–666.

MOOREN, H.W.D., KRAMPS, J.A., FRANKEN, C., MEIJER, C.J.L.M. & DICKMAN, J.A. (1983). Localisation of low molecular weight bronchial protease inhibitor in the peripheral human lung. *Thorax*, **38**, 180–183.

NAKAJIMA, K., POWERS, J.C., ASHE, B.M. & ZIMMERMAN, M. (1979). Mapping the extended binding site of cathepsin G and human leukocyte elastase. Studies with peptide substrates related to the alpha$_1$-protease inhibitor reactive site. *J. Biol. Chem.*, **254**, 4027–4032.

OHLSSON, K., OHLSSON, I. & TYNELIUS-BRATTHAL, G. (1973). Neutrophil leukocyte collagenase, elastase and serum protease inhibitors in human gingival crevices. *Acta Odontol. Scand.*, **31**, 145–153.

PUCHELLE, E.J., HINNRASKI, J., TOURNIER, J.M. & ADNET, J.J. (1985). Ultrastructural localisation of bronchial inhibitor in human airways using protein A-gold technique. *Biol. Cell.*, **55**, 151–154.

RENESTO, P., FERRER-LOPEZ, P. & CHIGNARD, M. (1990). Interference of recombinant eglin C, a proteinase inhibitor extracted from leeches, with neutrophil-mediated platelet activation. *Lab. Invest.*, **62**, 409–416.

RICE, W.G. & WEISS, S.J. (1990). Regulation of proteolysis at the neutrophil-substrate interface by secretory leukoprotease inhibitor. *Science*, **249**, 178–181.

SAKLATVALA, J. & BARRET, A.J. (1980). Identification of proteinase in rheumatoid synovium; detection of leukocyte elastase, cathepsin G and another serine proteinase. *Biochem. Biophys. Acta*, **615**, 167–177.

SELAK, M.A., CHIGNARD, M. & SMITH, J.B. (1988). Cathepsin G is a strong platelet agonist released by neutrophils. *Biochem. J.*, **251**, 293–299.

SELAK, M.A. & SMITH, J.B. (1990). Cathepsin G binding to human platelets. Evidence for a specific receptor. *Biochem. J.*, **266**, 55–62.

SENIOR, R.M., TEGNER, H., KUHN, C., OHLSSON, K., STARCHER, B.C. & PIERCE, J.A. (1977). The induction of pulmonary emphysema with human leukocyte elastase. *Am. Rev. Respir. Dis.*, **116**, 469–475.

SMITH, C.E. & JOHNSON, D.A. (1985). Human bronchial leucocyte proteinase inhibitor. Rapid isolation and kinetic analysis with human leucocyte proteinases. *Biochem. J.*, **225**, 463–472.

SMITH, C.E., STACK, M.S. & JOHNSON, D.A. (1987). Ozone effects on inhibitors of human neutrophil proteinases. *Arch. Biochem. Biophys.*, **253**, 146–155.

SNIDER, G.L. (1989). Chronic obstructive pulmonary disease: risk factors, pathophysiology and pathogenesis. *Annu. Rev. Med.*, **40**, 411–429.

SNIDER, G.L., LUCEY, E.C., CHRISTENSEN, T.G., STONE, P.J., CALORE, J.D., CATANESE, A. & FRANZBLAU, C. (1984). Emphysema and bronchial secretory cell metaplasia in hamsters by human neutrophil products. *Am. Rev. Respir. Dis.*, **129**, 155–160.

SUTER, S., SCHAAD, U.B., TEGNER, H., OHLSSON, K., DESGRAND-CHAMPS, D. & WALDVOGEL, F.A. (1986). Level of free granulocyte elastase in bronchial secretions from patients with cystic fibrosis: effect of antimicrobial treatment against pseudomonas aeruginosa. *J. Infect. Dis.*, **153**, 902–909.

THOMPSON, R.C. & OHLSSON, K. (1986). Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 6692–6696.

VOLGELMEIER, C., BULH, R., HOYT, R.F., WILSON, E., FELLS, G.A., HUBBARD, R.C., SCHNEBLI, H.P., THOMPSON, R.C. & CRYSTAL, R.G. (1990). Aerosolization of recombinant SLPI to augment antineutrophil elastase protection from pulmonary epithelium. *J. Appl. Physiol.*, **69**, 1843–1848.

WARSHAWSKI, F.J., SIBBALD, W.J., DRIEDGER, A.A. & CHEUNG, H. (1986). Abnormal neutrophil-pulmonary interaction in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.*, **133**, 797–804.

WEISS, S.J. & REGIANI, S. (1984). Neutrophils degrade subendothelial matrices in the presence of alpha-1-proteinase inhibitor. Cooperative use of lysosomal proteinases and oxygen metabolites. *J. Clin. Invest.*, **73**, 1297–1303.

WEITZ, J.I., HUANG, A.J., LANDMAN, S.L., NICHOLSON, S.C. & SILVERSTEIN, S.C. (1987). Elastase-mediated fibrigenolysis by chemoattractant-stimulated neutrophils occurs in the presence of physiologic concentrations of antiproteinases. *J. Exp. Med.*, **166**, 1836–1850.

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Porcine ventricular endocardial cells in culture express the inducible form of nitric oxide synthase

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1 We have investigated whether porcine endocardial cells in culture express the inducible, Ca^{2+}–independent form of nitric oxide (NO) synthase.

2 NO synthase activity in cytosolic extracts of endocardial cells was measured by estimation of the rate of formation of L-[^{14}C]-citrulline from L-[^{14}C]-arginine.

3 Treatment of the cells in culture with lipopolysaccharide or cytokines induced a Ca^{2+}-independent NO synthase activity in the cell cytosol. The combination of tumour necrosis factor (TNFα, 10 ng ml^{-1}) and interleukin-1β (IL-1β, 10 ng ml^{-1}) induced the greatest enzyme activity.

4 The increased Ca^{2+}-independent NO synthase activity following exposure to cytokines was parallelled by an increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels in the endocardial cell cytosol.

5 Simultaneous addition of dexamethasone (0.01–1 μM) or cycloheximide (0.03–3 μM) inhibited in a concentration-dependent manner TNFα- and IL-1β-induced expression of Ca^{2+}-independent NO synthase activity. Neither dexamethasone (1 μM) nor cycloheximide (3 μM) had any effect on the activity of the constitutive NO synthase.

6 The possible pathophysiological consequences of endocardial expression of the inducible NO synthase are discussed.

Keywords: Nitric oxide synthase; lipopolysaccharide; tumour necrosis factor; interleukin-1β; endothelium; interferon-γ; dexamethasone; cycloheximide; cyclic GMP; heart

Introduction

The role of endothelial cells in the regulation of vascular smooth muscle tone is now well established (Henderson, 1991; Moncada *et al.*, 1991). Endocardial cells are ontogenetically similar to vascular endothelial cells and in a manner analogous to endothelial control of blood vessel tone, endocardial cells play a role in modulating the contractility of cardiac muscle (Lewis *et al.*, 1990; Smith *et al.*, 1991). Endocardial cells, like endothelial cells, contain a calcium-dependent constitutive nitric oxide (NO) synthase (Schulz *et al.*, 1991). NO released by endocardial cells elevates myocardial guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels in isolated papillary muscles, abbreviating the twitch contraction (Smith *et al.*, 1991). Cultured endocardial cells in addition to releasing NO, also release a factor, putatively termed 'endocardin', which exerts an opposite twitch prolonging effect to NO in papillary muscles selectively denuded of endocardium (Smith *et al.*, 1991).

Vascular endothelial cells have been shown to express an inducible, calcium-independent NO synthase when exposed to endotoxin or some cytokines (Radomski *et al.*, 1990; Kilbourn & Belloni, 1990). NO synthesized by this inducible enzyme appears to be responsible for the hypotension observed in endotoxic shock (Rees *et al.*, 1990). If endocardial cells also express the inducible form of NO synthase this may contribute to the depression of cardiac contractility observed in endotoxaemia. In the present study we demonstrate that endocardial cells in culture are also able to express an inducible, cytosolic, calcium-independent NO synthase.

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Methods

Endocardial cell culture

Endocardial cells were isolated from freshly obtained pig hearts as previously described (Smith *et al.*, 1991). Briefly, cells were harvested by exposure of the right ventricular cavity to 0.2% collagenase in medium E199 and cultured in complete medium (medium E199 supplemented with 10% foetal bovine serum, 10% newborn bovine serum, glutamine (6 mM), benzyl penicillin (200 u ml^{-1}) and streptomycin (200 μg ml^{-1})) in fibronectin-coated 75 cm^2 plastic tissue culture flasks. Flasks were coated with fibronectin by exposure to a sterile solution of fibronectin (10 μg ml^{-1}) in 10 mM HEPES (pH 7.3) for 1 h. Confluent cells were subcultured by a brief (3–5 min) exposure to a 0.85% saline solution containing 0.05% trypsin and 0.02% EDTA. Newborn calf serum was added to inactivate the trypsin and the cell suspension was centrifuged (200 *g* for 5 min) and resuspended in complete medium. Cells used in this study were either primary or up to third passage, exhibited cobblestone morphology, and incorporated fluorescent labelled acetylated low density lipoprotein (Smith *et al.*, 1991).

Preparation of endocardial cell cytosol

Following incubation, culture medium was removed and the cells were washed three times with Dulbecco's phosphate buffered saline (PBS) without calcium, magnesium and sodium bicarbonate. Cells from 3 or 4 (75 cm^2) tissue culture flasks were removed by scraping, combined, placed in an equal volume of culture medium and centrifuged (200 *g* for 10 min). The cell pellet was washed twice with PBS before resuspending the cells at a concentration of approximately 30–70 × 10^6 cells ml^{-1} in ice-cold homogenization buffer con-

taining 320 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, 1 mM DL-dithiothreitol, 10 $\mu g\ ml^{-1}$ leupeptin, 10 $\mu g\ ml^{-1}$ soybean trypsin inhibitor and 2 $\mu g\ ml^{-1}$ aprotinin (adjusted to pH 7.2 at 20°C with HCl). The cells were homogenized by two cycles of freezing in liquid nitrogen and thawing on ice. The homogenate was centrifuged at 150,000 *g* for 35 min at 4°C and the supernatant was incubated with cation-exchange resin (AG 50W- X8, Na^+ form) for 5 min at 4°C to deplete endogenous L-arginine. The Na^+-form of Dowex-50 was prepared by washing the H^+-form of the resin with 1 M NaOH four times and then washing with H_2O until the pH was less than 7.5. Upon settling of the resin, the supernatant was kept on ice and used within 2 h for determination of NO synthase activity or cyclic GMP content.

Induction of NO synthase

Cultured endocardial cells in culture medium containing 10 μM sepiapterin, were incubated for 18 h with LPS (10 $\mu g\ ml^{-1}$) and/or IFNγ (150 u ml^{-1}), IL-1β (10 ng ml^{-1}) or TNFα (10 or 100 ng ml^{-1}). Sepiapterin was included to stimulate the pterin salvage pathway in the endocardial cells to ensure full activity of the enzyme in the assay (Gross *et al.*, 1991). In some experiments, endocardial cells were stimulated with a combination of TNFα (10 ng ml^{-1}) and IL-1β (10 ng ml^{-1}) in the presence or absence of dexamethasone (0.01–1 μM) or cycloheximide (0.03–3 μM) added at the same time as the cytokines.

Assay of nitric oxide synthase activity

NO synthase activity was assayed as described by Salter *et al.* (1991). Briefly, 20 µl of tissue extract was added to plastic Eppendorff tubes prewarmed to 37°C containing 100 µl of a buffer consisting of 50 mM potassium phosphate, pH 7.2, 60 mM L-valine (to inhibit L-arginase), 120 μM NADPH, 1.2 mM L-citrulline, 24 μM L-arginine and L-[U-^{14}C] arginine (150 000 d.p.m.), 1.2 mM $MgCl_2$ and 0.24 mM $CaCl_2$. Duplicate or triplicate incubations for 10 min at 37°C were performed for each sample in the presence of either EGTA (1 mM), or EGTA plus L-N^G-monomethyl-arginine (L-NMMA) (1 mM each), to determine the level of the Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities respectively, before terminating the reaction by removal of substrate and dilution by addition of 1 ml of 1:1 (v/v) H_2O/Dowex AG 50W-X8, Na^+-form, pH 7.5. H_2O (0.5 ml) was added to the incubate mix, left to settle for 10 min and 0.4 ml of supernatant was removed and examined for the presence of [^{14}C]-citrulline by liquid-scintillation counting. L-[U-^{14}C]-Arginine was purified before use by adding the radiolabel in 20 mM HEPES (2 ml), pH 5.5, to a 1 ml column of Dowex resin (prepared as above), washing the column with 8 ml of H_2O and eluting with 4 ml of 0.5 M ammonia. The eluent was freeze-dried and resuspended in 2% ethanol.

Assay of cyclic GMP

Samples of endocardial cell cytosol were prepared and incubated as described above. The reaction was terminated by addition of 50 µl 20% $HClO_4$. The samples were neutralized with 100 µl of 1.08 M K_3PO_4, centrifuged at 12 000 *g* for 2 min and the supernatant assayed in triplicate for cyclic GMP content with a commercially available cyclic GMP kit (see Materials).

Materials

The following were used (sources in parentheses): aprotinin (Sigma), L-[U-^{14}C]-arginine (Amersham International), cation exchange resin (Dowex AG 50W-X8, 200–400 mesh; Bio-Rad), cell culture materials (Flow and Sigma), cyclic GMP enzyme immunoassay (Amersham International, cat. no. RPN226), cycloheximide (Sigma), dexamethasone (Merck, Sharp and Dohme), DL-dithiothreitol (Sigma), EGTA (Sigma), interferon-γ (Genzyme, Boston, U.S.A.) interleukin-1β (IL-1β, British Biotechnology), leupeptin (Sigma), Salmonella type IV lipopolysaccharide (LPS, Difco), L-N^G-monomethyl arginine (L-NMMA, Wellcome), sepiapterin (Dr B. Shircks Laboratories, Jona, Switzerland), soybean trypsin inhibitor (Sigma), tumour necrosis factor (TNFα, British Biotechnology).

Statistics

Results are expressed as mean ± s.e.mean for *n* experiments. Student's unpaired *t* test was used to compare individual means. $P < 0.05$ was considered to be statistically significant.

Results

In the presence of calcium (0.24 mM) cytosol prepared from control cultures of endocardial cells converted L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline at a rate of 1.4 ± 0.17 pmol $min^{-1}\ mg^{-1}$ protein ($n = 3$). In the absence of calcium and in the presence of 1 mM EGTA, the rate of L-[U-^{14}C]-citrulline formation was significantly reduced to 0.07 ± 0.03 pmol $min^{-1}\ mg^{-1}$ protein, ($n = 3$, $P < 0.005$). In the absence of calcium and the presence of EGTA and L-NMMA, there was no detectable conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline (data not shown). The basal activity of the guanylate cyclase in the endocardial cell cytosol was also significantly reduced by removal of calcium and addition of 1 mM EGTA (Table 1). Pretreatment of the cells for 18 h with 10 ng ml^{-1} TNFα did not significantly increase the calcium-independent NO synthase activity in the endocardial cells. However, incubation of the cells with 100 ng ml^{-1} TNFα or 10 $\mu g\ ml^{-1}$ LPS, or 10 ng ml^{-1} IL-1β produced small but significant increases ($P < 0.001$, 0.005 and 0.005

Table 1 Effect of 18 h cytokine pretreatment on the cyclic GMP content of endocardial cell cytosol

Pretreatment	*Calcium concentration*	*Cyclic GMP concentration* (fmol mg^{-1} protein min^{-1})
None (control)	0.24 mM	11.8 ± 1.39
None	0 mM + 1 mM EGTA	1.0 ± 0.14†
TNFα + IL-1β	0 mM + 1 mM EGTA	42.6 ± 3.97*
IL-1β	0 mM + 1 mM EGTA	10.9 ± 1.29*
IL-1β + IFNγ	0 mM + 1 mM EGTA	15.9 ± 1.82*

Pretreatment with interleukin-1β (IL-1β, 10 ng ml^{-1}), or IL-1β combined with either tumour necrosis factor (TNFα, 100 ng ml^{-1}) or interferon γ (IFNγ, 50 u ml^{-1}) increased the cyclic GMP content of the endocardial cell cytosol in the absence of calcium. Each treatment was assayed in triplicate in each experiment, and the results from three separate experiments averaged. Results are expressed as mean ± s.e.mean.

†Indicates significantly different from controls in the presence of calcium: $P < 0.005$; *indicates significantly different from untreated group in the absence of calcium, $P < 0.001$.

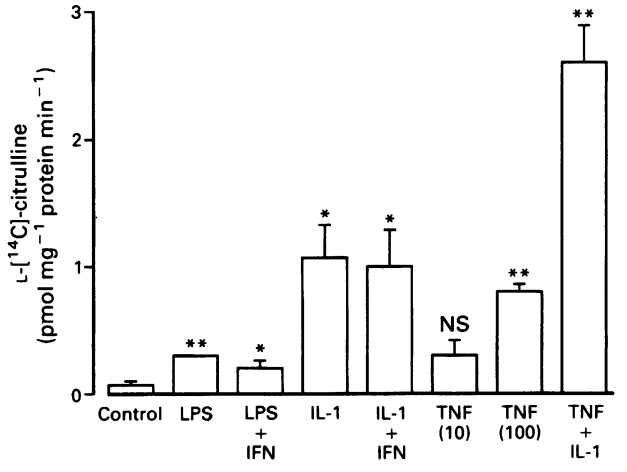


Figure 1 Effect of lipopolysaccharide (LPS) or cytokine pretreatment on cytosolic, Ca^{2+}-independent, inducible NO synthase activity in cultured endocardial cells. Cells were incubated for 18 h with LPS (10 $\mu g\ ml^{-1}$), alone or in combination with interferon-γ (IFN, 150 u ml^{-1}); interleukin-1β (IL-1, 10 ng ml^{-1}) or IL-1 + IFN; tumour necrosis factor (TNF, 10 or 100 ng ml^{-1}), or TNF (10 ng ml^{-1}) + IL-1 as indicated below the columns. Each treatment was assayed in triplicate in each experiment, and the results from three separate experiments averaged. Results are expressed as mean ± s.e.mean. *and **indicate significantly greater than controls at the $P < 0.05$ and $P < 0.005$ levels respectively; NS indicates not significantly different from controls.

respectively) in the Ca^{2+}-independent NO synthase activity in the cytosols of endocardial cells (Figure 1). Combinations of IFNγ (150 u ml^{-1}) with LPS or IL-1β did not stimulate significantly more inducible NO synthase activity than LPS or IL-1β alone. The total NO synthase activity (i.e. Ca^{2+}-dependent plus Ca^{2+}-independent) with all these treatments was between 1 and 2 fold that of untreated cells. The combination of TNFα (10 ng ml^{-1}) with IL-1β (10 ng ml^{-1}) produced significantly more inducible NO synthase activity than either agent alone ($P < 0.05$, cf. IL-1β, $P < 0.005$ cf. TNFα, 100 ng ml^{-1}; Figure 1), increasing the total NO synthase activity of the cells by 3 fold. Pretreatment of the cells with TNFα and/or IL-1β also significantly elevated the cytokine-induced cyclic GMP formation in the absence of calcium in the endocardial cell cytosols (Table 1).

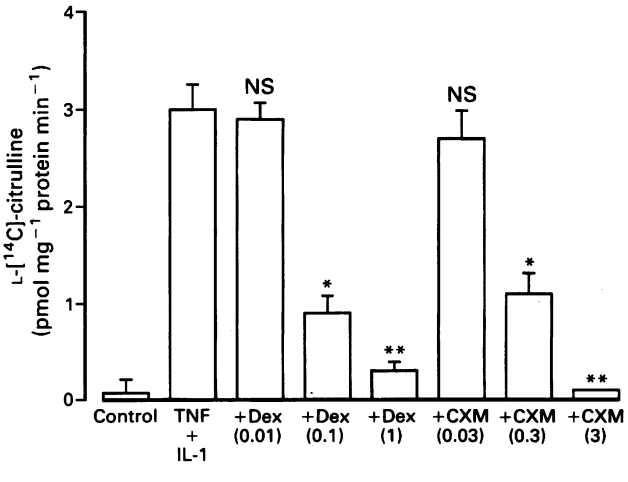


Figure 2 Effect of dexamethasone (Dex, 0.01–1 μM) or cycloheximide (CXM, 0.03–3 μM) on tumour necrosis factor (TNF, 10 ng ml^{-1}) + interleukin-1β (IL-1, 150 u ml^{-1})-induced expression of cytosolic, Ca^{2+}-independent NO synthase activity in cultured endocardial cells. Each treatment was assayed in duplicate or triplicate in each experiment, and the results from three separate experiments averaged. Results are expressed as mean ± s.e.mean. NS indicates not significantly different from TNF + IL-1; *and **indicate significantly less than TNF + IL-1 at the $P < 0.005$ and $P < 0.001$ levels respectively.

Treatment of the endocardial cells for 18 h with dexamethasone (1 μM) or cycloheximide (3 μM) in the absence of cytokines did not affect the Ca^{2+}-dependent constitutive NO synthase activity (NO synthase activities were 1.3 ± 0.18, 1.4 ± 0.24 and 1.3 ± 0.09 pmol mg^{-1} protein min^{-1} in control, dexamethasone and cycloheximide treated cells respectively). However, simultaneous treatment of the cells with 0.01 to 1 μM dexamethasone or 0.03 to 3 μM cycloheximide produced a concentration-dependent inhibition of the induction of NO synthase by TNFα and IL-1β (Figure 2).

Discussion

Previous studies have demonstrated the induction of a calcium-independent NO synthase in endothelial cells, smooth muscle cells and cardiac myocytes (Radomski *et al.*, 1990; Rees *et al.*, 1990; Busse & Mulsch, 1990; Schulz *et al.*, 1991; 1992). This study shows for the first time that endocardial cells, the cells lining the chambers of the heart, are also capable of expressing a calcium-independent NO synthase. As in other cell types, induction of the calcium-independent NO synthase can be inhibited in a concentration-dependent manner by dexamethasone or the protein synthesis inhibitor, cycloheximide (Radomski *et al.*, 1990; Rees *et al.*, 1990; Schulz *et al.*, 1992).

Direct comparison of NO synthase levels in different cell types is not readily achieved because of the multiplicity of methods used to assess enzyme activity. The constitutive NO synthase levels in pig endocardial cells in culture reported in this study are similar to the constitutive NO synthase levels we have previously obtained, using the same techniques, for rat left ventricular wall (Schulz *et al.*, 1991). Rat myocytes in culture by contrast contained more constitutive enzyme than samples of left ventricular wall, and were less responsive to the Ca^{2+}-independent NO synthase inducing effects of LPS (Schulz *et al.*, 1992). The constitutive NO synthase levels in cultured endocardial cell cytosol previously reported by us using a different technique (Schulz *et al.*, 1991) were approximately 50% of those seen in pig aortic endothelial cell cultures. Further comparisons of NO synthase activity with other cell types cannot be made readily.

As has previously been reported for vascular endothelium and other cell types, neither dexamethasone nor cycloheximide affected the activity of the calcium-dependent constitutive NO synthase of the endocardial cells, indicating that the effects of dexamethasone and cycloheximide are not due to a cytotoxic action. Indeed cycloheximide has been shown to be non-toxic (by trypan blue exclusion) to endothelial cells at a concentration of 890 μM (Gross *et al.*, 1991).

The physiological and pathophysiological implications of these findings remain to be established. It is known, however, that NO released from endocardial cells of papillary muscle preparations can elevate papillary muscle cyclic GMP levels, and influence the contractile state of the underlying myocardium (Shah *et al.*, 1991; Smith *et al.*, 1991). Furthermore it has recently been demonstrated that endocardial endothelium, like vascular endothelium, has platelet anti-adhesive properties (Siney & Lewis, 1992). Whether NO released from endocardium in the whole heart is capable of influencing myocardial contractility is however not yet established. The half-life of NO is short and it is not known whether it can diffuse into the ventricular walls sufficiently far and in sufficient concentrations to affect contractile performance. High concentrations of sodium nitroprusside (10^{-5} M), a donor of NO, infused into the left ventricular cavity of an isolated Langendorff heart preparation failed to induce changes in contractile performance or myocardial cyclic GMP levels. However, intracoronary infusion of sodium nitroprusside resulted in the characteristic abbreviation of twitch contraction and elevation of cyclic GMP levels previously described in papillary muscles (Fort & Lewis, 1991).

As far as pathophysiology is concerned, it is possible that increased production of NO by the Ca^{2+}-independent NO synthase in the endocardium myocardium (Schulz *et al.*, 1992) may play a role in the cardiac dysfunction of septic shock and other immunologically based conditions such as idiopathic dilated cardiomyopathy and during the treatment of cancer with cytokines.

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References

BUSSE, R. & MULSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.*, **275**, 87–90.

FORT, S. & LEWIS, M.J. (1991). Regulation of myocardial contractile performance by sodium nitroprusside in the isolated perfused heart of the ferret. *Br. J. Pharmacol.*, **102**, 351P.

GROSS, S.S., JAFFE, E.A., LEVI, R. & KILBOURN, R.G. (1991). Cytokine-activated cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin-dependent, calmodulin-independent and inhibited by arginine analogues with a rank-order of potency characteristic of macrophages. *Biochem. Biophys. Res. Commun.*, **178**, 823–829.

HENDERSON, A.H. (1991). Endothelium in control. *Br. Heart J.*, **65**, 116–125.

KILBOURN, R.G. & BELLONI, P. (1990). Endothelial cell production of nitrogen oxides in response to interferon-gamma in combination with tumour necrosis factor, interleukin-1 or endotoxin. *J. Natl. Cancer Inst.*, **82**, 772–776.

LEWIS, M.J., SHAH, M.J., SMITH, J.A. & HENDERSON, A.H. (1990). Does endocardium modulate myocardial contractile performance? *Cardioscience*, **1**, 83–87.

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

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., CELLEK, S., PALMER, R.M.J. & MONCADA, S. (1990). Dexamethasone inhibits the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. *Biochem. Biophys. Res. Commun.*, **173**, 541–547.

SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distribution and changes in activity of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthases. *FEBS Lett.*, **291**, 145–149.

SCHULZ, R., NAVA, E. & MONCADA, S. (1992). Induction and potential biological relevance of a Ca^{2+}-indendent nitric oxide synthase in the myocardium. *Br. J. Pharmacol.*, **105**, 575–580.

SCHULZ, R., SMITH, J.A., LEWIS, M.J. & MONCADA, S. (1991). Nitric oxide synthase in cultured endocardial cells of the pig. *Br. J. Pharmacol.*, **104**, 21–24.

SHAH, M.J., LEWIS, M.J. & HENDERSON, A.H. (1991). Effects of 8-bromo cyclic GMP on contraction and on inotropic response of ferret cardiac muscle. *J. Mol. Cell. Cardiol.*, **23**, 55–64.

SINEY, L. & LEWIS, M.J. (1992). Endothelium-derived relaxing factor inhibits the platelet adhesion to cultured porcine endocardial endothelium. *Eur. J. Pharmacol.*, (in press).

SMITH, J.A., SHAH, A.M. & LEWIS, M.J. (1991). Factors released from the endocardium of the ferret and the pig modulate myocardial contraction. *J. Physiol.*, **439**, 1–14.

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Effect of flosequinan on ischaemia-induced arrhythmias and on ventricular cyclic nucleotide content in the anaesthetized rat

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1 Flosequinan, milrinone, isoprenaline and forskolin given intravenously at similarly hypotensive doses have been evaluated in separate studies for their effect on ischaemia-induced arrhythmias and on ventricular cyclic nucleotide content following coronary artery ligation in the pentobarbitone anaesthetized rat.

2 Flosequinan did not affect mortality or arrhythmias following coronary artery ligation in either study and no change in ventricular cyclic nucleotide content was observed.

3 Isoprenaline caused a significant increase in mortality ($P<0.05$) in both studies whereas milrinone and forskolin caused a significant increase in mortality in only one of the two studies conducted. All three agents caused significant increases in cyclic AMP which were associated with increased incidence of arrhythmias.

4 When compared at similarly hypotensive doses, flosequinan, in contrast to milrinone, isoprenaline and forskolin, did not influence ischaemia-induced arrhythmias or raise ventricular cyclic nucleotide levels in the anaesthetized rat.

Keywords: Ischaemia-induced arrhythmias; anaesthetized rat; cyclic AMP; cyclic GMP; flosequinan

Introduction

Flosequinan is currently undergoing clinical evaluation for the treatment of heart failure, a condition associated with a high incidence of ventricular arrhythmias and sudden cardiac death (Wilson, 1987; Podrid *et al.*, 1990). Elevated levels of cyclic AMP have been implicated in the genesis of ischaemia-induced arrhythmias and the lowering of fibrillation threshold in the dog, pig, and baboon heart (Podzuweit, 1982) and in the rat isolated heart (Lubbe *et al.*, 1983). Elevation of ventricular guanosine 3':5'-cyclic monophosphate (cyclic GMP) as well as adenosine 3':5'-cyclic monophosphate (cyclic AMP) has also been shown to increase ischaemia-induced ventricular arrhythmias in anaesthetized rats (Kane *et al.*, 1985). If such changes in cyclic nucleotides were to occur in heart failure patients this could prove detrimental in the treatment of a condition where ventricular arrhythmias are already prevalent (Packer, 1989). Flosequinan has previously been shown to possess only non-selective phosphodiesterase inhibitory activity of low potency (Frodsham & Jones, 1992) and to elevate ventricular cyclic AMP and cyclic GMP in *in vitro* experiments at high concentrations (Yates, 1991) although the relevance of these observations to the therapeutic plasma concentration of flosequinan is questionable. In contrast however, Gristwood *et al.* (1992) have suggested that flosequinan behaves like a phosphodiesterase type III inhibitor.

The aim of this study was to determine whether flosequinan affected ventricular arrhythmias and cyclic nucleotide content by use of a model of ischaemia-induced arrhythmias in the pentobarbitone anaesthetized rat at a dose causing substantial hypotension. Also evaluated in this model at similarly hypotensive doses, were milrinone, isoprenaline and forskolin, compounds which raise cyclic AMP by selective phosphodiesterase type III inhibition, β-adrenoceptor activation and direct activation of adenylate cyclase respectively. Preliminary accounts of this work have been presented previously (Jones & Sim, 1990; Jones *et al.*, 1991).

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Methods

Surgical and recording procedures

The method used to produce coronary artery occlusion in anaesthetized rats has been fully described by Clark *et al.* (1980). Briefly, male Wistar rats (250–400 g, Olac UK) were anaesthetized with sodium pentobarbitione, 60 mg kg^{-1}, i.p. The trachea was cannulated and the animals artificially ventilated with a miniature Ideal respiration pump (47 strokes per minute, 4 ml tidal volume). The jugular vein and carotid artery were cannulated for drug administration and for the recording of blood pressure, using a pressure transducer, respectively. Blood pressure and a limb lead ECG were continuously recorded on a Grass model 7 polygraph. Mean blood pressure was calculated as diastolic blood pressure + 1/3 pulse pressure. A left thoractomy was performed, and the fourth rib removed. After the pericardium was opened the heart was exteriorized and a fine silk ligature (Mersilk W595, Ethicon Ltd) placed around the left anterior descending coronary artery as described by Selye *et al.* (1960). The heart was then put back into the chest cavity and left to stabilize for 10 min before starting the experimental protocol.

Animals with a mean blood pressure of less than 70 mmHg after placement of the ligature were excluded from the experiment as were those which demonstrated arrhythmias prior to the ligature being tied. Following coronary ligation animals which died within the first 5 min period due to a progressive but complete loss of blood pressure without demonstrating arrhythmias were also excluded. Overall 34 animals were excluded for the above reasons representing a 14.4% loss rate.

Experimental protocol

Three separate sets of experiments were performed. In the first experimental group sham-operated rats (the coronary ligature being positioned but not tied) were dosed intravenously with compounds or vehicle to determine the degree and duration of haemodynamic effects. Over the following

35 min period, heart rate and mean blood pressure were measured at 5 min intervals. Doses were titrated to produce an approximate 50% fall in mean blood pressure for the duration of the experiment. These doses were then used in the subsequent experiments.

In a second series of experiments to study ischaemia-induced arrhythmias, rats were dosed intravenously with vehicle or drug and the coronary artery ligature permanently tied 5 min later. Over a 30 min ischaemic period, heart rate and mean blood pressure were recorded as above at 5 min intervals, and the total number of arrhythmias observed were counted in 5 min periods.

In a third series of experiments to study ventricular cyclic nucleotide content, the same protocol as in the second experiment was followed, with the exception that at 15 min following coronary artery ligation, at a time when peak arrhythmia is normally observed, the heart was removed. Hearts were also removed for cyclic nucleotide analysis if during the 15 min ischaemic period sustained ventricular fibrillation (VF) lasting more than 30 s was observed. Normally animals fail to recover sinus rhythm after such sustained periods of VF and were therefore classified as having died due to VF. Hearts were rapidly washed in ice-cold saline, the free wall of the left ventricle (i.e. ischaemic tissue) and the right ventricle (comprising the right ventricle and the intraventricular septum; i.e. non-ischaemic tissue) were freeze clamped with Wollenberger tongs precooled to the temperature of liquid nitrogen. Samples of left and right ventricle were wrapped in aluminium foil and stored in liquid nitrogen until assayed. The time taken to excise the heart, dissect and freeze clamp was approximately 15 s. Arrhythmias were also counted in this study.

For the arrhythmia and cyclic nucleotide studies, animals of similar age and weight from the same batch were caged and studied in pairs. One animal from each pair was given vehicle and the other the drug under test. Thus each test animal had its corresponding control.

Determination of cyclic nucleotide content

The frozen ventricular samples were trimmed of surplus tissue and powdered at the temperature of liquid nitrogen with a Braun Mikrodismembrator. Cyclic AMP and cyclic GMP in samples of powdered tissue (200 mg) were extracted with 3 ml of 6% w/v trichloroacetic acid (TCA). Following centrifugation (10 000 *g* for 5 min), the supernatant was removed and a 1 ml sample of the TCA extract washed 5 times with 4 ml of water saturated ether to remove TCA. Cyclic AMP and cyclic GMP were assayed in triplicate by radioimmunoassay (Amersham International PLC, Cat. No. RPA 538 and IMK 8821 or RPA 540 respectively). Any interference with the assay caused by residual TCA contamination was corrected for by the inclusion of a solution of ether extracted TCA in the standard curve. The protein pellet was solubilized by incubation in 1 M NaOH at 37°C for 24 h and the protein content determined by a modification of the Lowry procedure (Markwell *et al.*, 1978). Values are expressed as pmol cyclic AMP or cyclic GMP per mg protein.

The limit of detection of cyclic AMP and cyclic GMP was, as defined by Amersham, that level of nucleotide necessary to reduce the zero dose binding by two standard deviations and was approximately 2 fmol/tube for cyclic AMP and 4 fmol/tube for cyclic GMP. These levels, using an undiluted extract, correspond to tissue concentrations of 2.7 fmol cyclic AMP mg^{-1} protein and 5.4 fmol cyclic GMP mg^{-1} protein. However, in practice, sample extracts were diluted 200 fold for cyclic AMP and 4 fold for cyclic GMP so that unknown determinations fell approximately in the middle of the standard curves.

The recovery of cyclic nucleotides was assessed by the addition of a known amount of cyclic AMP and cyclic GMP to powdered heart tissue and subjecting the sample to the entire extraction and radioimmunoassay procedure. The recovery of cyclic AMP and cyclic GMP was 92 ± 11% and 105 ± 7% respectively (mean ± s.d., $n = 6$). Within assay variation for the radioimmunoassay determination was 2.2% for cyclic AMP and 3.9% for cyclic GMP (coefficient of variation, $n = 18$ for both nucleotides). Between assay variation was obtained by assaying the same standard each time an assay was conducted and yielded a coefficient of variation of 13% for cyclic AMP and 8% for cyclic GMP ($n = 6$).

Determination of arrhythmias

The number of premature ventricular beats (PVBs) and the incidence and duration of ventricular tachycardia (VT, a succession of seven or more PVBs) and ventricular fibrillation (VF, chaotic ventricular contraction associated with a complete collapse of blood pressure) were counted over 5 min periods during the experiment. The PVB count includes the number of beats observed during periods of VT.

Drugs

Flosequinan and milrinone, synthesized by the Medicinal Chemistry Department, Boots Pharmaceuticals, were dissolved in saline and given as an i.v. bolus. Isoprenaline (Sigma, dissolved in saline containing ascorbic acid to prevent oxidation) and forskolin (Sigma, dissolved in ethanol) were continuously infused (0.03 ml min^{-1}) to maintain their hypotensive effect. Control animals were treated with the appropriate drug vehicle.

Statistical analysis

All data are presented as the mean ± the standard error of the mean. The cyclic AMP and cyclic GMP levels in treated and control groups have been compared separately for the left and right ventricles and mean values (average of right and left ventricular values). Data were evaluated by the Wilks Shapiro test and *F* test for variance ratio to ensure normality of data and equal variances of samples prior to parametric analysis using two sample *t* tests. If evidence was found that data were not normally distributed, data were analysed via the nonparametric Mann Whitney test after checking for equal dispersions by the Ansari Bradley test. The observed mortality in drug-treated groups was compared with the corresponding control groups by Fisher's exact test. The PVB count between control and drug-treated groups was compared by Wilcoxon's rank sum test and the PVB counts between individual control groups were compared by a Kruskal Wallis test. The comparison between the pre-dose, 5 min after dose and percentage fall in mean blood pressure was made by a oneway analysis of variance followed by a Newman-Keuls multiple range test.

Results

Haemodynamic effects in sham-operated rats

Flosequinan at 3.0 and 6.0 mg kg^{-1} caused a fall in mean blood pressure of 44% and 43% respectively, 5 min after dosing, the fall in blood pressure being maintained only at the higher dose used (Table 1). A slight fall in heart rate at 3.0 mg kg^{-1} and a slight rise in heart rate at 6.0 mg kg^{-1} flosequinan was observed. Milrinone at 5 and 35 min after dosing produced a fall in mean blood pressure of 29% and 21% at 0.2 mg kg^{-1} and a fall of 59% and 52% at 0.3 mg kg^{-1} respectively, with no significant effect on heart rate. Isoprenaline (0.1 μg kg^{-1} min^{-1}) caused a 47% and 44% fall in forskolin (10 μg kg^{-1} min^{-1}), a 55% and 62% fall in mean blood pressure 5 and 35 min following dosing respectively. Both isoprenaline and forskolin significantly increased heart rate by 14% and 12% and by 9% and 19%, 5 and 35 min

Table 1 Effect of treatment on mean blood pressure and heart rate in sham-operated anaesthetized rats

			Mean blood pressure (mmHg)			*Heart rate* (beats min^{-1})		
Treatment	*Dose*	n	Pre-dose	5 min	35 min	Pre-dose	5 min	35 min
Flosequinan	3.0 mg kg^{-1}	6	110 ± 9.3	62 ± 9.2**	87 ± 10.4	421 ± 33.3	400 ± 30.4*	413 ± 27.0
Flosequinan	6.0 mg kg^{-1}	4	140 ± 2.5	80 ± 14.7*	80 ± 18.2*	465 ± 15.5	482 ± 8.5	470 ± 11.5
Milrinone	0.3 mg kg^{-1}	4	106 ± 10.0	43 ± 4.0**	51 ± 3.1*	420 ± 14.7	440 ± 30.2	435 ± 14.4
Milrinone	0.2 mg kg^{-1}	4	127 ± 3.4	90 ± 10.7	100 ± 8.2	515 ± 11.9	532 ± 16.5	505 ± 21.8
Isoprenaline	0.1 μg kg^{-1} min^{-1}	4	118 ± 15.2	62 ± 11.0*	66 ± 7.8	457 ± 11.0	520 ± 10.0*	510 ± 5.7*
Forskolin	10.0 μg kg^{-1} min^{-1}	4	111 ± 12.3	50 ± 3.0*	42 ± 9.3*	455 ± 22.6	497 ± 23.6*	542 ± 8.5*
Saline vehicle	1.0 ml kg^{-1}	6	109 ± 10.4	123 ± 10.4	90 ± 9.4	435 ± 16.3	436 ± 17.8	395 ± 15.2
Ethanol vehicle	1.0 ml kg^{-1} min^{-1}	5	130 ± 18.0	120 ± 8.1	131 ± 14.1	482 ± 7.4	466 ± 9.3	466 ± 5.1

* $P<0.05$ Paired t test compared to '0' min reading
** $P<0.01$ Paired t test compared to '0' min reading

after dosing respectively. Dosing with saline (vehicle for flosequinan, milrinone and isoprenaline) or ethanol (vehicle for forskolin) caused little change in blood pressure or heart rate. The falls in mean blood pressure observed 5 min after dosing with the various drugs were not significantly different from each other and were substantially maintained for the duration of the experiment.

Effect upon ischaemia-induced arrhythmias

In the second series of experiments a 25.9% mortality due to ventricular fibrillation was observed following coronary artery ligation in the combined control group, although in individual control groups mortality varied considerably (from 0% to 50%; Table 2b). All mortality due to VF occurred within the first 15 min following coronary artery ligation (93% in the first 5–10 min period). In survivors of the 30 min ischaemic period, a total PVB count in the combined group of 751 ± 117 ($n = 43$) was observed, although considerable variation was again shown between the individual control groups. PVB count in the combined control group 0–5 min following ligation was 43 ± 11 and increased markedly between 5–10 min (333 ± 53) and 10–15 min (273 ± 64) after coronary artery ligation and then fell to almost zero towards the end of the 30 min observation period (3 ± 2). Although initial studies indicated that peak arrhythmogenesis occurred at 15 min following coronary ligation, overall analysis revealed peak arrhythmias at 10 min following ligation as shown above. However, for consistency with the arrhythmia study, all samples for the subsequent cyclic nucleotide analysis were taken at 15 min or earlier as appropriate.

Mean blood pressure and heart rate were not significantly different between the control groups and were largely unaffected by dosing with vehicle (Table 2a). Following coronary artery ligation, blood pressure decreased in the various control groups by 22% to 48% (mean 34%) whereas heart rate was unaffected.

Neither flosequinan nor milrinone caused any significant change in mortalitty or arrhythmias compared to their respective control or combined saline control groups (Table 2b). Flosequinan (3.0 and 6.0 mg kg^{-1}) caused a 31% and 32% fall in blood pressure and a 1.5% and 5.6% rise in heart rate respectively, which was smaller than expected from sham-operated data, and milrinone (0.3 mg kg^{-1}) caused mean blood pressure to fall by 56% with no change in heart rate. Both isoprenaline and forskolin significantly increased the mortality compared to their respective control groups although the PVB count was not significantly affected, possibly due to the low number of survivors. Isoprenaline and forskolin produced a 39% and 48% fall in mean blood pressure accompanied by a tachycardia of 17.8% and 7.1% respectively. Although analysis of variance showed that the pre-dose mean blood pressures of the drug-treated groups were not significantly different, 5 min after dosing the mean blood pressure in the milrinone group was significantly different ($P<0.05$) from both the flosequinan dosed groups.

Effect of cyclic nucleotide levels

In the third series of experiments no significant difference in cyclic AMP or cyclic GMP content was observed between the ischaemic left ventricle and the non-ischaemic right ventricle in any control or drug treated group (Table 3b). Statistical analysis has therefore been conducted on the values for left, right and average ventricular nucleotide levels. In addition, in sham-operated rats, left and right ventricular cyclic AMP (6.74 ± 0.26 and 6.71 ± 0.31, pmol mg^{-1} protein, $n = 6$) and cyclic GMP content (0.23 ± 0.05 and 0.36 ± 0.04, pmol mg^{-1} protein, $n = 6$) were not significantly different from the values in the control treated coronary artery-ligated rats.

The higher cyclic GMP values observed in the flosequinan and isoprenaline groups were due to a different antibody used in earlier batches of the pre-production assay kits employed. Representative samples from both groups were

Table 2a Effect of treatment on mean blood pressure and heart rate in coronary ligated anaesthetized rats

			Mean blood pressure (mmHg)		*Heart rate* (beats min^{-1})	
Treatment	*Dose*	n	Pre-dose	5 min	Pre-dose	5 min
Flosequinan	3.0 mg kg^{-1}	13	119 ± 6.1	82 ± 6.6	457 ± 9.1	466 ± 9.0
Saline	1.0 ml kg^{-1}	13	116 ± 7.9	120 ± 8.0	462 ± 11.6	463 ± 12.0
Flosequinan	6.0 mg kg^{-1}	12	109 ± 10.3	74 ± 8.6	440 ± 16.6	465 ± 12.9
Saline	1.0 ml kg^{-1}	10	114 ± 13.3	106 ± 13.3	459 ± 11.9	450 ± 10.7
Milrinone	0.3 mg kg^{-1}	13	107 ± 9.1	47 ± 5.0	460 ± 12.2	464 ± 10.7
Saline	1.0 ml kg^{-1}	13	115 ± 9.1	118 ± 8.5	457 ± 12.0	456 ± 12.8
Isoprenaline	0.1 μg kg^{-1} min^{-1}	11	107 ± 14.4	65 ± 7.0	420 ± 14.3	495 ± 11.5
Saline	0.3 ml kg^{-1} min^{-1}	9	95 ± 16.3	102 ± 16.5	407 ± 19.5	424 ± 17.3
Forskolin	10.0 μg kg^{-1} min^{-1}	12	118 ± 5.6	61 ± 6.8	464 ± 13.5	497 ± 5.0
Ethanol	0.3 ml kg^{-1} min^{-1}	13	120 ± 8.7	103 ± 10.1	451 ± 11.1	424 ± 11.4
Combined controls		58	113 ± 8.7	110 ± 4.8	449 ± 6.1	445 ± 5.9

Table 2b Effect of treatment on mortality and ischaemia-induced arrhythmias in coronary ligated anaesthetized rats

Treatment	Dose	n	% mortality	n	PVB count	Survivors Incidence of (%) VT	Survivors Incidence of (%) VF	Duration of (s)† VT	Duration of (s)† VF
Flosequinan	3.0 mg kg^{-1}	13	7.7	12	315 ± 67	92	17	12 ± 5	45,49
Saline	1.0 ml kg^{-1}	13	15.4	11	361 ± 119	89	9	21 ± 8	68
Flosequinan	6.0 mg kg^{-1}	12	33.3	8	679 ± 196	88	50	52 ± 15	27 ± 11
Saline	1.0 ml kg^{-1}	10	50.0	6	598 ± 224	67	17	42 ± 20	5
Milrinone	0.3 mg kg^{-1}	13	23.1	10	767 ± 211	90	20	55 ± 17	4,18
Saline	1.0 ml kg^{-1}	13	30.7	9	743 ± 196	100	56	42 ± 11	41 ± 10
Isoprenaline	0.1 μg kg^{-1} min^{-1}	11	72.7*	3	370 ± 140	100	100	17 ± 5	39 ± 17
Saline	0.3 ml kg^{-1} min^{-1}	9	0	9	899 ± 263	89	67	68 ± 32	56 ± 21
Forskolin	10.0 μg kg^{-1} min^{-1}	12	75.0*	3	1264 ± 282	100	67	64 ± 11	46,55
Ethanol	0.3 ml kg^{-1} min^{-1}	13	30.7	9	1172 ± 392	89	22	70 ± 30	18,4
Combined controls		58	25.9	44	751 ± 117	89	34	40 ± 10	56 ± 13

* $P<0.05$ Fisher's exact test compared to corresponding control group.
† Individual values shown where $n<3$

subsequently reassayed with a later version of the kit and compared to that of the milrinone group which had been previously assayed with the later version of the kit. The values of cyclic GMP changed from 1.26 ± 0.02 to 0.45 ± 0.09 in the flosequinan samples and from 1.96 ± 0.03 to 0.39 ± 0.05 pmol mg^{-1} protein in the isoprenaline samples whilst that of the milrinone samples were unchanged (0.28 ± 0.03 compared to 0.25 ± 0.01 mean ± s.e.mean, $n = 5$). Therefore the variation in absolute cyclic GMP levels observed between the various groups was due to the different versions of the assay kit. However, since the assay of cyclic GMP for both drug-treated and corresponding control groups were assayed simultaneously with the same kit, it is not considered that the variation of cyclic GMP values observed from kit to kit has any bearing on the interpretation of the results.

Flosequinan (6.0 mg kg^{-1}) did not significantly affect either mortality (Table 3a) or cyclic nucleotide levels compared with control, but caused a 43.8% fall in mean blood pressure and a 2% increase in heart rate. The increase in mortality after 0.3 mg kg^{-1} milrinone was not significant ($P = 0.21$ and $P = 0.06$ versus corresponding and combined controls respectively), but left ventricular and mean cyclic AMP levels were significantly increased ($P<0.05$). Milrinone (0.3 mg kg^{-1}) caused a 60.5% fall in blood pressure associated with a slight fall in heart rate of 2%. Milrinone at 0.2 mg kg^{-1} caused a lower reduction in blood pressure of 43% with no effect on heart rate and significantly increased both mean cyclic AMP levels ($P<0.05$) and mortality compared to the combined control data ($P = 0.02$) but not against the corresponding controls ($P = 0.09$). Isoprenaline caused a 41.3% fall in blood pressure and a 9.2% tachycardia and significantly increased mortality compared to its corresponding control group, the mean and right ventricular cyclic AMP being significantly increased. Forskolin, which caused a 42.9% fall in mean blood pressure and a 5.7% increase in heart rate, significantly raised mean and right ventricular cyclic AMP compared to corresponding control values. Mortality in the forskolin dosed group was not significantly higher than the corresponding or combined control group.

In the various drug-treated groups, mean blood pressure both before and 5 min after dosing were not significantly different from each other. Similarly the percentage falls in mean blood pressure were also not significantly different from each other (oneway analysis of variance, $F = 0.97$, $P = 0.43$; $F = 0.89$, $P = 0.47$ and $F = 1.67$, $P = 0.17$, for pre-dose, post-dose and percentage fall in blood pressure respectively).

Table 3a Effect of treatment on mean blood pressure, heart rate and mortality in coronary ligated anaesthetized rats

Treatment	Dose	n	Mean blood pressure (mmHg) Pre-dose	Mean blood pressure (mmHg) 5 min	Heart rate (beats min^{-1}) Pre-dose	Heart rate (beats min^{-1}) 5 min	% mortality
Flosequinan	6.0 mg kg^{-1}	11	114 ± 8.9	64 ± 9.6	479 ± 10.0	489 ± 17.9	9.1
Saline	1.0 ml kg^{-1}	13	104 ± 9.9	99 ± 11.2	464 ± 13.8	468 ± 14.3	38.5
Milrinone	0.3 mg kg^{-1}	11	119 ± 5.4	47 ± 8.0	446 ± 10.7	436 ± 14.5	72.7
Saline	1.0 ml kg^{-1}	12	107 ± 5.9	114 ± 7.3	435 ± 10.1	434 ± 10.9	41.6
Milrinone	0.2 mg kg^{-1}	11	122 ± 4.4	70 ± 7.0	474 ± 8.3	474 ± 12.3	81.8[b]
Saline	1.0 ml kg^{-1}	12	109 ± 6.5	115 ± 5.5	459 ± 12.0	465 ± 11.0	41.6
Isoprelanine	0.1 μg kg^{-1} min^{-1}	12	104 ± 6.8	61 ± 9.1	424 ± 8.6	463 ± 7.0	75.0[a]
Saline	0.3 ml kg^{-1} min^{-1}	11	94 ± 11.4	87 ± 9.2	403 ± 13.2	402 ± 14.8	27.3
Forskolin	10.0 μg kg^{-1} min^{-1}	12	114 ± 8.1	65 ± 10.2	459 ± 10.4	485 ± 13.3	50.0
Ethanol	0.3 ml kg^{-1} min^{-1}	12	118 ± 6.2	106 ± 7.5	460 ± 9.7	444 ± 8.4	50.0
Combined controls		60	107 ± 3.7	104 ± 3.9	445 ± 5.9	444 ± 6.1	40.0

[a] $P<0.05$ Fisher's exact test compared with corresponding control group
[b] $P<0.05$ Fisher's exact test compared with combined control group

Table 3b Effect of treatment on mortality and ventricular cyclic nucleotides in coronary ligated anaesthetized rats

			Cyclic AMP (pmol mg^{-1}protein†)			*Cyclic GMP* (pmol mg^{-1} protein†)		
Treatment	*Dose*	n	*LV*	*RV*	*Mean*	*LV*	*RV*	*Mean*
Flosequinan	6.0 mg kg^{-1}	11	7.00 ± 0.41	6.87 ± 0.41	6.94 ± 0.34	1.47 ± 0.79	1.28 ± 0.13	1.33 ± 0.14
Saline	1.0 ml kg^{-1}	13	6.70 ± 0.31	6.34 ± 0.22	6.52 ± 0.21	1.37 ± 0.10	1.39 ± 0.20	1.29 ± 0.08
Milrinone	0.3 mg kg^{-1}	11	8.44 ± 0.55[b]	8.51 ± 0.33	8.47 ± 0.38[bc]	0.38 ± 0.03	0.36 ± 0.03	0.37 ± 0.02
Saline	1.0 ml kg^{-1}	12	6.94 ± 0.27	7.62 ± 0.34	7.27 ± 0.79	0.39 ± 0.06	0.35 ± 0.05	0.37 ± 0.05
Milrinone	0.2 mg kg^{-1}	11	8.54 ± 0.23[c]	9.54 ± 0.25[d]	9.04 ± 0.14[bd]	0.65 ± 0.42	0.68 ± 0.38	0.67 ± 0.40
Saline	1.0 ml kg^{-1}	12	8.10 ± 0.17	8.76 ± 0.31	8.43 ± 0.22	0.34 ± 0.05	0.40 ± 0.07	0.37 ± 0.05
Isoprenaline	0.1 μg kg^{-1} min^{-1}	12	8.54 ± 0.39[c]	9.01 ± 0.55[bd]	8.77 ± 0.42[bd]	2.81 ± 0.28	2.19 ± 0.19	2.60 ± 0.25
Saline	0.3 ml kg^{-1} min^{-1}	11	7.57 ± 0.53	7.36 ± 0.39	7.47 ± 0.38	2.60 ± 0.19	2.08 ± 0.11	2.38 ± 0.16
Forskolin	10.0 μg kg^{-1} min^{-1}	12	10.89 ± 1.07[d]	10.85 ± 1.08[bd]	10.87 ± 1.08[bd]	0.26 ± 0.04	0.25 ± 0.04	0.26 ± 0.04
Ethanol	0.3 ml kg^{-1} min^{-1}	12	6.98 ± 0.98	7.32 ± 0.83	7.15 ± 0.91	0.24 ± 0.02	0.33 ± 0.05	0.28 ± 0.03
Combined controls		60	7.25 ± 0.24	7.46 ± 0.23	7.35 ± 0.22	0.93 ± 0.12	0.89 ± 0.10	0.86 ± 0.10

[b] $P<0.05$ compared to corresponding control group.
[c] $P<0.05$ compared to combined control group.
[d] $P<0.01$ compared to combined control group.
† 1 g wet ventricular weight is equivalent to 119 mg protein

None of the treatments significantly altered cyclic GMP levels.

With respect to the mean cyclic AMP levels of ventricular tissue (pmol mg^{-1} protein) from animals which survived or those which fibrillated during the experiment, no significant difference was observed in either the control treated (7.05 ± 0.19, $n = 35$ vs 7.39 ± 0.22, $n = 24$) or drug-treated animals (8.49 ± 0.61, $n = 23$ vs 9.06 ± 0.27, $n = 33$) respectively.

PVB counts were also recorded in this cyclic nucleotide study and were unaffected by flosequinan (326 ± 68 ($n = 11$) for flosequinan vs 443 ± 212 ($n = 8$) for vehicle). The PVB counts after milrinone, isoprenaline and forskolin were milrinone 0.3 mg kg^{-1} 1103 ± 752 ($n = 3$) vs 244 ± 60 ($n = 7$); milrinone 0.2 mg kg^{-1} 257 ± 131 ($n = 2$) vs 751 ± 334 ($n = 7$); isoprenaline, 524 ± 253 ($n = 4$) vs 443 ± 80 ($n = 7$) and forskolin 994 ± 306 ($n = 6$) vs 630 ± 208 ($n = 6$) (for drug-treated groups versus corresponding control groups respectively) and although differences were observed, none was significantly different due to the low number of survivors.

Discussion

In anaesthetized rats undergoing coronary artery ligation, an overall mortality due to VF of 25.9% was observed in the combined control group. In survivors of the 30 min post ischaemic period the PVB count of 751 ± 117 was lower than that found by others (about 1200) although the overall mortality and incidence of arrhythmias was similar to that previously reported (Clark *et al.*, 1980; Marshall *et al.*, 1981; Daugherty *et al.*, 1986; Kane *et al.*, 1985). In individual control groups the mortality, PVB count and incidence of VT and VF varied considerably. Although the reasons for these variations are unknown, it emphasises the need to run concurrent groups of test and control animals rather than rely on a single control group against which all other treatments are compared, particularly when experiments are conducted over a period of time.

Flosequinan at 3.0 and 6.0 mg kg^{-1} i.v. did not influence mortality or ischaemia-induced arrhythmias. The hypotensive effects of flosequinan in the arrhythmia study were less than that expected from the sham-operated control studies due to some animals demonstrating only transient hypotension in both dosage groups. Since all deaths due to VF occurred within the first 15 min after coronary ligation, mortality data from the cyclic nucleotide study can also be considered with the mortality data in the arrhythmias study. In the subsequent cyclic nucleotide study, flosequinan (at 6.0 mg kg^{-1}) again had no effect on mortality or arrhythmia count in survivors and no changes in cyclic nucleotide levels were observed.

Of the other drugs tested, isoprenaline demonstrated similar effects in both arrhythmia and cyclic nucleotide studies in that mortality was significantly raised in both studies and was associated with an increase in cyclic AMP. Although both milrinone (at both 0.2 and 0.3 mg kg^{-1}) and forskolin significantly elevated cyclic AMP they did not have a consistent effect upon mortality or arrhythmias in the two studies conducted. Milrinone was without effect on mortality or PVB count in the arrhythmia study whereas in the two subsequent cyclic nucleotide studies a significant increase in mortality was observed with the lower dose (0.2 mg kg^{-1}) but this did not quite reach significance with the higher dose (0.3 mg kg^{-1}). Forskolin significantly increased mortality in the arrhythmia study although mortality was unaltered in the cyclic nucleotide study.

The results presented indicate that isoprenaline and forskolin are associated with an increase in mortality following coronary artery ligation and also increased heart rate. Tosaki *et al.* (1990) observed that in the rat isolated heart isoprenaline exacerbated reperfusion-induced arrhythmias primarily as a result of β_1-receptor mediated tachycardia. It was concluded therefore that heart rate during reperfusion was a major determinant of VF although the incidence of VF appears to plateau off at pacing rates above 350 beats per minute. The higher heart rate of anaesthetized animals compared to rat isolated hearts is due to a high sympathetic tone induced by barbiturate anaesthesia. In coronary artery ligated anaesthetized rats, Marshall *et al.* (1981) showed that a single intravenous dose of 10 μg kg^{-1} isoprenaline, which produced a significant 10% increase in heart rate and a 43% decrease in blood pressure, increased mortality from 7% to 50% and markedly increased arrhythmias. Interestingly Marshall *et al.* (1981) also showed that pressor doses of adrenaline, noradrenaline and phenylephrine reduced the number of arrhythmias and it was suggested that this was due to the reflex increase in vagal tone modifying sympathetic tone, although this was not associated with bradycardia. Increased sympathetic tone, and high level of catecholamines have been associated with the increased severity of ventricular arrhythmias but in rats the circulating catecholamine levels do not appear to influence significantly ischaemia-induced arrhythmias (Daugherty *et al.*, 1986).

In the present study a comparison of the initial heart rate of control animals which survived or died due to VF revealed no significant differences (445 ± 5.3 ($n = 78$) and 452 ± 7.3 ($n = 40$) beats per minute respectively, $P = 0.41$, NS, combined data from the arrhythmia and cyclic nucleotide

studies). Similarly in the drug-treated animals, heart rate following dosing was again not related to incidence of VF (477 ± 5.4 ($n = 60$) in survivors and 470 ± 6.0 ($n = 58$) in animals dying of VF). Heart rate does not therefore appear to be related to mortality in this study.

With respect to cyclic nucleotide levels in arrhythmogenesis, previous studies in the cat (Corr *et al.*, 1978), pig (Podzuweit *et al.*, 1981), and baboon (Podzuweit, 1982) suggested that cyclic AMP levels rose in ischaemic tissue and that this rise preceded the onset of fibrillation. However, Podzuweit (1982) observed that in rat isolated hearts increases in cyclic AMP were observed 30–45 min after ischaemia and that the rise in cyclic AMP was substrate-dependent, being inhibited by glucose. In isolated perfused hearts of the rat Lubbe *et al.* (1983) showed a significant rise in cyclic AMP content in the ischaemic ventricle 15 min post ligation which was related to a reduction in fibrillation threshold. Similarly, in anaesthetized rats, Kane *et al.* (1985) showed that the cyclic AMP content of left (ischaemic) and right (non-ischaemic) ventricular tissue was increased 5 min following coronary artery ligation, returning to baseline levels thereafter. This also suggested that the rise in cyclic AMP following ischaemia may be a brief and transitory effect which preceded the onset of arrhythmias. Manning *et al.* (1985) also observed only a marginal and transient increase in cyclic AMP in rat isolated hearts 3 min following coronary artery ligation which fell back to normal within 10 min. These authors also showed that although forskolin markedly raised cyclic AMP levels it did not induce arrhythmias in this model, calling into question the link between cyclic AMP and ischaemia-induced arrhythmias. Recently Worthington & Opie (1992) have reported that the mechanism by which cyclic AMP is elevated has a profound influence on the fibrillation threshold in isolated rat hearts. Large increases in cyclic AMP caused by forskolin did not cause such a large decrease in fibrillation threshold as that produced following the smaller increases in cyclic AMP caused by isoprenaline. Compartmentalisation of cyclic AMP may be an explanation for these differing effects and may help to explain the observations in this study of a consistent increase in mortality caused by isoprenaline compared to the inconsistent effects on mortality of forskolin and milrinone even though these agents caused an increase in cyclic AMP greater than or similar to that observed with isoprenaline.

In this study no significant difference in cyclic nucleotides was observed between either sham-operated rats and coronary artery-ligated controls or between ischaemic and non-ischaemic ventricular tissue in control or treated groups. We present no evidence therefore to support the hypothesis that ischaemia *per se* is related to elevation in cyclic AMP although it is possible that tissue samples were taken too late after coronary ligation to measure an early transient rise. There is however, evidence to suggest that drug-induced elevations in cyclic AMP caused by either selective phosphodiesterase type III inhibition, β-adrenoceptor activation or direct activation of adenylate cyclase are associated with an increased incidence of arrhythmias. Since flosequinan causes neither an elevation of cyclic AMP nor an increase in arrhythmias this suggests that cyclic AMP is not involved in its pharmacological activity in this model.

In conclusion, when compared at similarly hypotensive doses, flosequinan, in contrast to milrinone, isoprenaline and forskolin, does not influence ischaemia-induced arrhythmias or raise ventricular cyclic nucleotide levels in the anaesthetized rat.

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References

CLARK, C., FOREMAN, M.I., KANE, K.A., McDONALD, F.M. & PARRATT, J.R. (1980). Coronary artery ligation in anaesthetised rats as a method for the production of experimental dysrhythmias and for the determination of infarct size. *J. Pharmacol. Methods*, **3**, 357–368.

CORR, P.B., WITKOWSKI, F.X. & SOBEL, B.E. (1978). Mechanisms contributing to malignant dysrhythmias induced by ischaemia in the cat. *J. Clin. Invest.*, **61**, 109–119.

DAUGHERTY, A., FRAYN, K.N., REDFERN, W.S. & WOODWARD, B. (1986). The role of catecholamines in the production of ischaemia-induced ventricular arrhythmias in the rat *in vivo* and *in vitro*. *Br. J. Pharmacol.*, **87**, 265–277.

FRODSHAM, G. & JONES, R.B. (1992). Effect of flosequinan upon isoenzymes of phosphodiesterase from guinea-pig cardiac and vascular smooth muscle. *Eur. J. Pharmacol.*, **211**, 383–391.

GRISTWOOD, R.W., BELETA, J., BOU, J., CARDELUS, I., FERNANDEZ, A.G., LLENAS, J. & BERGA, P. (1992). Studies on the cardiac actions of flosequinan *in vitro*. *Br. J. Pharmacol.*, **105**, 985–991.

JONES, R.B., FRODSHAM, G., DICKINSON, K. & FOSTER, G.A. (1991). Effect of flosequinan on cGMP and cAMP levels in non-ischaemic and ischaemic rat ventricle. *Br. J. Pharmacol.*, **104**, 173P.

JONES, R.B. & SIM, M.F. (1990). Effect of flosequinan on ischaemic arrhythmias in anaesthetised rats. *Br. J. Pharmacol.*, **100**, 440P.

KANE, K.A., MORCILLO-SANCHEZ, E.J., PARRATT, J.R., RODGER, I.W. & SHAHID, M. (1985). The relationship between coronary artery occlusion-induced arrhythmias and myocardial cyclic nucleotide levels in the anaesthetised rat. *Br. J. Pharmacol.*, **84**, 139–145.

LUBBE, W.F., NGUYEN, T. & WEST, E.J. (1983). Modulation of myocardial cyclic AMP and vulnerability to fibrillation in the rat heart. *Fed. Proc.*, **42**, 2460–2464.

MANNING, A.S., KINOSHITA, K., BUSCHMANS, E., COLTART, D.J. & HEARSE, D.J. (1985). The genesis of arrhythmias during myocardial ischaemia. Dissociation between changes in cyclic adenosine monophosphate and electrical instability in the rat. *Circ. Res.*, **57**, 668–675.

MARKWELL, M.A.K., HAAS, S.M., BIEBER, L.L. & TOLBERT, N.E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.*, **87**, 206–210.

MARSHALL, R.J., MUIR, A.W. & WINSLOW, E. (1981). Development of a severe model of early coronary artery ligation-induced dysrhythmias in the anaesthetised rat. *Br. J. Pharmacol.*, **73**, 951–959.

PACKER, M. (1989). Effect of phosphodiesterase inhibitors on survival of patients with chronic congestive heart failure. *Am. J. Cardiol.*, **63**, 41A–45A.

PODRID, P.J., FUCHS, T. & CANDINAS, R. (1990). Role of the sympathetic nervous system in the genesis of ventricular arrhythmias. *Circulation*, **82** (suppl 1), I-103–I-113.

PODZUWEIT, T. (1982). Early arrhythmias resulting from acute myocardial ischaemia; possible role of cyclic AMP. In *Early Arrhythmias Resulting from Myocardial Ischaemia, Mechanisms and Prevention by Drugs*. ed Parratt, J.R. pp. 171–198. London: Macmillan Press.

PODZUWEIT, T., ELS, D.J. & McCARTHY, J. (1981). Cyclic AMP mediated arrhythmias induced in the ischaemic pig heart. *Basic. Res. Cardiol.*, **76**, 443–448.

SELYE, H., BAJUSZ, E., GRASSO, S. & MENDELL, P. (1960). Simple techniques for the surgical occlusion of coronary vessels in the rat. *Angiology*, **11**, 398–407.

WILSON, J.R. (1987). Use of antiarrhythmic drugs in patients with heart failure: clinical efficiacy, haemodynamic results, and relation to survival. *Circulation*, **75**, (suppl IV), IV-64–IV-73.

WORTHINGTON, M.G. & OPIE, L.H. (1992). Contrasting effects of cyclic AMP increase caused by β-adrenergic stimulation or by adenylate cyclase activation on ventricular fibrillation threshold of isolated rat heart. *J. Cardiovasc. Pharmacol.*, **20**, 595–600.

TOSAKI, A., WOODWARD, B., YAMAMOTO, F. & HEARSE, D.J. (1990). Isoproterenol and the genesis of reperfusion-induced arrhythmias in isolated rat heart: adrenoceptor or free radical-mediated mechanisms? *J. Cardiovasc. Pharmacol.*, **15**, 398–407.

YATES, D.B. (1991). Pharmacology of flosequinan. *Am. Heart. J.*, **121**, 974–983.

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Antiepileptic drug pharmacokinetics and neuropharmacokinetics in individual rats by repetitive withdrawal of blood and cerebrospinal fluid: milacemide

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1 The kinetics and metabolism of milacemide have been studied in an animal model which allows the simultaneous investigation of the temporal inter-relationships of drugs and metabolites in blood (pharmacokinetics) and cerebrospinal fluid (CSF, neuropharmacokinetics) in individual freely moving rats.

2 Milacemide dose-dependently increased CSF glycine and glycinamide (intermediary metabolite) concentrations. This confirms that milacemide is a CNS glycine prodrug.

3 Pretreatment with L-deprenyl (2 mg kg^{-1}), a specific inhibitor of monoamine oxidase type B (MAO-B), almost completely prevented the formation of glycinamide and increased milacemide accumulation in CSF. T_{max} and $t_{1/2}$ were significantly increased and C_{max} and AUC values were decreased for glycinamide compared to controls. Pretreatment with clorgyline (5 mg kg^{-1}), a specific inhibitor of MAO-type A, only moderately decreased glycinamide C_{max} and AUC values.

4 After milacemide administration (100, 200 and 400 mg kg^{-1}, i.p.) serum and CSF milacemide concentrations rose linearly and dose-dependently. Serum glycinamide concentrations exhibited small dose-dependent rises but these were not linearly related. In contrast, CSF glycinamide concentrations rose linearly and dose-dependently with C_{max} values 2.5, 3.2 and 4.1 times greater than the corresponding values for serum glycinamide after giving 100, 200 and 400 mg kg^{-1} respectively of milacemide.

5 Serum glycine concentrations were unaffected but CSF concentrations increased dose-dependently and these were significant at the higher milacemide doses (200 and 400 mg kg^{-1}). Animals given 400 mg kg^{-1} milacemide had glycine values which were still significantly elevated 7 h later.

6 In conclusion, serum milacemide rapidly enters and equilibrates with the CNS compartment where it is metabolised primarily by MAO-B to glycinamide and finally to glycine. Metabolism in the peripheral compartment is negligible.

Keywords: Milacemide; glycine; cerebrospinal fluid; pharmacokinetics; neuropharmacokinetics; monoamine oxidase

Introduction

Glycine, a non-essential and the simplest amino acid known, was first proposed as a spinal inhibitory neurotransmitter by Aprison & Werman, (1965) and subsequently has been found to have numerous functions within the central nervous system (CNS). It is a major postsynaptic inhibitor, an essential modulator of the N-methyl-D-aspartate (NMDA) receptor and it is also involved in the metabolism of proteins, peptides, one-carbon units and nucleotides (Brody *et al.*, 1976; Borman *et al.*, 1987; Johnson & Asher, 1987). As glycine is considered to play a role in disorders such as epilepsy, Alzheimer's disease, Huntington's disease, stroke and schizophrenia, its pharmacological manipulation may have therapeutic potential (Greenamyre *et al.*, 1985; Rothman & Olney, 1987; Deutsch *et al.*, 1989).

The influx of glycine into the brain from the blood under physiological conditions is negligible (Oldendorf, 1971) but very large oral or intraperitoneal doses can increase brain glycine concentrations significantly (Richter & Wainer, 1971; Toth & Lajtha, 1981) and have an anticonvulsant effect in numerous seizure models (Toth *et al.*, 1983; Spenos & Darly, 1984). Glycine also enhances the anticonvulsant effect of other drugs (Liu *et al.*, 1990; Paterson, 1991). However, in man it is not known whether the accompanying elevation in extracerebral glycine is harmful (Truong & Fahn, 1988; Waziri, 1988).

Milacemide (2-n-pentylaminoacetamide), a glycinamide derivative, is considered to be a glycine prodrug and has anticonvulsant, mood elevating and cognitive enhancing properties (van Dorsser *et al.*, 1983; Houtkooper *et al.*, 1986; Norton *et al.*, 1986; Saletu *et al.*, 1986; Handelmann *et al.*, 1989; Schwartz *et al.*, 1992). Its exact mechanism of action is unclear but glycine, γ-aminobutyric acid (GABA) and dopamine may play a role (de Varebeke *et al.*, 1983; Semba *et al.*, 1992). Milacamide readily crosses the blood-brain-barrier and is converted by N-dealkylation to glycinamide and then to glycine. The first step is considered to be catalysed by the enzyme monamine oxidase type B (MAO-B; de Varebeke *et al.*, 1989). As rat brain glycine levels can be elevated by oral milacemide administration (Christophe *et al.*, 1983) it may have potential in the treatment of disorders associated with glycine dysfunction.

In order that the therapeutic potential of a drug can be optimumly realised, it is essential to devise a rational dosing regimen based on its kinetics. Therefore, we have used a procedure (Patsalos *et al.*, 1992) based on earlier CSF studies (Hutson *et al.*, 1985; Sokomba *et al.*, 1988) which permits simultaneous investigation of the temporal inter-relationship of drugs and metabolites in blood (pharmacokinetics) and cerebrospinal fluid (CSF; neuropharmacokinetics) in individual freely moving rats to study the kinetics of milacemide and its metabolism. Furthermore, using selective inhibitors of MAO-B and MAO type A (MAO-A) we have determined their respective contributions to the metabolism of milacemide and thus their possible importance in modulating its pharmacological activity.

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Methods

Male Sprague-Dawley rats (Charles River, Margate, Kent) weighing 250–300 g were individually housed under a 12 h light-dark cycle (lights on 06 h 00 min) with free access to a normal laboratory diet (SDS R and M number 1 expanded, Scientific Dietary Services, Witham, Essex) and water.

Surgical procedures, CSF and blood sampling

Rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) and catheters implanted in the cisterna magna, for CSF sampling, and the right jugular vein, for blood sampling, as previously described (Patsalos *et al.*, 1992).

Three days after surgery, CSF and blood catheters were checked for patency and CSF (30 µl) and blood (200 µl) samples simultaneously collected every 30 min for 1.5 h; the first two samples were not used for analysis and were discarded.

Animals were then injected with milacemide (100, 200 or 400 mg kg^{-1} dissolved in 0.154 M NaCl; Continental Pharma Inc., Belgium) intraperitoneally and CSF and blood were withdrawn at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4, 5, 6, 7 and 8 h later for milacemide, glycinamide and glycine determination. CSF samples were collected in 0.5 ml polypropylene tubes, (Treff Lab, Dagersheim, Switzerland) and blood samples in 1.5 ml polypropylene tubes (Bio-Medical Laboratories Supplies, Birmingham, UK). After clot retraction, sera were separated by centrifugation. Sera and CSF were stored at −20°C until analysis.

Milacemide and glycinamide analysis

Milacemide and glycinamide concentrations in sera (10 µl) and CSF (5 µl) were determined by high performance liquid chromatography (h.p.l.c.) with u.v. and spectrofluorometric detection respectively as described by Semba *et al.* (1991).

Glycine analysis

Glycine concentrations in CSF and sera were determined by h.p.l.c. and based on a modification of the method of Turnell & Cooper (1982) as follows:

CSF By use of a Gilson autoinjector (model 231), 2.5 µl CSF, 10 µl homoserine (internal standard; 60 mM constituted in 100 mM borate buffer pH 9.5 and containing mercaptoethanol 60 µl per 25 ml) and 10 µl *o*-phthalaldehyde solution (50 mg *o*-phthalaldehyde dissolved in 1 ml methanol and made up to 10 ml with 50 µl mercaptoethanol and 100 mM borate buffer pH 9.5) were pipetted into a plastic autosampling vial. After mixing, derivatisation was allowed to proceed for 1 min and then 10 µl of the mixture was injected into a Gilson liquid chromatography apparatus controlled by a Hewlett-Packard Vectra EC/12 computer with data processing capability. A 20 µl loop and a Kratos fluorescence detector (model GM970, Ramsey, NJ, U.S.A.) set at excitation wavelength 395 nm and emission filter cut-off 470 nm were used. The analytical column was Microsorb C18 (5 µm particle size, 15 cm × 4.6 mm i.d., Rainin Instrument Company Inc, Woburn, MA, U.S.A.) attached to a 1.5 cm × 4.6 mm i.d. guard column packed with the same material (Rainin Instrument Company, Inc). The mobile phase comprised: Solvent A–640 ml of 250 mM sodium dihydrogen orthophosphate, 190 ml acetonitrile made up to 1900 ml with water (h.p.l.c. grade), buffered to pH 6.5 with 5 M NaOH and made to a final volume of 2 litres with water. Solvent B–560 ml acetonitrile, 480 ml methanol and 460 ml water. All solvents were filtered (0.45 µm membrane, HVLP 04700, Millipore U.K. Ltd, Watford, U.K.) and degassed with helium (15–20 min) before use. The gradient programme used was: 100%A (0–10 min), 92% A/ 8%B (10–22 min) and the retention times for homoserine and glycine were 15 and 17 min respectively. The flow rate was set at 2.0 ml min^{-1}.

Serum The procedures used for serum were essentially the same as for CSF except that deproteinisation was first undertaken as follows: 5 µl serum, 10 µl homoserine (internal standard) and 50 µl deproteinisation reagent (comprising 5 ml acetonitrile and 10 µl mercaptoethanol), were pipetted into a plastic microcentrifuge tube and shaken for 2 min. After centrifugation for 2 min the supernatant was removed and 10 µl were mixed with 10 µl *o*-phthalaldehyde derivatisation mixture (described above) and 7 µl injected into the h.p.l.c. apparatus. The solvent gradient programme used was: 100%A (0–4 min), 85%A/15%B (4–22.2 min) and the retention times for homoserine and glycine were 14 and 16 min respectively.

Effect of selective monoamine oxidase inhibitors on milacemide metabolism

By use of clorgyline (May and Baker, Dagenham, U.K.) and L-deprenyl, specific inhibitors of MAO-A and MAO-B respectively, the contribution of these enzymes to the metabolism of milacemide to glycinamide was examined. Rats were cannulated for CSF sampling only and clorgyline (5 mg kg^{-1}), L-deprenyl (2 mg kg^{-1}) or saline vehicle (0.154 M NaCl) were administered intraperitoneally ($n = 4–7$ per treatment group). Two hours later the rats were given milacemide (400 mg kg^{-1}, i.p.) and CSF (30 µl) samples collected at 30 min intervals for 4 h.

Pharmacokinetic analysis

Milacemide and glycinamide versus time data for both sera and CSF were analysed according to a one compartment model using PCNONLIN 3.0 programme (Metzler & Weiner, 1988) with a weighting factor of 1/concentration. Initial parameter estimates were obtained using JANA (Dunn, 1985) and both programmes were implemented on an Amstrad 2286/40 computer. The following parameters were computed: time to maximum concentration (T_{max}), maximum concentration (C_{max}), area under the concentration versus time curve (AUC) and apparent elimination half-life ($t_{1/2}$).

Statistics

Statistical analysis of the pharmacokinetic parameters was made by summary measures and analysed by one-way ANOVA followed by Neuman-Keuls multiple comparison test.

Results

Blood pharmacokinetics

Figure 1 shows the serum concentration-time profiles of milacemide and glycinamide after milacemide administration (100, 200 and 400 mg kg^{-1}). Milacemide concentrations rose linearly and dose-dependently and had already peaked at the time of the first blood sample (0.25 h). Concentrations then fell exponentially. Glycinamide concentrations exhibited small dose-dependent rises, but these were not linearly related.

Table I shows the apparent pharmacokinetic constants for individual rats together with the mean values, as calculated from log concentration versus time plots. The pharmacokinetic constants for individual rats showed moderate variability within each dose group with values mostly varying over a 2–3 fold range. Mean C_{max} values for milacemide increased proportionately with increasing dose. However, whilst mean $t_{1/2}$ values were indistinguishable for the groups

Table 1 Pharmacokinetic constants for blood (serum)

	Milacemide				*Glycinamide*		
Rat No.	C_{max} (μmol l^{-1})	AUC (μmol l^{-1} h^{-1})	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (μmol l^{-1})	AUC (μmol l^{-1} h)	$t_{1/2}$ (h)
A 100 mg kg^{-1}							
1	118	92	0.6	–	–	–	–
2	199	90	0.3	0.3	85	61	0.6
3	71	104	0.1	0.6	69	98	0.5
4	42	54	0.9	0.5	66	92	0.7
5	191	105	0.4	0.6	51	84	0.9
6	143	77	0.4	0.3	54	75	1.0
7	108	71	0.5	0.3	61	70	0.7
8	76	169	1.5	0.9	47	105	1.1
Mean	118	95	0.7	0.5	62	84	0.8
± s.e.mean	± 20	± 12	± 0.1	0.2	5	6	0.1
B 200 mg kg^{-1}							
1	139	104	0.5	0.3	75	79	0.8
2	111	56	0.3	0.3	28	31	0.8
3	257	327	0.9	0.5	58	128	1.4
4	171	247	1.0	0.6	80	174	1.6
5	274	266	0.7	0.6	96	160	0.6
6	211	180	0.6	0.4	98	131	0.9
7	152	130	0.6	0.9	47	105	1.1
Mean	188	187	0.7	0.5	69	126	1.0
± s.e.mean	± 23	± 37	± 0.1	0.1	10	13	0.1
C 400 mg kg^{-1}							
1	387	845	1.5	1.2	49	189	1.4
2	443	692	1.1	0.6	68	208	1.9
3	193	458	1.6	0.9	77	237	1.5
4	550	947	1.2	0.3	98	263	1.9
5	300	582	1.3	0.5	79	211	2.0
6	475	1166	1.7	0.6	82	261	2.0
7	391	1002	1.8	1.3	70	259	1.4
8	487	769	1.1	0.8	55	214	2.4
Mean	403	807	1.4**	0.8	72	230	1.8**
± s.e.mean	± 40	± 8	± 0.1	0.1	5	10	0.1

T_{max} = time to maximum concentration; C_{max} = maximum concentration; AUC = area under the serum concentration versus time curve; $t_{1/2}$ = apparent elimination half-life. $^{**}P<0.01$ compared with 100 mg kg^{-1} and 200 mg kg^{-1} milacemide-treated groups: one-way ANOVA followed by Neuman-Keuls multiple comparison test.

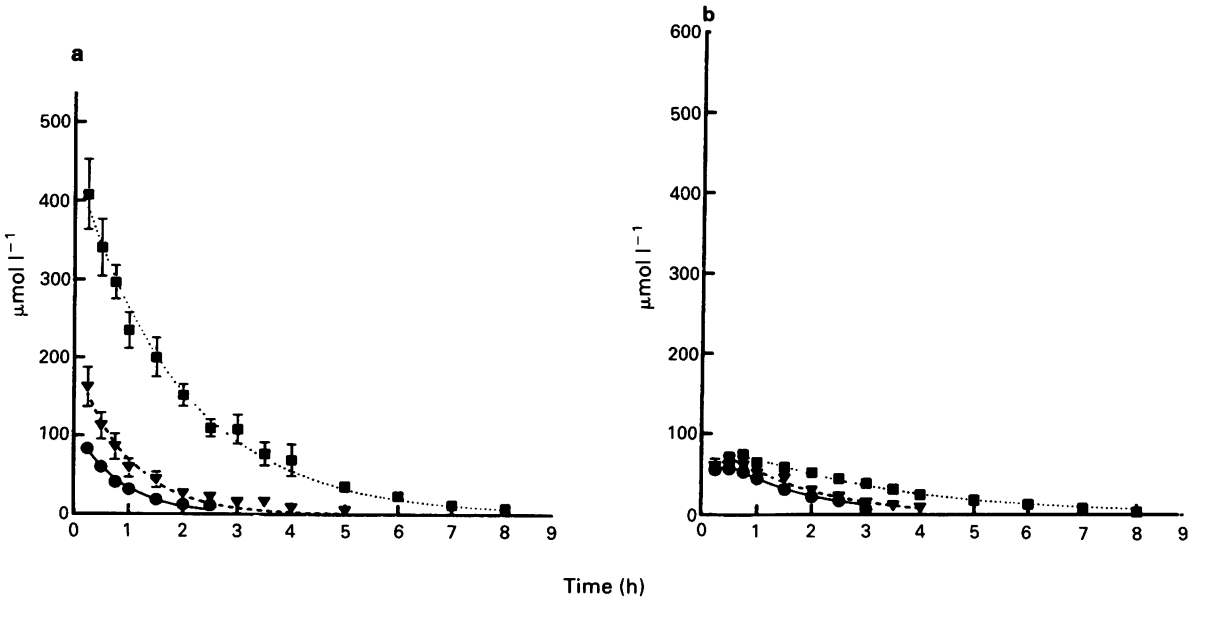


Figure 1 Serum milacemide (a) and glycinamide (b) after i.p. administration of milacemide. Values are mean ± s.e.mean of 7–8 rats. Milacemide 100 mg kg^{-1} (●). 200 mg kg^{-1} (▼) and 400 mg kg^{-1} (■).

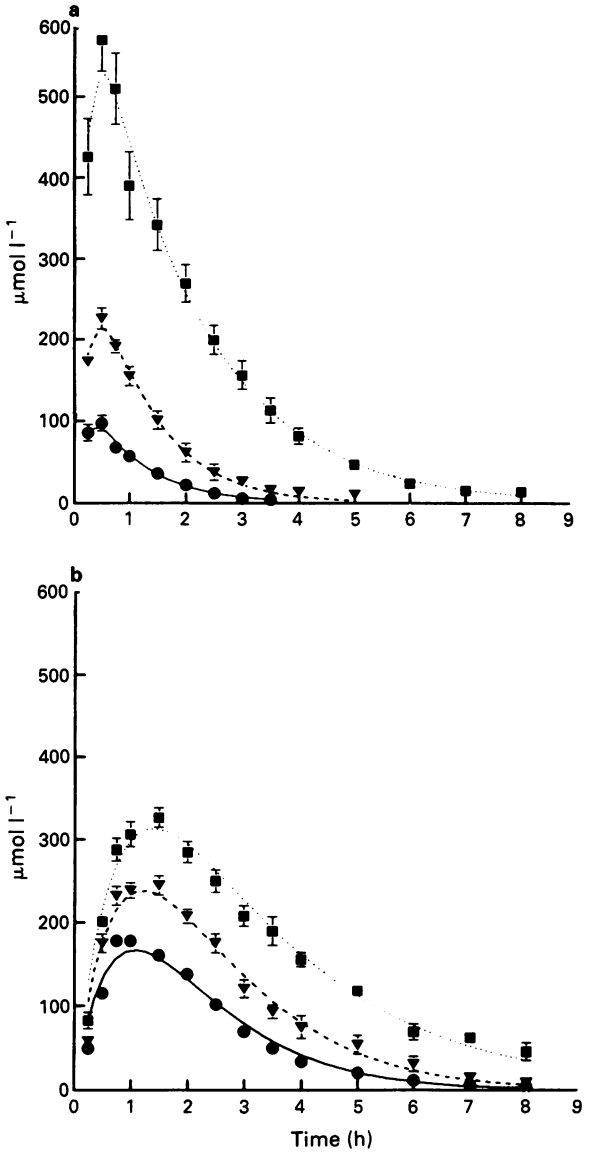


Figure 2 Cerebrospinal fluid milacemide (a) and glycinamide (b) after i.p. administration of milacemide. Values are mean ± s.e.mean of 7–8 rats; Milacemide 100 mg kg^{-1} (●); 200 mg kg^{-1} (▼) and 400 mg kg^{-1} (■).

given 100 and 200 mg kg^{-1} milacemide, the value was substantially and significantly higher ($P<0.001$) in the group given 400 mg kg^{-1} milacemide. This difference was reflected in the high AUC value for the latter group.

Mean serum glycinamide concentrations peaked a little later than milacemide (mean T_{max} range, 0.5–0.8 h). The C_{max} value was approximately half that of milacemide in the group given 100 mg kg^{-1} milacemide. As glycinamide concentrations rose only slightly with increasing milacemide dose (Figure 1b), mean C_{max} values for glycinamide after 200 and 400 mg kg^{-1} milacemide were respectively 0.37 and 0.18 of the corresponding values for the precursor. Mean $t_{1/2}$ values for glycinamide increased with dose but statistical significance ($P<0.001$) was only achieved at the highest dose.

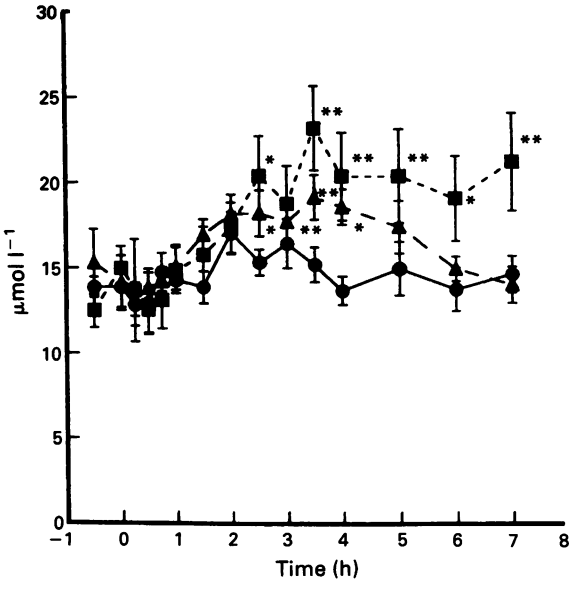


Figure 3 Cerebrospinal fluid glycine after i.p. administration of milacemide. Values are mean ± s.e.mean of 7–8 rats; Milacemide 100 mg kg^{-1} (●); 200 mg kg^{-1} (▼) and 400 mg kg^{-1} (■). *$P<0.05$; **$P<0.01$ compared with baseline values (one-way ANOVA followed by Neuman-Keuls multiple comparison test).

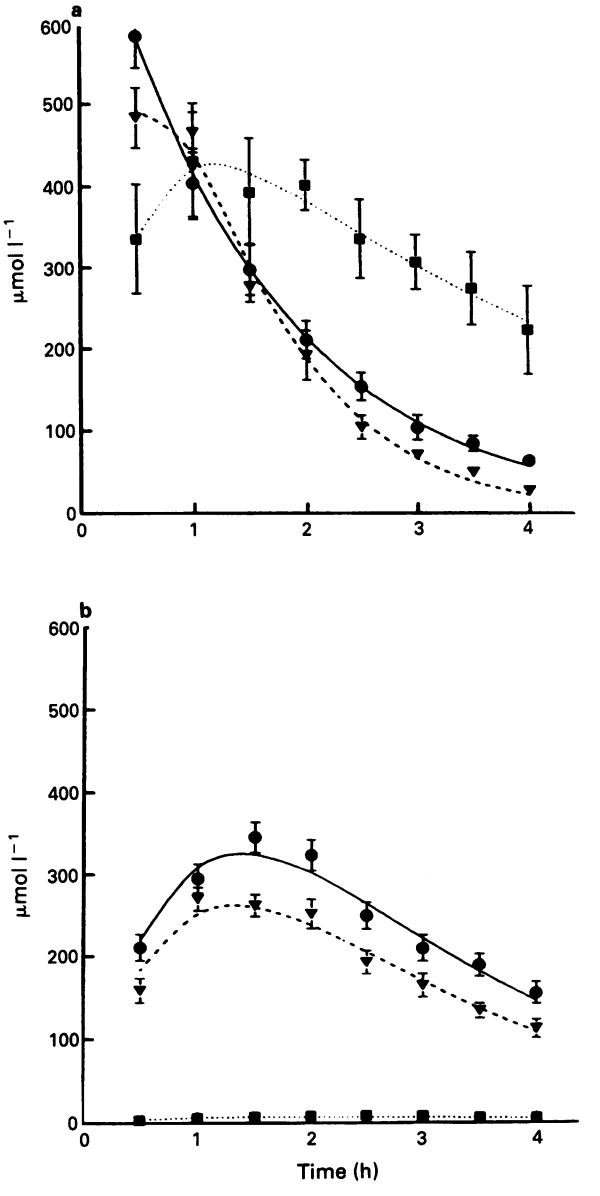


Figure 4 Cerebrospinal fluid milacemide (a) and glycinamide (b) after i.p. administration of milacemide (400 mg kg^{-1}) to rats pretreated with L-deprenyl (■); clorgyline (▼) or 0.154 M saline (●). Values are mean of 7–8 rats.

Table 2 Neuropharmacokinetic constants for cerebrospinal fluid

	Milacemide				*Glycinamide*			
Rat No.	T_{max} (h)	C_{max} (μmol l^{-1})	AUC (μmol l^{-1} h^{-1})	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (μmol l^{-1})	AUC (μmol l^{-1} h)	$t_{1/2}$ (h)
A 100 mg kg^{-1}								
1	0.4	94	111	0.5	1.0	167	469	0.5
2	0.2	135	113	0.5	1.1	437	437	0.6
3	0.3	99	97	0.6	1.2	165	559	0.7
4	0.6	41	71	0.7	1.2	112	395	0.8
5	0.4	96	124	0.6	1.1	161	487	0.8
6	0.3	109	129	0.6	1.1	171	492	0.7
7	0.3	95	108	0.6	1.1	141	423	0.7
8	0.7	95	158	0.5	1.1	157	493	0.8
Mean	0.4	95	114	0.6	1.1	153	469	0.7
± s.e.mean	0.1	9	9	0.1	0.1	7	18	0.04
B 200 mg kg^{-1}								
1	0.5	197	251	0.5	1.1	219	687	0.8
2	0.4	194	178	0.4	1.1	215	640	0.7
3	0.5	212	332	0.6	1.3	244	837	0.9
4	0.5	196	432	1.3	1.6	234	1025	1.3
5	0.5	213	267	0.5	1.2	227	610	0.8
6	0.5	243	330	0.5	1.2	233	800	0.9
7	0.5	233	350	0.6	1.4	175	666	0.9
Mean	0.5	213	306	0.6	1.3	221	752	0.9
± s.e.mean	0.1	7	31	0.1	0.1	8	55	0.1
C 400 mg kg^{-1}								
1	0.7	621	1583	1.2	1.3	217	982	0.4
2	0.2	690	1028	1.0	1.9	275	1376	1.3
3	0.4	471	854	1.0	1.4	271	1305	0.5
4	0.7	604	1422	1.1	1.5	289	1189	0.7
5	0.7	436	1083	1.2	1.4	328	1250	0.6
7	0.5	587	1176	1.1	1.5	327	1397	0.7
8	0.5	407	905	1.2	1.3	323	1239	0.6
Mean	0.6	533	1149	1.1**	1.5*,**	294	1255	0.7
± s.e.mean	0.1	37	88	0.1	0.1	14	46	0.1

For abbreviations see Table 1.
*$P<0.05$; **$P<0.05$; **$P<0.01$, compared with 200 mg kg^{-1}, 100 mg kg^{-1} and 100 mg kg^{-1} milacemide-treated groups respectively: one-way ANOVA followed by Neuman-Keuls multiple comparison test.

CSF neuropharmacokinetics

Figure 2 shows the corresponding CSF concentration time profiles of milacemide and glycinamide. As observed for sera (Figure 1a), CSF milacemide concentrations rose linearly and dose-dependently peaking (T_{max}) at approximately 0.5 h after milacemide administration and then falling exponentially. In contrast to sera (Figure 1b), CSF glycinamide concentrations also rose linearly and dose-dependently.

The apparent neuropharmacokinetic constants for individual rats, and the mean values, are shown in Table 2. Mean C_{max} values for CSF milacemide were fairly comparable to those in serum, though the CSF/serum ratio rose with milacemide dosage (0.81, 1.13 and 1.32 at 100, 200 and 400 mg kg^{-1}). CSF mean $t_{1/2}$ and AUC values mirrored those observed in serum.

CSF glycinamide concentrations peaked somewhat later (mean T_{max}, 1.1–1.5 h) than milacemide (mean T_{max}, 0.4–0.5 h) and C_{max} values were 2.5, 3.2 and 4.1 times greater than the corresponding values for serum glycinamide after giving 100, 200 or 400 mg kg^{-1} respectively of milacemide. Further, C_{max} values for CSF glycinamide were approximately 1.5 fold higher, equivalent and almost half those of milacemide after the same doses. Mean $t_{1/2}$ values for glycinamide did not vary appreciably with milacemide dosage.

Glycine concentrations

Glycine concentrations in sera were unaffected by milacemide administration (data not shown). Figure 3 shows the glycine concentration profiles in CSF for the animals described previously. Values rose linearly and dose-dependently, but were only significantly elevated at the higher milacemide doses (200 and 400 mg kg^{-1}), with a T_{max} of 3.5 h. Glycine values for rats given milacemide (400 mg kg^{-1}) rose to about 150% of control values. Glycine concentrations after 200 mg kg^{-1} milacemide returned to baseline by 6 h after administration but animals given 400 mg kg^{-1} of the drug had values that were still significantly elevated ($P<0.001$) 7 h later.

Effect of monoamine oxidase inhibition

Figure 4 shows milacemide and glycinamide concentration profiles in CSF of rats given 400 mg kg^{-1} milacemide and given saline (vehicle), 5 mg kg^{-1} clorgyline or 2 mg kg^{-1} L-deprenyl 2 h previously. L-Deprenyl prevented almost completely the formation of glycinamide and increased the accumulation of milacemide (Table 3). In contrast, clorgyline did not significantly affect milacemide concentrations. However, it caused a small but significant reduction in mean glycinamide C_{max} though mean AUC was unaffected.

Table 3 shows a comparison of the various apparent neuropharmacokinetic constants obtained. L-Deprenyl pretreatment significantly ($P<0.01$) increased T_{max} and $t_{1/2}$ and significantly decreased C_{max} and AUC for glycinamide compared to controls. Milacemide $t_{1/2}$ and AUC values were also significantly increased by L-deprenyl pretreatment. In contrast clorgyline pretreatment affected only glycinamide C_{max} and AUC values ($P<0.05$).

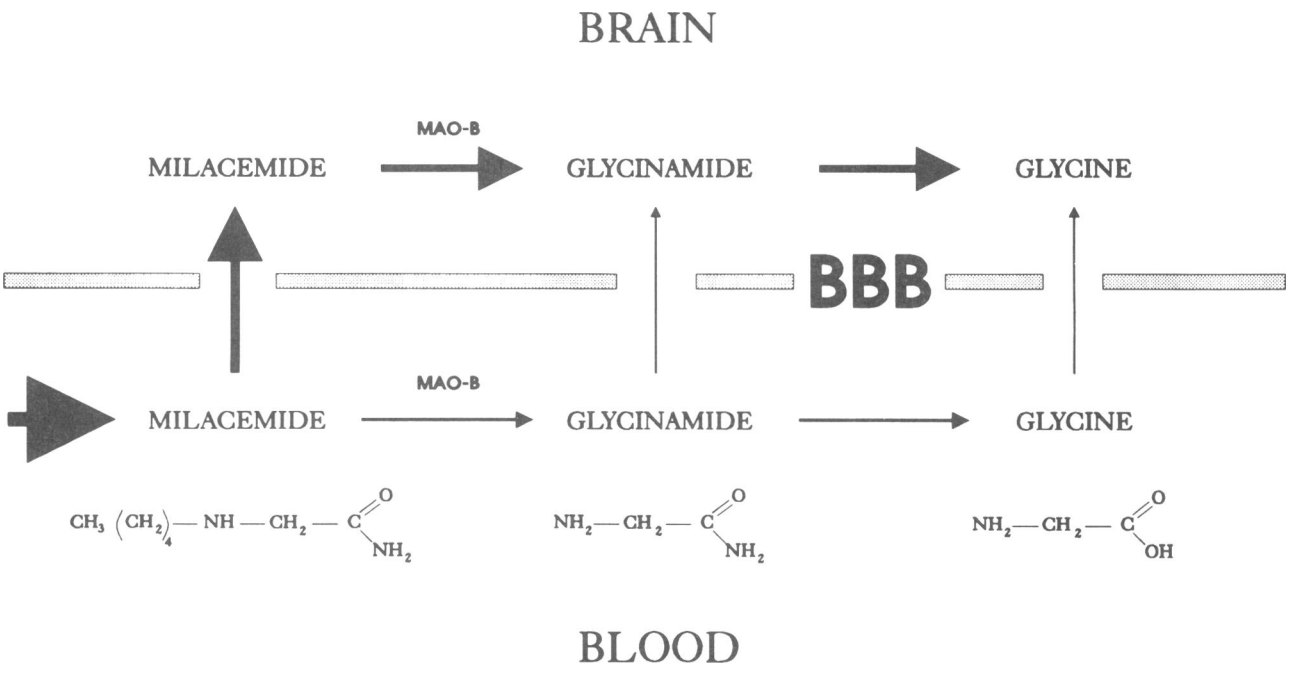


Figure 5 Schematic representation of the inter-relationship of milacemide, glycinamide and glycine in blood and brain (cerebrospinal fluid). BBB = blood-brain-barrier.

Table 3 Effect of pretreatment with selective monoamine oxidase type A (MAO-A) and type B (MOA-B) inhibitors on cerebrospinal fluid milacemide and glycinamide neuropharmacokinetic constants

	n	T_{max} (h)	C_{max} (μmol l^{-1})	AUC (μmol l^{-1} h)	$t_{1/2}$ (h)
Milacemide					
Vehicle	(4)	0.3 ± 0.1	568 ± 53	639 ± 50	0.9 ± 0.1
Clorgyline	(5)	0.6 ± 0.1	513 ± 35	716 ± 52	0.7 ± 0.1
L-Deprenyl	(7)	1.2 ± 0.3	457 ± 50	1150 ± 140*	1.9 ± 0.4*
Glycinamide					
Vehicle	(4)	1.4 ± 0.1	326 ± 40	1253 ± 99	1.0 ± 0.1
Clorgyline	(5)	1.3 ± 0.2	263 ± 14**	976 ± 66*	0.9 ± 0.1
L-Deprenyl	(7)	3.0 ± 0.3**	8 ± 1**	81 ± 23**	1.5 ± 0.3

Values are expressed as mean ± s.e.mean.
*$P<0.05$; **$P<0.01$; compared with vehicle-treated (0.154 M saline) group; one-way ANOVA followed by Neuman-Keuls multiple comparison test. For abbreviations see Table 1.

Discussion

Pharmacokinetic considerations are important for the design of dosage regimens in clinical pharmacotherapy, including therapeutic drug monitoring which is performed routinely for several antiepileptic drugs. The adjustment of drug dosage based on the monitoring of serum or plasma concentration of antiepileptic drugs, assumes that these concentrations reflect those at the CNS site of action or biophase. As this assumption is not easy to investigate in man, relevant animal studies are useful.

As concentrations of drugs in CSF are indices of their concentrations at central sites of action and the CSF is kinetically indistinguishable from the central site of action of various drugs, including numerous antiepileptic drugs (Danhof & Levy, 1984; Dingemanse *et al.*, 1987; Klockowski & Levy, 1988; Sokomba *et al.*, 1988), studies of the inter-relationship between CSF and blood kinetics are desirable. The technique for the serial and simultaneous sampling of CSF and blood compartments in freely behaving rats (Patsalos *et al.*, 1992) has been found to be useful for the evaluation of the antiepileptic drug, milacemide, as much useful kinetic data was obtained on a small number of rats. Furthermore, kinetic values were obtained for individual animals.

Our main findings concern the temporal distribution, kinetics and metabolism of milacemide in serum and CSF. Significant divergences between the blood and CSF compartments were observed. The dose-dependent increase in CSF glycinamide and glycine concentrations (Figures 2b and 3) after milacemide administration (100, 200 and 400 mg kg^{-1}) largely confirms that milacemide is a CNS glycine prodrug with intermediary metabolism to glycinamide.

After intraperitoneal administration, milacemide rapidly appeared in both serum ($T_{max}<0.25$ h) and CSF (mean T_{max} 0.5 h; range 0.2–1.0 h). These fairly comparable values suggest ready pentration of the blood-brain-barrier.

The dosages of the MAO inhibitors L-deprenyl (2 mg kg^{-1}) and clorgyline (5 mg kg^{-1}) were chosen to inhibit selectively MAO-B and MAO-A activity respectively (Johnston, 1968; Kalir *et al.*, 1981; Knoll & Magyar, 1972). Therefore, as L-deprenyl but not clorgyline significantly increased CSF milacemide $t_{1/2}$ and AUC (Figure 4, Table 3) and decreased CSF glycinamide AUC by 93% while clorgyline only decreased it by 22%, it can be concluded that the conversion of milacemide to glycinamide is largely mediated by MAO-B.

These CNS data confirm *in vitro*, *ex vivo* and indirect urine findings (de Varebeke *et al.*, 1983; 1989). Also, CSF glycinamide C_{max} values (Tables 1 and 2) after 100 mg kg^{-1} milacemide administration were 2.5 fold higher than serum values which is consistent with peripheral (liver) MAO-B activity being lower than in the CNS. This agrees with *in vitro* evidence that K_m and V_{max} values for glycinamide formation from milacemide were 37% higher for rat brain than liver MAO-B (de Varebeke *et al.*, 1988). The dose-dependent increase in CSF but not in serum glycinamide, and the similar glycinamide $t_{1/2}$ values for both sera and CSF suggest that peripheral but not central MAO-B activity is rate-limiting and essentially saturated after 100 mg kg^{-1} milacemide administration. Further, the similar $t_{1/2}$ values for milacemide and glycinamide suggests that glycinamide elimination is formation rate-limiting (Rowland & Tozer, 1989).

The distribution of milacemide and glycinamide and the contribution of the peripheral blood compartment to the CSF compartment need some comment. The rapid and dose-dependent increase in CSF milacemide indicates that blood-brain-barrier transport occurs readily and is not rate-limiting over the concentration-range used. Glycinamide can cross the blood-brain-barrier (Christophe *et al.*, 1983). However, since its concentrations in sera were 60–75% lower than in CSF (Figures 1b and 2b), blood glycinamide probably contributes minimally to CSF glycinamide and the latter is largely produced by brain MAO-B.

Previous *in vitro* data suggest that milacemide may be both a substrate and an inhibitor of MAO-B (de Varebeke *et al.*, 1989). The disproportionate increase and divergence in CSF milacemide neuropharmacokinetic parameters (C_{max} and AUC) compared to the serum pharmacokinetic parameters is consistent with this. Inhibition of liver MAO-B by milacemide would result in more milacemide becoming available for transport into the brain.

An increase of serum glycine after milacemide administration was not detected. In contrast, CSF glycine increased dose-dependently and significantly at the higher doses (200 and 400 mg kg^{-1}). After intraperitoneal administration at high doses, glycine (40 mmol kg^{-1}) readily enters the brain (presumably by diffusion) and increases significantly in the CSF for up to 8 h (unpublished data). Further, the apparent elimination $t_{1/2}$ for glycine in blood and CSF in these experiments were essentially the same (approximately 2 h) suggesting that the elimination of glycine in both compartments is similar. Thus the absence of a significant increase in serum glycine in the present study further suggests that peripheral metabolism to glycine plays a minor role in the elimination of milacemide. As might be expected the $t_{1/2}$ for glycine as a metabolite of milacemide is larger (approximately 6 h, Figure 3) than that for glycine as a substrate (approximately 2 h, unpublished data).

Whether an elevation of brain glycine concentrations when milacemide is used clinically has pharmacologically relevant effects is unknown. Although milacemide has been used to treat patients with epilepsy or myoclonus, the overall response was marginal (Houtkooper *et al.*, 1986; Norton *et al.*, 1986; Brown *et al.*, 1991). Its evaluation in the management of schizophrenia and Huntington's Chorea has similarly been disappointing (Rosse *et al.*, 1991; Giuffra *et al.*, 1992). The maximum dose used in these patients was approximately 35 mg kg^{-1} but as oral bioavailability of milacemide in man is limited by a first pass effect of 90% (Roba *et al.*, 1986), its effective maximum dose used was probably not more than 3.5 mg kg^{-1}. Further, as MAO-B is present in endothelial cells of the blood-brain-barrier (Minn *et al.*, 1991), the low milacemide doses used clinically probably lead to milacemide blood concentrations too low for effective blood-brain-barrier transport. As milacemide is generally well tolerated clinically, the present data suggest that milacemide might be evaluated clinically at a much higher dose and that its measurement in blood would be helpful in the optimization of therapy since this is an index of CNS concentrations.

In summary, Figure 5 shows schematically the interrelationship between blood and brain of milacemide, glycinamide and glycine. Milacemide rapidly equilibrates between blood and brain and it is largely metabolized by MAO-B to glycinamide which is subsequently metabolized to glycine. Cerebral metabolism via MAO-B is more important than peripheral metabolism and although peripherally produced glycinamide can cross the blood-brain-barrier, its contribution to cerebral glycinamide is small. As serum glycine was not increased in the present study, the rise in CSF glycine on milacemide administration is derived exclusively from cerebral metabolism.

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References

APRISON, M.H. & WERMAN, R. (1965). The distribution of glycine in cat spinal cord and roots. *Life Sci.*, **4**, 2075–2083.

BORMAN, J., HAMILL, O.P. & SAKMANN, B. (1987). Mechanism of anion permeation through channels gated by glycine and γ-aminobutyric acid in mouse cultured neurones. *J. Physiol*, **385**, 243–288

BRODY, T., SHIN, Y.S. & STOKSTAD, E.L. (1976). Rat brain folate identification. *J. Neurochem.*, **27**, 409–413.

BROWN, P., THOMPSON, P.D., ROTHWELL, J.C., DAY, B.L. & MARSDEN, C.D. (1991). A therapeutic trial of milacemide in myoclonus and Stiff-Man syndrome. *Movement Disord.*, **6**, 73–75.

CHRISTOPHE, J., KUTZNER, R., NGUYEN-BUI, W.D., DAMIEN, C., CHATELAIN, P. & GILLET, L. (1983). Conversion of orally administered 2-n-pentylaminoacetamide into glycinamide and glycine in the rat brain. *Life Sci.*, **33**, 533–541.

DANHOF, M. & LEVY, G. (1984). Kinetics of drug action in disease states. I. Effect of infusion rate on phenobarbital concentrations in serum, brain and cerebrospinal fluid of normal rats at onset of loss of righting reflex. *J. Pharmacol. Exp. Ther.*, **229**, 44–50, 1984.

DEUTSCH, S.I., MASTROPAOLO, J., SCHWARTZ, B.L., ROSSE, R.B. & MORIHISA, J.M. (1989). A 'glutamatergic' hypthesis of schizophrenia. Rationale for pharmacotheraphy with glycine. *Clin. Neuropharmacol.*, **12**, 1–13.

DE VAREBEKE, J.P., CAVALIER, R., DAVID-REMACLE, M. & YOUDIM, M.B.H. (1988). Formation of the neurotransmitter glycine from the anticonvulsant milacemide is mediated by brain monoamine oxidase B. *J. Neurochem.*, **50**, 1011–1016.

DE VAREBEKE, J.P., NIEBES, P., PAUWELS, G., ROBA, J. & KORF, J. (1983). Effect of milacemide, a glycinamide derivative, on the rat brain γ-aminobutyric acid system. *Biochem. Pharmacol.*, **32**, 2751–2755.

DE VAREBEKE, J.P., PAUWELS, G., BUYSE, C., DAVID-REMACLE, M., DE MEY, J., ROBA, J. & YOUDIM, M.B.H. (1989). The novel neuropsychotropic agent milacemide is a specific enzyme-activated inhibitor of brain monoamine oxidase B. *J. Neurochem.*, **53**, 1109–1116.

DINGEMANSE, J., VAN BREE, J.B.M.M. & DANHOF, M. (1987). Pharmacokinetic modelling of the anticonvulsant action of phenobarbital in rats. *J. Pharmacol. Exp. Ther.*, **249**, 601–608.

DUNN, A. (1985). A new iterative polyexponential curve fitting program. *Comput. Meth. Prog. Biomed.*, **20**, 269–275.

GIUFFRA, M.E., MOURADIAN, M.M. & CHASE, T.N. (1992). Glutamatergic therapy for Huntington's Chorea. *Clin. Neuropharmacol.*, **15**, 148–151.

GREENAMYRE, J.D., PENNEY, J.B., YOUNG, A.B., D'AMATO, C.J., HICKS, S.P. & SHOULSON, I. (1985). Alterations in L-glutamate binding in Alzheimer's and Huntington's diseases. *Science*, **227**, 1496–1498.

HANDELMANN, G.E., NEVINS, M.E., MUELLER, L.L., AANOUDE, S.M. & CORDI, A.A. (1989). Milacemide, a glycine prodrug, enhances performance of learning tasks in normal amnesic rodents. *Pharmacol. Biochem. Behav.*, **34**, 823–828.

HOUTKOOPER, M.A., VAN OORSCHOT, C.A.E.H., RENTMEESTER, T.W., HOPPENER, P.J.E.A. & ONKELINX, C. (1986). Double-blind study of milacemide in hospitalised therapy-resistant patients with epilepsy. *Epilepsia*, **27**, 255–262.

HUTSON, P.H., SARNA, G.S., KANTAMANENI, B.D. & CURZON, G. (1985). Monitoring the effect of a tryptophan load on brain indole metabolism in freely moving rats by simultaneous cerebrospinal fluid monitoring and brain dialysis. *J. Neurochem.*, **44**, 1266–1274.

JOHNSON, J.W. & ASCHER, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, **325**, 529–531.

JOHNSTON, J.P. (1968). Some observations upon a new inhibitor of monoamine oxidase in brain tissues. *Biochem. Pharmacol.*, **17**, 1285–1297.

KALIR, A., SABBAGH, A. & YOUDIM, M.B.H. (1981). Selective acetylenic 'suicide' and reversible inhibitors of monoamine oxidase type A and type B. *Br. J. Pharmacol.*, **73**, 55–64.

KLOCKOWSKI, P.M. & LEVY, G. (1988). Kinetics of drug action in disease states. XXIV. Pharmacodynamics of diazepam and its active metabolites in rats. *J. Pharmacol. Exp. Ther.*, **244**, 912–918.

KNOLL, J. & MAGYAR, K. (1972). Some puzzling pharmacological effects of monoamine oxidase inhibitors. *Adv. Biochem. Psychopharmacol.*, **5**, 393–408.

LIU, Z., SEILER, N., MARESCAUX, C., DEPAULIS, A. & VERGNES, M. (1990). Potentiation of γ-vinyl GABA (vigabatrin) effects by glycine. *Eur. J. Pharmacol.*, **182**, 109–115.

MINN, A., GHERSI-EGEA, J.F., PERRIN, R., LEININGER, B. & SIEST, G. (1991). Drug metabolising enzymes in the brain and cerebral microvessels. *Brain Res. Rev.*, **16**, 65–82.

NORTON, L.C., LAXER, K.D., SCHOMER, D. & OSBORN, P. (1986). Phase II study to evaluate the efficacy and safety of milacemide (CP 1552S) in the treatment of complex partial seizures in adult patients. *Epilepsia*, **27**, 648.

OLDENDORF, W.H. (1971). Brain uptake of radiolabeled amino acids, amines and hexoses after arterial injection. *Am. J. Physiol.*, **221**, 1629–1639.

PATERSON, S.L. (1991). Glycine potentiation of anticonvulsant drugs in pentylenetetrazol seizures in rats. *Brain Res. Bull.*, **26**, 43–47.

PATSALOS, P.N., ALAVIJEH, M.S., SEMBA, J. & LOLIN, Y.I. (1992). A freely moving and behaving rat model for the chronic and simultaneous study of drug pharmacokinetics (blood) and neuropharmacokinetics (cerebrospinal fluid): hematological and biochemical characterisation and kinetic evaluation using carbamazepine. *J. Pharmacol. Toxicol. Method*, **28**, 21–28.

RICHTER, J.J. & WAINER, A. (1971). Evidence for separate systems for the tranpsort of neutral and basic amino acids across the blood-brain-barrier. *J. Neurochem.*, **18**, 613–620.

ROBA, J., CAVALIER, R., CORDI, A., GORISSEN, H., HERIN, M., DE VAREBEKE, P.J., ONKELINX, C., REMACLE, M. & VAN DORSSER, W. (1986). Milacemide. In *New Antiepileptic Drugs*. Meldrum, B.S. & Porter, R.J. pp.179–190. London: John Libbey & Company Ltd.

ROSSE, R.B., SCHWARTZ, B.L., DAVIS, R.E. & DEUTSCH, S.I. (1991). An NMDA intervention strategy in schizophrenia with 'low dose' milacemide. *Clin. Neuropharmacol.*, **14**, 268–272.

ROTHMAN, S.M. & OLNEY, J.W. (1987). Excitotoxicity and the NMDA receptor. *Trends Neurosci.*, **10**, 299–302.

ROWLAND, M. & TOZER, T.N. (1989). *Clinical Pharmacokinetics. Concepts and Application*. 2nd Ed. Philadelphia: Lea and Febiger.

SALETU, B., GRUNBERGER, J. & LINZMAYER, L. (1988). Acute and subacute central nervous system effects of milacemide in elderly people. Double-blind placebo controlled quantitative electroencephalography and psychometric investigation. *Arch. Gerentol. Geriat.*, **5**, 165–182.

SCHWARTZ, B.L., HASHTROUDI, S., HERTING, R.L. & DEUTSCH, S.I. (1992). The effects of milacemide on item and source memory. *Clin. Neuropharmacol.*, **15**, 114–119.

SEMBA, J., DOHENY, M., PATSALOS, P.N., SARNA, G. & CURZON, G. (1992). Effect of milacemide on extracellular and tissue concentrations of dopamine and 5-hydroxytryptamine in rat frontal cortex. *Br. J. Pharmacol.*, **105**, 59–62.

SEMBA, J., RATNARAJ, N. & PATSALOS, P.N. (1991). Simple and rapid micro-analytical procedures for the estimation of milacemide and its metabolite glycinamide in rat plasma and cerebrospinal fluid by high-performance liquid chromatography. *J. Chromatogr. Biomed-Applic.*, **565**, 357–362.

SOKOMBA, E.N., PATSALOS, P.N., LOLIN, Y.I. & CURZON, G. (1988). Concurrent monitoring of central carbamazepine and transmitter amine metabolism and motor activity in individual unrestrained rats using repetitive withdrawal of cerebrospinal fluid. *Neuropharmacol.*, **27**, 409–415.

SPENOS, G. & DALY, E.C. (1984). Activity of glycine, dimethylglycine and their metabolites against 3-mercaptopropionic acid induced seizures in mice. *Neurology*, **34**, (Suppl. 1) 22.

TOTH, E. & LAJTHA, A. (1981). Elevation of cerebral levels of non-essential amino acids in vivo by administration of large doses. *Neurochem. Res.*, **6**, 1309–1317.

TOTH, E., LAJTHA, A., SARHAN, S. & SEILER, N. (1983). Anticonvulsant effects of some inhibitory neurotransmitter amino acids. *Neurochem. Res.*, **8**, 291–302.

TRUONG, D.D. & FAHN, S. (1988). Therapeutic trial with glycine in myoclonus. *Movement Disord.*, **3**, 222–232.

TURNELL, D.C. & COOPER, J.C.H., (1982). Rapid assay for amino acids in serum or urine by pre-column derivatisation and reversed-phase liquid chromatrography. *Clin. Chem.*, **28**, 527–531.

VAN DORSSER, W., BARRIS, D., CORDI, A. & ROBA, J. (1983). Anticonvulsant activity of milacemide. *Arch. Int. Pharmacodyn.*, **266**, 234–249.

WAZIRI, R. (1988). Glycine therapy of schizophrenia. *Biol. Psychiatry*, **23**, 210–211.

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Endothelin-3-induced relaxation of rat thoracic aorta: a role for nitric oxide formation

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1 Endothelin-3 (ET-3) at concentrations below those which caused contraction (30 nM) elicited endothelium-dependent relaxation followed by rebound contraction in rat isolated thoracic aorta.

2 Endothelin-1 also relaxed the rat aorta with a similar potency.

3 The nitric oxide (NO) synthase inhibitor, N^G-nitro L-arginine, the radical scavenger, haemoglobin and the soluble guanylate cyclase inhibitor, methylene blue, each inhibited the ET-3-induced relaxation.

4 The calmodulin inhibitor, calmidazolium, considerably attenuated the relaxation caused by ET-3 without affecting that to nitroprusside.

5 Concentrations of ET-3 that were necessary to induce the relaxation also caused concentration-dependent elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels.

6 N^G-nitro L-arginine, haemoglobin, methylene blue, calmidazolium and removal of the endothelium completely abolished ET-3-stimulated cyclic GMP production.

7 These results suggest that ET-3 triggers NO formation possibly via ET_B receptors on the endothelium to activate soluble guanylate cyclase, which in turn stimulates cyclic GMP production and smooth muscle relaxation. The enzyme contributing to the NO formation may be of the calcium/calmodulin-dependent, constitutive type.

Keywords: Endothelin-3; relaxation; rat aorta; endothelium; nitric oxide (NO); calmodulin; NO synthase; cyclic GMP

Introduction

Soon after their discovery (Yanagisawa *et al.*, 1988), endothelins were shown to have dual actions. Besides contracting isolated blood vessels, they dilate perfused vascular beds such as the rat mesentery (Warner *et al.*, 1989; Hasunuma *et al.*, 1990), and cause a depressor response following an increase in blood pressure in the anaesthetized rat (De Nucci *et al.*, 1988) and cat (Lippton *et al.*, 1989). It has been speculated that the depressor effects may be mediated by endothelium-derived relaxing factor (EDRF), on the basis of circumstantial evidence that N^G-monomethyl L-arginine, haemoglobin (Oxy-Hb) and methylene blue (MB) can attenuate these effects (Fukuda *et al.*, 1990; 1991; Fozard & Part, 1992). In addition, in endothelial cells in culture, endothelin-3 (ET-3) stimulates guanosine 3':5'-cyclic monophosphate (cyclic GMP) formation (Emori *et al.*, 1991). However, the relaxant effect and precise mechanism(s) of action of ET-3 on isolated blood vessels have not been described.

In the present study, we examined whether ET-3-induced relaxation of rat thoracic aorta ring preparations was mediated by nitric oxide (NO), using cyclic GMP production as a marker. While this work was in progress, reports were published showing that endothelin-1-induced relaxation of rat aorta was possibly mediated by EDRF or cyclo-oxygenase products (Mehta *et al.*, 1992; Namiki *et al.*, 1992).

Methods

Organ bath experiments

Male Wistar rats aged 10 weeks (8–12 weeks, 305.4 ± 11.8 g) were used. The thoracic aortae were removed and cleaned of adjacent tissue. Ring segments of the artery of 3 mm length were cut with parallel razors and mounted in 10 ml organ baths, essentially by the method previously described (Bevan *et al.*, 1975). Briefly, a U-shaped stainless steel wire was passed through the lumen of the ring and the ends of this wire anchored to a holder. A second wire was passed through the vessel and connected to an isometric transducer (Nihon Kohden TB-651T). The Krebs solution had the following composition (mM): NaCl 115.3, KCl 4.9, $CaCl_2$ 1.46, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 11.1, EDTA disodium salt 0.03 and ascorbic acid 0.11. This solution was maintained at 34°C and bubbled with O_2 containing 5% CO_2 (pH 7.4).

The artery preparations were equilibrated for 2 h before the administration of drugs at the optimal resting tension for producing the largest response to phenylephrine. For the aortae from 10 week old rats, the optimal value was found to be 1.0 g. After confirming that the concentration-response curve for phenylephrine had become constant, the experiments were started. For measurement of relaxation, the arteries were first contracted with phenylephrine at concentrations corresponding to the EC_{80} value (0.3 μM for the arteries with endothelium and 0.1 μM for endothelium-denuded arteries). Concentration-response curves were constructed by adding ET-3 cumulatively to the 10 ml organ bath in volumes of 7–20 μl, and relaxations were plotted as percentages of the contractions induced by the EC_{80} concentration of phenylephrine. To avoid problems due to desensitization of the aortae to the 2nd application of ET-3, responses in the presence of inhibitors were compared with those of paired preparations. The integrity of the endothelium was confirmed by testing the relaxations induced by cumulative applciation of acetylcholine.

Removal of the endothelium was achieved mechanically by passing a cotton thread through the lumen of the artery and sliding it in and out several times, and its removal was confirmed by observing the loss of the relaxant response of

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the arteries to acetylcholine at concentrations of up to 10 μM.

Oxy-Hb was prepared as described by Martin et al. (1985).

Assay of cyclic GMP

Segments of aorta were incubated in Krebs solution bubbled with 95% O_2 and 5% CO_2 for 2 h at 34°C, and phenylephrine was cumulatively applied every 60 min until consistent relaxation to acetylcholine was obtained. Phenylephrine at the EC_{80} concentration was added 5 min before exposure to ET-3. The preparations were incubated with 3 nM ET-3 for 30 s, and then quickly frozen in liquid nitrogen and homogenized in ice-cold 6% trichloroacetic acid in ice. The homogenates were centrifuged at 1700 *g* for 15 min at 4°C and the supernatants were extracted with 3 volumes of water-saturated ether. Cyclic GMP was measured by radioimmunoassay (Honma et al., 1977) with commercially available kits.

Statistical analysis

Values are expressed as means ± s.e.mean. The statistical significance between means was analysed by Student's unpaired *t* test, and *P* values of less than 0.05 were considered as significant.

Materials

The drugs used were endothelin-1, endothelin-3, N^G-nitro L-arginine, N^G-nitro D-arginine (all from Peptide Institute, Osaka, Japan), acetylcholine chloride, ATP, isoprenaline hydrochloride, sodium nitroprusside, phenylephrine hydrochloride, methylene blue, haemoglobin and calmidazolium (all from Sigma Chemical Co., St Louis, Mo, U.S.A.). Kits for the radioimmunoassay of cyclic GMP were obtained from Yamasa Shoyu Co., Ltd. (Choshi, Japan).

Results

Characteristics of endothelin-3-induced relaxation

After confirming that the thoracic aorta ring preparation precontracted with an EC_{80} concentration of phenylephrine (0.3 μM) showed consistent relaxation in response to acetylcholine or ATP, ET-3 was cumulatively applied. ET-3 at concentrations of above 1 nM, induced a concentration-dependent relaxation of the arteries obtained from 10 week old rats (Figure 1). When the concentration of ET-3 was increased to 30 nM, a contractile effect became apparent; the relaxation reversed towards the initial precontracted level. However, relaxation caused by the EC_{50} concentration of ET-3 (3 nM) persisted over 60 min. Usually on the 2nd application (60 min after washing), ET-3 at concentrations of up to 30 nM failed to relax the arteries. However, at this time, acetylcholine-induced relaxation was not affected (EC_{50} values: 77.7 ± 19.7 nM before ET-3 *vs.* 62.8 ± 21.3 nM after ET-3, $n = 5$). Loss of sensitivity of the arteries to ET-3 was reversible, but more than 4 h were necessary for the arteries to recover initial sensitivity to ET-3.

Removal of the endothelium by rubbing the lumen of the aortae with cotton thread abolished ET-3-induced relaxation (Figure 1b), without affecting that induced by nitroprusside (EC_{50} values: 0.52 ± 0.14 nM with endothelium *vs.* 0.75 ± 0.19 nM without endothelium, $n = 5$) or isoprenaline (EC_{50} values: 26.4 ± 2.4 nM with endothelium *vs.* 30.7 ± 8.1 nM without endothelium, $n = 5$).

In the preparations treated with indomethacin (100 μM for 60 min and then washed for 30 min) or glibenclamide (up to 10 μM for 30 min), neither ET-3-induced relaxation nor rebound contraction were affected.

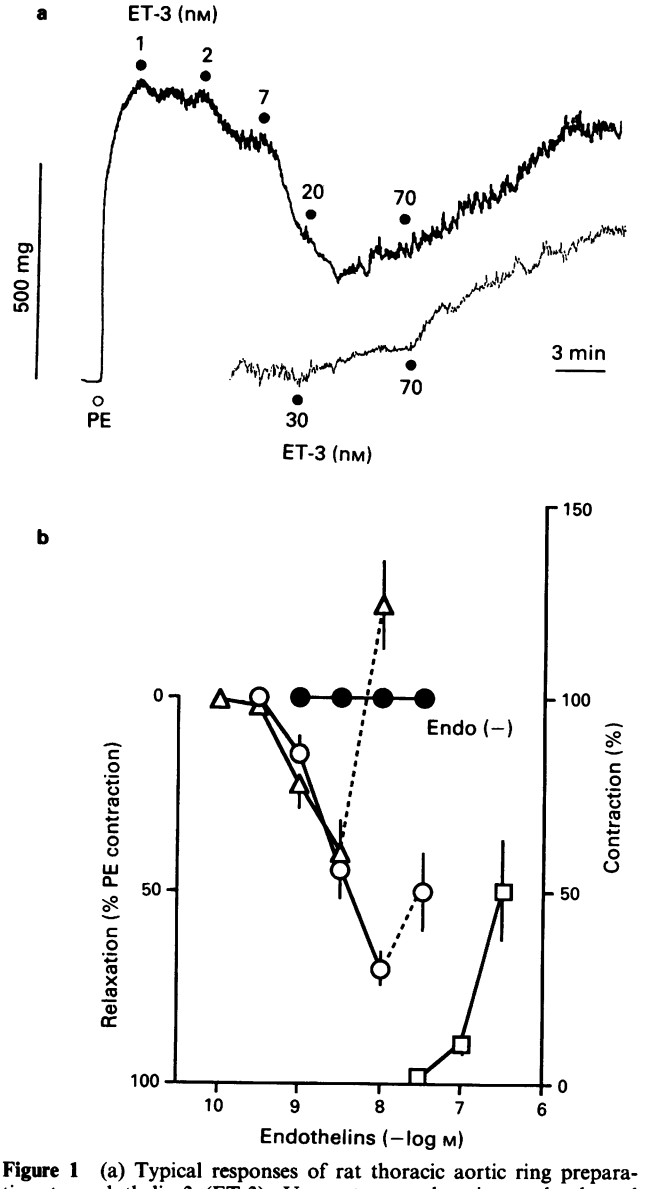


Figure 1 (a) Typical responses of rat thoracic aortic ring preparation to endothelin-3 (ET-3). Upper trace; relaxation and rebound contraction induced by ET-3. The aorta was first contracted with the EC_{80} concentration of phenylephrine (PE, 0.3 μM) to produce tone, and ET-3 was applied cumulatively. Lower trace; contraction elicited by ET-3 in aorta without phenylephrine-induced tone. (b) Effect of ET-3 on thoracic aortae from 10 week old rats. (○), ET-3 control; (●), ET-3 on endothelium-denuded arteries; (△), ET-1-induced relaxation is also shown for comparison. Dotted lines indicate rebound contractions following relaxations; (□), ET-3-induced contraction of the aorta in resting state. Left and right ordinates show the relaxation and contraction of the artries, respectively, as percentages of the contractions induced by the EC_{80} concentrations of phenylephrine (PE, 0.3 μM). Other experimental conditions were as for (a). The values are means with s.e.mean of preparations from 5 to 9 rats.

In the quiescent preparations (when the tone was not produced with PE), ET-3 at higher concentrations (above 30 nM) than those inducing relaxation (1–30 nM) evoked contraction of the arteries (Figures 1a and b).

Endothelin-1 (ET-1) elicited substantially similar responses to ET-3, the threshold concentration of ET-1 for inducing relaxation was the same as that for ET-3, but rebound contraction following relaxation developed at a lower concentration (10 nM) than with ET-3 (30 nM). The vasorelaxation to ET-1 showed tachyphylaxis, and cross desensitization occurred between responses to ET-1 and ET-3.

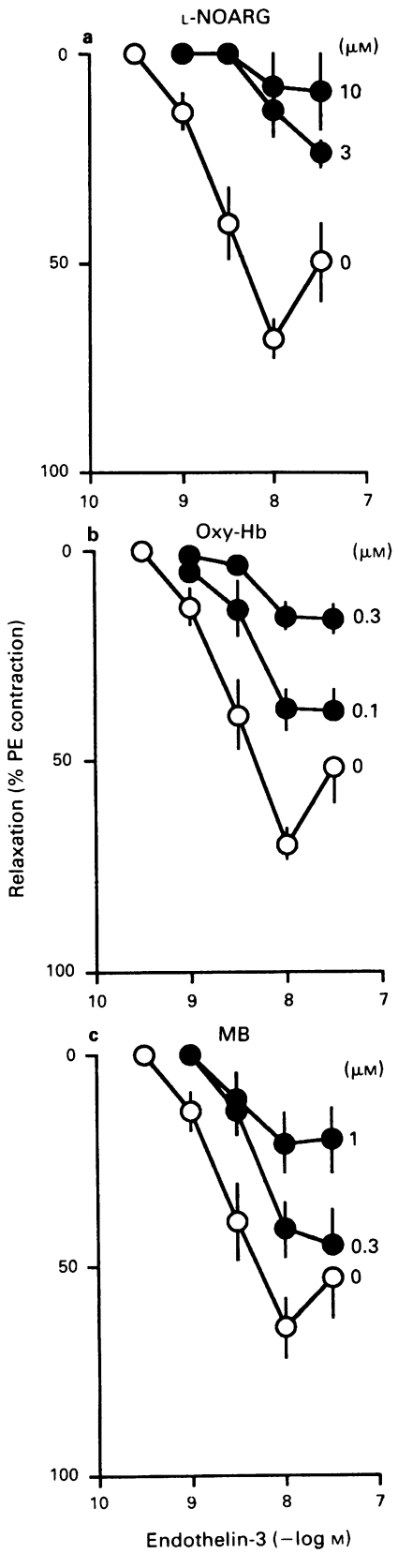


Figure 2 Inhibitory effects of N^G-nitro L-arginine (L-NOARG, a), haemoglobin (Oxy-Hb, b) and methylene blue (MB, c) on the endothelin-3 (ET-3)-induced relaxation of rat thoracic aorta. The agents were added 20 min before application of ET-3. (○), control curves; (●), in the presence of the inhibitors. Other experimental conditions were as for Figure 1. The values are means with s.e.mean of preparations from 5 rats.

Effects of compounds affecting NO pathway

Pretreatment with N^G-nitro L-arginine (L-NOARG, 3–10 μM), Oxy-Hb (0.1–0.3 μM) or MB (0.1–3 μM) for 20 min attenuated the ET-3-induced relaxation. Concentration-response curves for ET-3 in the presence of these inhibitors are shown in Figure 2.

In addition, when the relaxation induced by 30 nM ET-3 had reached a steady level, it was reversed rapidly and dose-dependently by L-NOARG at concentrations above 1 μM (data not shown). In contrast to the inhibitory effect of L-NOARG, N^G-nitro D-arginine was without effect on the ET-3-induced relaxation. Similar reversal of the relaxation to ET-3 was observed following application of either Oxy-Hb (0.1–0.3 μM) or MB (0.3–1 μM).

Effect of calmidazolium

Pretreatment for 30 min with the calmodulin inhibitor calmidazolium, at a concentration which did not affect phenylephrine-induced initial tone (10 μM), suppressed ET-3-induced relaxation (Figure 3). This concentration of calmidazolium also attenuated acetylcholine-induced relaxation, without affecting that to nitroprusside.

Stimulation of cyclic GMP production

ET-3 at a concentration of 10 nM caused a transient increase in the cyclic GMP level from a basal concentration of 1.58 ± 0.30 pmol mg^{-1} protein to 10.60 ± 1.30 pmol mg^{-1} protein ($n = 7$) in 30 s. The concentrations then rapidly decreased to the basal level by 2 min (Figure 4a). ET-3-induced cyclic GMP production was increased concentration-dependently (Figure 4b).

Removal of the endothelium or treatment of the preparations for 20 min with L-NOARG (10 μM), Oxy-Hb (1 μM) or MB (1 μM), at concentrations that inhibited the relaxant effect of ET-3, decreased the basal level of cyclic GMP and completely abolished the increase in cyclic GMP production stimulated by 10 nM ET-3 (Figure 5a).

In addition, treatment with 10 μM calmidazolium for 30 min abolished the ET-3-stimulated increase in cyclic GMP formation (Figure 5b). Under these conditions, calmidazolium also suppressed acetylcholine-stimulated cyclic GMP production, without affecting the nitroprusside-induced increase in cyclic GMP.

Discussion

Endothelins have been reported to have dual actions; they cause contraction of perfused arteries or induce a pressor response in anaesthetized animals and, in lower doses, they reduce perfusion pressure (Warner *et al.*, 1989; Hasunuma *et al.*, 1990) or elicit a depressor response (De Nucci *et al.*, 1988; Lippton *et al.*, 1989). However, the precise mechanism of vasorelaxant effect of ET-3 in the rat isolated aortic ring or helical preparations has not been demonstrated. In the present study, we found that ET-3 elicited a endothelium-dependent relaxation and stimulated the production of cyclic GMP in phenylephrine-precontracted rat aortae.

A complete loss of relaxation of the arteries to ET-3 was observed following a second application of ET-3. In addition, the vasorelaxant response to ET-1 was also subjected to tachyphylaxis. Furthermore, tachyphylaxis to one ET resulted in cross tachyphylaxis to another ET, as has been observed with the systemic depressor responses to ET-1 and ET-3 (Le Monnier de Goubille, 1990). The desensitization to ET-3 or ET-1 was specific, because under these conditions, acetylcholine-induced relaxation was largely unaffected.

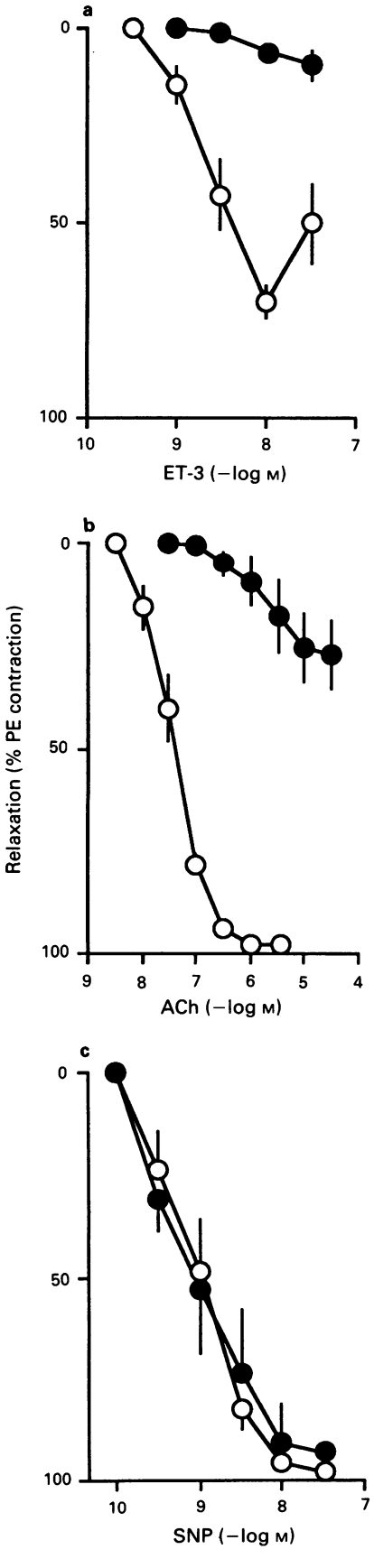


Figure 3 Selective inhibition by calmidazolium (CMZ) of the relaxation induced by endothelin-3 (ET-3). CMZ (10 μM, ●) was applied for 30 min before the application of ET-3 (a). Acetylcholine (ACh, b) and nitroprusside (SNP, c) were used for comparison. (○), respective control. Other experimental conditions were as for Figures 1a and b. The values are means with s.e.mean of preparations from 5 rats.

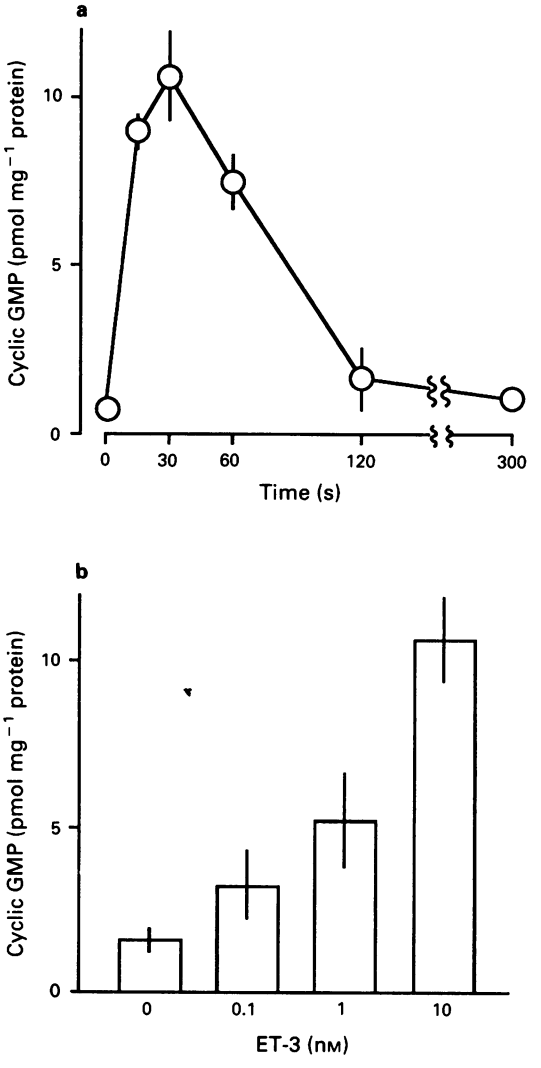


Figure 4 Time course of the change in cyclic GMP concentrations induced by 10 nM endothelin-3 (ET-3) (a), and dose-dependent production of cyclic GMP (b) in rat thoracic aorta. The aortic preparations were incubated with the EC_{80} concentration of phenylephrine (0.3 μM) for 5 min before exposure to ET-3. Amounts of cyclic GMP in the presence of various concentrations of ET-3 (b) were determined after 30 s. Values are means with s.e.mean of 5 to 7 preparations from 5 rats.

Therefore, it is suggested that the desensitization occurs probably at the level of ET-3 or ET-1 receptors. Following the relaxation caused by high concentrations of ET-3, at which the contractile effect became dominant, a rebound-like contraction developed, which had a similar time course to the contraction elicited by high concentrations of ET-3 in unstimulated aortae. Therefore, it is possible that the vasocontractile action of high concentrations of ET-3 could attenuate its vasorelaxant action. Conversely, the vasocontractile action could also be masked or attenuated by its dominant relaxant effect. In this respect, it has been reported that ET-1 stimulated EDRF release and resultant formation of cyclic GMP, which in turn modulated the contractile response to ET-1 (Topouzis *et al.*, 1991). However, the possibility cannot be ruled out that, as with the endothelium-dependent relaxation induced by platelet activating factor (Chiba *et al.*, 1990; Moritoki *et al.*, 1992), attenuation of relaxation reflects rapid desensitization to high concentrations of ET-3, which is in part responsible for the rebound-like contraction.

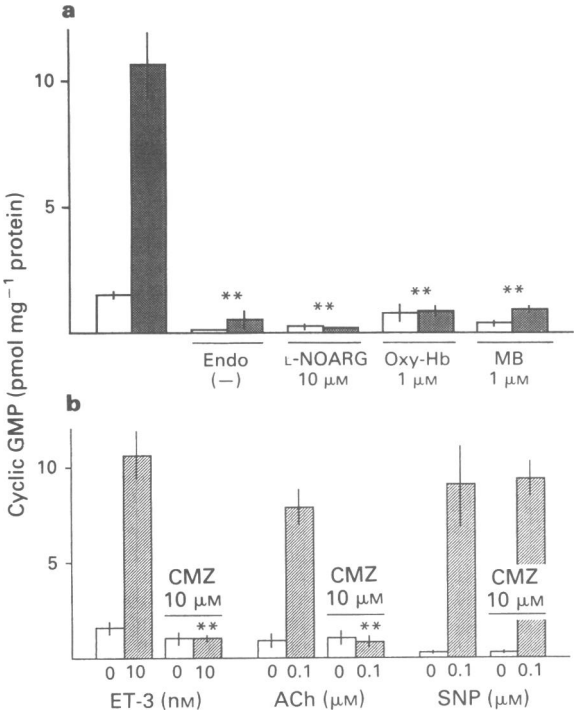


Figure 5 (a) Effects of N^G-nitro L-arginine (L-NOARG), haemoglobin (Oxy-Hb), methylene blue (MB) and removal of the endothelium (E−) on the cyclic GMP production induced by endothelin-3 (ET-3). The inhibitors were applied 20 min before and during incubation with 10 nM ET-3. Amounts of cyclic GMP were determined after 30 s. Open columns, basal level of cyclic GMP; closed columns, ET-3-stimulated production of cyclic GMP. Other experimental conditions were as for Figure 4. Columns represent means (with s.e.mean) of values ($n = 6$) in the arteries from 5 to 7 rats. $^{**}P < 0.01$, significantly different from the control value. (b) Effect of calmidazolium (CMZ) on the cyclic GMP production induced by ET-3, acetylcholine (ACh) and nitroprusside (SNP). CMZ was applied for 30 min before and during incubation with ET-3, ACh and SNP. ACh and SNP were used for comparison. Other experimental conditions were as for Figures 4 and 5(a). Columns represent means (s.e.mean) of values ($n = 5$) in the arteries from 5 to 6 rats. $^{**}P < 0.01$, significantly different from the respective control values without CMZ.

The relaxation to ET-3 was entirely dependent on the presence of the endothelium, suggesting the involvement of the NO pathway. Therefore, we examined the influence of the NO synthase inhibitor L-NOARG (Moore *et al.*, 1990) on the ET-3-induced relaxation. L-NOARG, when applied before ET-3, inhibited relaxation. When applied after the relaxation had reached a plateau, it reversed the relaxation toward the precontracted level. In addition, Oxy-Hb also inhibited the relaxation. These results suggest that ET-3 produces NO in the endothelium. The idea that the NO thus formed, activates soluble guanylate cyclase in the vascular smooth muscle (Rapoport & Murad, 1983; Rapoport *et al.*, 1983) came from the result that MB, a soluble guanylate cyclase inhibitor (Gruetter *et al.*, 1981; Martin *et al.*, 1985), attenuated the relaxation to ET-3. Further evidence supporting the contribution of NO to the ET-3-induced relaxation is that ET-3, at concentrations that caused relaxation, also increased production of cyclic GMP, and that removal of the endothelium, L-NOARG, Oxy-Hb and MB all decreased the ET-3 stimulated increase in cyclic GMP level.

Endothelin receptors have recently been classified as ET_A (Arai *et al.*, 1990) and ET_B (Sakurai *et al.*, 1990). ET_A receptors selectively bind ET-1, and ET_B receptors bind ET-1 and ET-3, nonselectively (Williams *et al.*, 1991). The idea that the endothelin receptors on the endothelium are of the ET_B subtype came from the observation that ET_B receptors mRNAs are expressed in the intact porcine aortic intimal cells (Sakamoto *et al.*, 1991). In rat thoracic aorta ring preparations, both ET-1 and ET-3 were found to cause similar degrees of relaxation, within the same concentration-range. Therefore, the receptors mediating NO formation and subsequent relaxation may be of the ET_B subtype.

Two types of constitutive NO synthase have been identified in endothelial cells: one is particulate and the other cytosolic, but both are Ca^{2+}- and calmodulin-dependent (Busse & Mülsch, 1990; Förstermann *et al.*, 1991). In this respect calmodulin antagonists, such as W-7 and calmidazolium, have been shown to abolish endothelium-dependent relaxations induced by acetylcholine, histamine, A23187 or ATP (Weinheimer & Osswald, 1986; Adeagbo & Triggle, 1991; Schini & Vanhoutte, 1992) and cyclic GMP increases stimulated by acetylcholine or ATP (Förstermann *et al.*, 1991; Schini & Vanhoutte, 1992). From the present results, we speculate that a calcium/calmodulin-dependent NO synthase and the formation of NO are involved in the ET-3-induced vasorelaxation and cyclic GMP production. The evidence for this idea comes from the result that both effects were found to be considerably attenuated by the calmodulin antagonist, calmidazolium, at a concentration suppressing acetylcholine-induced relaxation. This effect of calmidazolium was specific for the endothelium-dependent relaxations in that it did not affect endothelium-independent nitroprusside-induced relaxation and cyclic GMP formation.

Although the ET-3-stimulated increase in cyclic GMP formation was transient and decreased to the basal level in 2 min, ET-3-induced relaxation was still maintained. This may imply that mediators, such as endothelium-derived hyperpolarizing factor (EDHF) and prostanoids are released simultaneously with NO or following activation of the NO pathway. In this respect, it has been reported that endothelin-1 stimulates prostacyclin release from bovine cultured aortic endothelial cells, possibly by activation of phospholipase A_2 (Filep *et al.*, 1991). The relaxations induced by ET-1 and ET-3 in rat thoracic aorta are probably not due to prostaglandins, because the cyclo-oxygenase inhibitor, indomethacin, did not attenuate the relaxation. It has been reported that the K^+-channel antagonist, glibenclamide (Standen *et al.*, 1989), can inhibit pulmonary vasodilatation induced by ET-1 in the rat (Hasunuma *et al.*, 1990). In the present study, however, glibenclamide failed to attenuate ET-3-induced relaxation of rat thoracic aorta, suggesting that the effect is not mediated at least by glibenclamide-sensitive K^+-channel activation. While this manuscript was in preparation, it was reported that in canine coronary artery, calmidazolium inhibited relaxation mediated by EDHF but did not inhibit those induced by endothelium-derived NO (Nagao *et al.*, 1992; Illiano *et al.*, 1992). However, we found that ET-3-induced relaxation, which was inhibited by calmidazolium, was almost completely blocked by NO pathway inhibitors, suggesting that EDHF does not contribute to ET-3-induced relaxation. Nevertheless, the possibility cannot be ruled out that ET-3 activates the NO pathway, which in turn stimulates production of EDHF, and EDHF contributes to sustaining component of ET-3-induced relaxation.

It is concluded that in the rat isolated thoracic aorta ring preparation, ET-3 induces endothelium-dependent relaxations that are mediated through the release of NO, the resultant activation of soluble guanylate cyclase and subsequent production of cyclic GMP. The enzyme contributing to NO formation may be of the calcium-calmodulin-dependent, constitutive type.

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References

ADEAGBO, A.S.O. & TRIGGLE, C.R. (1991). Effects of some inorganic divalent cations and protein kinase C inhibitors on endothelium-dependent relaxation in the rat isolated aorta and mesenteric arteries. *J. Cardiovasc. Pharmacol.*, **18**, 511–521.

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

BEVAN, J.A., DUCKLES, S.P. & LEE, T.J.-F. (1975). Histamine potentiation of nerve- and drug-induced response of a rabbit cerebral artery. *Circ. Res.*, **36**, 647–653.

BUSSE, R. & MÜLSCH, A. (1990). Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. *FEBS Lett.*, **265**, 133–136.

CHIBA, Y., MIKODA, N., KAWASAKI, H. & ITO, K. (1990). Endothelium-dependent relaxant action of platelet activating factor in the rat mesenteric artery. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **341**, 68–73.

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.

EMORI, T., HIRATA, Y., KANNO, K., OHTA, K., EGUCHI, S., IMAI, T., SHICHIRI, M. & MARUMO, F. (1991). Endothelin-3 stimulates production of endothelium-derived nitric oxide via phosphoinositide breakdown. *Biochem. Biophys. Res. Commun.*, **174**, 228–235.

FILEP, J.G., BATTISTINI, B., COTE, Y.P., BEAUDOIN, A.R. & SIROIS, P. (1991). Endothelin-1 induces prostacyclin release from bovine aortic endothelial cells. *Biochem. Biophys. Res. Commun.*, **177**, 171–176.

FÖRSTERMANN, 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.

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.

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., IZUMI, Y., SOMA, M., WATANABE, Y., WATANABE, M., HATANO, M., SAKUMA, I. & YASUDA, H. (1991). Effects of indomethacin, endothelium-denudation, methylene blue and L-N^G-monomethyl arginine on the vasoactive effects of endothelin-3. *Jpn. J. Pharmacol.*, **55**, 375–380.

GRUETTER, C.A., KADOWITZ, P.J. & IGNARRO, L.J. (1981). Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerin, sodium nitrite, and amyl nitrite. *Can. J. Physiol. Pharmacol.*, **59**, 150–162.

HASUNUMA, K., RODMAN, D.M., O'BRIEN, R.F. & MCMURTRY, I.F. (1990). Endothelin 1 causes pulmonary vasodilation in rats. *Am. J. Physiol.*, **259**, H48–H54.

HONMA, M., SATOH, T., TAKEZAWA, J. & UI, M. (1977). An ultrasensitive method for the simultaneous determination of cyclic AMP and cyclic GMP in small-volume samples from blood and tissue. *Biochem. Med.*, **18**, 257–273.

ILLIANO, S., NAGAO, T. & VANHOUTTE, P.M. (1992). Calmidazolium, a calmodulin inhibitor, inhibits endothelium-dependent relaxations resistant to nitro-L-arginine in the canine coronary artery. *Br. J. Pharmacol.*, **107**, 387–392.

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

LIPPTON, H.L., COHEN, G.A., MCMURTRY, I.F. & HYMAN, A.L. (1991). Pulmonary vasodilation to endothelin isopeptides in vivo is mediated by potassium channel activation. *J. Appl. Physiol.*, **70**, 947–952.

LIPPTON, H.L., HAUTH, T.A., SUMMER, W.R. & HYMAN, A.L. (1989). Endothelin produces pulmonary vasoconstriction and systemic vasodilation. *J. Appl. Physiol.*, **66**, 1008–1012.

MARTIN, W., VILLIANI, 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.

MEHTA, J.L., LAWSON, D.L., YANG, B.C., MEHTA, P. & NICHOLS, W.W. (1992). Modulation of vascular tone by endothelin-1: role of preload, endothelial integrity and concentration of endothelin-1. *Br. J. Pharmacol.*, **106**, 127–132.

MOORE, P.K., AL-SWAYEN, O.A., CHONG, N.W.S., 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.

MORITOKI, H., HISAYAMA, T., TAKEUCHI, S., MIYANO, H. & KONDOH, W. (1992). Involvement of nitric oxide pathway in the PAF-induced relaxation of rat thoracic aorta. *Br. J. Pharmacol.*, **107**, 196–201.

NAGAO, T., ILLIANO, S. & VANHOUTTE, P.M. (1992). Calmodulin antagonists inhibit endothelium-dependent hyperpolarization in the canine coronary artery. *Br. J. Pharmacol.*, **107**, 382–386.

NAMIKI, A., HIRATA, Y., ISHIKAWA, M., MOROI, M., AIKAWA, J. & MACHII, K. (1992). Endothelin-1- and endothelin-3-induced vasorelaxation via common generation of endothelium-derived nitric oxide. *Life Sci.*, **50**, 677–682.

RAPOPORT, R.M., DRAZNIN, M.B. & MURAD, F. (1983). Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. *Nature*, **306**, 174–176.

RAPOPORT, R.M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. *Circ. Res.*, **52**, 352–357.

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

SCHINI, V.B. & VANHOUTTE, P.M. (1992). Inhibitors of calmodulin impair the constitutive but not the inducible nitric oxide synthase activity in the rat aorta. *J. Pharmacol. Exp. Ther.*, **261**, 553–559.

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.

TOPOUZIS, S., HUGGINS, J.P., PELTON, J.T. & MILLER, R.C. (1991). Modulation by endothelium of the responses induced by endothelin-1 and by some of its analogues in rat isolated aorta. *Br. J. Pharmacol.*, **102**, 545–549.

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.

WEINHEIMER, G. & OSSWALD, H. (1986). Inhibition of endothelium-dependent smooth muscle relaxation by calmodulin antagonists. *Naunyn-Schmiedebergs. Arch. Pharmacol.*, **332**, 391–397.

WILLIAMS Jr., D.L., JONES, K.L., PETTIBONE, D.J., LIS, E.V. & CLINESCHMIDT, V. (1991). Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. *Biochem. Biophys. Res. Commun.*, **175**, 556–561.

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.

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Differential changes of adrenoceptor- and muscarinic receptor-linked prostacyclin synthesis by the aorta and urinary bladder of the diabetic rat

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1 The effect of experimental diabetes mellitus (DM; hyperglycaemic, non-ketotic; 2 months duration) in the rat on receptor-linked prostacyclin (PGI_2) synthesis (measured as 6-oxo-$PGF_{1\alpha}$ by radioimmunoassay) was studied in the aorta and urinary bladder using adrenaline, angiotensin II (AII) and acetylcholine (ACh). Signal transduction systems were studied via stimulation of PGI_2 synthesis with phorbol ester dibutyrate (PDBU; a protein kinase C activator [PKC]), Ca^{2+} ionophore A23187 (A23187) and thapsigargin (both elevate intracellular Ca^{2+}, activating phospholipase A_2 [PLA_2]) and arachidonate (AA; substrate for PGI_2 synthesis).

2 In response to adrenaline, AII and phorbol ester, aortic PGI_2 release was markedly reduced (all $>75\%$) in diabetic rats compared to controls. EC_{50}s of the dose-response curves for adrenaline, AII and PDBU were also markedly increased in aortae from DM rats compared to controls. Although there was decreased output of PGI_2 in response to A23187 by aortae from diabetic rats compared to controls, there was no difference in the EC_{50}s (mean ± s.e.mean: diabetic, $2.7 \pm 0.2 \times 10^{-6}$ M; controls $2 \pm 0.18 \times 10^{-6}$ M). There were no differences in PGI_2 release (or in the EC_{50}s) in response to thapsigargin or AA between aortae from diabetic and control rats.

3 In the urinary bladder, there was a marked increase in PGI_2 output in response to ACh and a marked decrease in EC_{50}s for the ACh-PGI_2 dose-response curves in diabetic rats ($EC_{50} = 5.8 \pm 0.32 \times 10^{-7}$ M) compared to controls ($EC_{50} = 2.2 \pm 0.15 \times 10^{-6}$ M). Although there was an increase in PGI_2 output in the urinary bladders from diabetic rats in response to A23187, there were no differences in the EC_{50}s (control, $1.8 \pm 0.2 \times 10^{-6}$ M; diabetic, $1.1 \pm 0.15 \times 10^{-6}$ M). In the urinary bladders, there were no differences in PGI_2 output (or the EC_{50}s) in response to PDBU, thapsigargin or AA between diabetic or control rats.

4 These data indicate that: (i) reduced PGI_2 synthesis coupled to adrenoceptors and AII receptors in the aortae of diabetic rats may be due to diminished PKC activity and not to changes in receptor density and/or affinity, Ca^{2+} stores, PLA_2, cyclo-oxygenase or PGI_2 synthase; (ii) the diametrically opposite effect of DM on ACh-stimulated PGI_2 synthesis is not due to an increase in PKC activity, but possibly to an increase in muscarine receptor number and/or affinity; (iii) changes in receptor-linked PGI_2 synthesis are not ubiquitous in experimental DM and may be organ-specific.

Keywords: Prostacyclin; diabetes mellitus; rat aorta; rat urinary bladder; adrenoceptors; muscarinic receptors

Introduction

Patients with diabetes mellitus (DM) are at greater risk than the healthy population of developing cardiovascular disease (CVD; Banga & Sixma, 1986; Mikhailidis *et al.*, 1988). Of the many aetiological factors associating CVD with DM, a marked attenuation of prostacyclin (PGI_2) synthesis by blood vessels from diabetic laboratory animals have been reported (Harrison *et al.*, 1978; Rogers *et al.*, 1981; Jeremy *et al.*, 1987a). Apart from being a vasodilator and inhibitor of platelet aggregation (Moncada & Vane, 1979), PGI_2 modulates other vascular functions including cell proliferation (Huttner *et al.*, 1977; Marui *et al.*, 1990; Pomerantz & Hajjar, 1989) and metabolism of cholesterol (Hajjar *et al.*, 1982). In turn, disruption of these processes have all been implicated in the aetiology of CVD, in particular, atherogenesis and thrombosis (Jeremy & Mikhailidis, 1990).

The precise mechanisms that determine reduced vascular PGI_2 synthesis are not clearly defined, although a reduction in substrate (arachidonate) levels in blood vessels has been suggested (Holman *et al.*, 1983; Jeremy *et al.*, 1987a; Dang *et al.*, 1988). It has also been established that vascular PGI_2 synthesis is stimulated by vasoactive agonists (e.g. noradrenaline, histamine, acetylcholine, angiotensin II; Jeremy *et al.*, 1988a) an effect which in turn is mediated by G proteins, protein kinase C (PKC) and inositol trisphosphate (IP_3; Griendling *et al.*, 1986; Hallam *et al.*, 1988; Jeremy *et al.*, 1988a). The net result of activation of these systems is transmembrane Ca^{2+} influx and an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Ca^{2+} then activates phospholipase A_2 (PLA_2) which in turn liberates PGI_2 substrate, arachidonic acid (AA), from phospholipid stores (Irvine, 1982). In this context, experimental DM in the rat is associated with altered receptor-linked functions in vascular tissue (Tomlinson *et al.*, 1992).

Thus, in order to elucidate further the mechanisms that determine diminished vascular PGI_2 synthesis, the effects of a range of stimulators that act at different sites in the receptor activation-PGI_2 synthesis pathway were studied on PGI_2 synthesis by the streptozotocin-induced diabetic rat: adrenaline (mediated by heterogeneous α-adrenoceptors; Jeremy *et al.*, 1985a) and angiotensin II (AII; Hassid & Williams, 1983; Stevens *et al.*, 1992), phorbol ester dibuytrate (PDBU; a PKC activator: Nishizuka, 1984; Jeremy & Dandona, 1986), thapsigargin (elevates intracellular Ca^{2+}; Thastrup *et al.*, 1990; Levine & Watanabe, 1991), Ca^{2+} ionophore A23187 (creates artificial Ca^{2+} channels; Reed & Lardy, 1972) and arachidonic acid (AA; substrate for PGI_2 synthesis). In order to examine whether other non-vascular smooth muscle tissues

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are affected in a similar or disparate manner to the aorta, PGI_2 synthesis by urinary bladder was investigated. It is also notable that the urinary bladder of the diabetic rat releases more PGI_2 than controls (Jeremy *et al.*, 1986a). Bladder PGI_2 synthesis was stimulated with acetylcholine (PGI_2 synthesis in the urinary bladder is mediated by muscarinic receptors; Jeremy *et al.*, 1986b) as well as PDBU, thapsigargin, A23187 and AA.

Methods

Induction of diabetes

All experiments were carried out on male Sprague Dawley rats of initial body weight of 250 g. Non-ketonuric, hyperglycaemic diabetes was induced by injecting streptozotocin (65 mg kg^{-1} body wt., i.v.). These rats developed glycosuria but not ketonuria or haematuria. Diabetic rats were fed either *ad libitum* with Diet 41B (Grain Harvester, Kent) and allowed free access to water. The study also included seven age-matched controls. Urine was monitored over the duration of diabetes for glucose, ketone bodies and proteins with Multistix (Ames Division, Miles Laboratories Ltd., Stoke Poges, Bucks). After 8 weeks, rats were anaesthetized with pentobarbitone (90 mg kg^{-1} intraperitoneally; Sagatal: May and Baker Ltd., Dagenham, Essex) and blood was collected by cardiac puncture for measurement of blood glucose. Thoracic aortae and urinary bladders were rapidly excised and placed in Dulbecco's Minimum Essential Medium (MEM), pregassed with 95% O_2/5% CO_2.

Preparation and incubation of aortic and urinary bladder tissue

After removal of adventitia and fatty tissue, the aortae were cut into 2 mm rings with a scalpel blade on a Teflon block. The bladders were cut longitudinally into four strips and each strip into six further segments. Tissues were then pooled and randomised in Dulbecco's Minimum Essential Medium (MEM: pregassed with 95% O_2:5% CO_2) and incubated for 6 h in MEM, at 37°C, with changes of medium every 30 min, to allow prostanoid release elicited by preparative handling to subside (Jeremy *et al.*, 1985a, 1987a). Following preincubation, tissues were placed in 1 ml MEM (per tube: 2 aortic rings, or 2 bladder segments, in duplicate [from each animal]). PGI_2 synthesis was then elicited in aortic tissue by the addition of varying concentrations of adrenaline, AII, PDBU, thapsigargin, AA and A23187 and in the bladder with ACh, PDBU, thapsigargin, AA and A23187. Tissues were then further incubated for 1 h at 37°C and aliquots of supernatant taken for estimation of 6-oxo-$PG_{1\alpha}$ (the stable spontaneous hydrolysate of PGI_2; Moncada & Vane, 1979) concentrations by radioimmunoassay as previously described (Jeremy *et al.*, 1985a; 1987a).

Materials and drugs

Adrenaline bitartrate, angiotensin II, acetylcholine chloride, arachidonic acid (calcium salt; 99.9% pure), phorbol ester dibutyrate, Ca^{2+} ionophore A23187, Dulbecco's Minimum Essential Medium and streptozotocin were purchased from Sigma Chemical Co. (Poole, Dorset). Thapsigargin was purchased from Novabiochem UK (Nottingham). Antisera against 6-oxo-$PGF_{1\alpha}$ of high serological specificity was purchased from Capell Laboratories (West Chester, PA, U.S.A.). ^{3}H-ligands were purchased from New England Nuclear (Dreieich, Germany) and unlabelled ligands from UpJohn Co. (Kalamazoo, MI, U.S.A.).

Statistical analysis

Values are expressed as mean ± s.d. of mean. A one-way analysis of variance was performed on the data and the significance determined by Student's *t* test. *P* values less than 0.001 were taken to be significant. Significant differences between responses are expressed graphically with an asterisk. EC_{50}s are expressed as mean ± s.e.mean, $n = 7$.

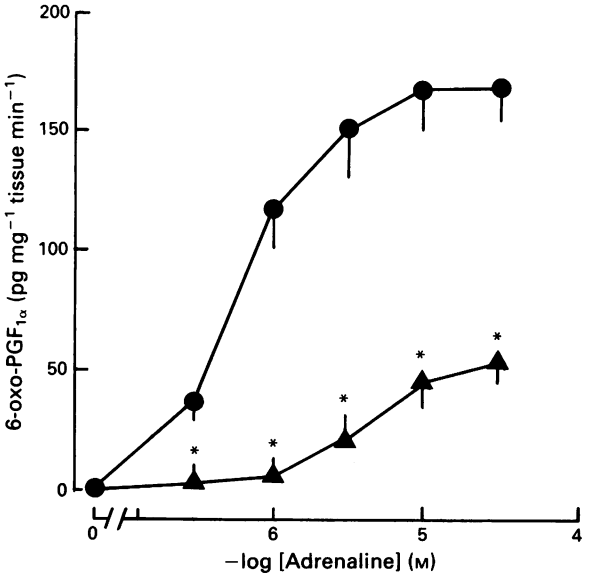


Figure 1 Effect of adrenaline on prostacyclin (PGI_2, as 6-oxo-$PGF_{1\alpha}$) release by the aortae of diabetic (▲) and age-matched control rats (●). Each point is the mean ± s.d., $n = 7$. *$P < 0.01$. EC_{50}s: control, $5.8 \pm 0.4 \times 10^{-7}$ M; diabetics, $4.1 \pm 0.3 \times 10^{-6}$ M.

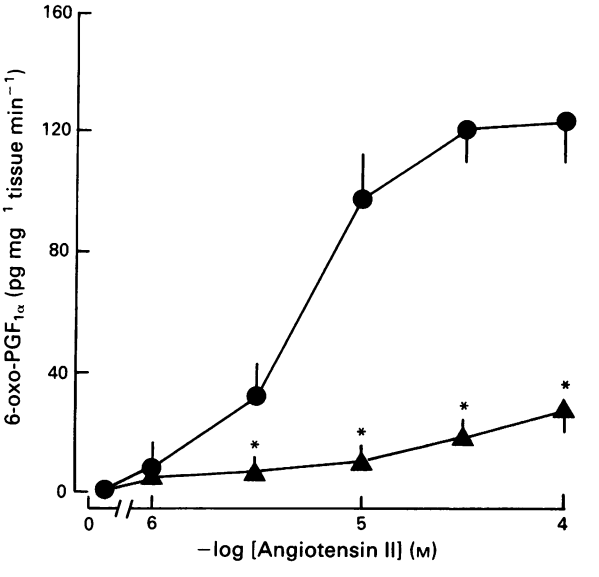


Figure 2 Effect of angiotensin II on prostacyclin (PGI_2) release (as 6-oxo-$PGF_{1\alpha}$) by the aortae of diabetic (▲) and age-matched control rats (●). Each point is the mean ± s.d., $n = 7$. *$P < 0.01$. EC_{50}s: control, $5.4 \pm 0.35 \times 10^{-7}$ M; diabetics, $4 \pm 0.28 \times 10^{-6}$ M.

Results

Body weights (median [range], $n = 35$) of the animal groups at the time they were killed were: controls, 475 g (449–516); diabetic, 239 g (220–280). At death, blood glucose concentrations (mmol l^{-1}; median [range]) were: controls, 8.4 (6.8–10.3); diabetic, 30.3 (28.2–32.7).

Aortic PGI_2 release (expressed as pg 6-oxo-$PGF_{1\alpha}$ liberated mg^{-1} tissue min^{-1}) was significantly reduced in diabetic rats compared to controls in response to adrenaline (Figure 1), AII (Figure 2) and PDBU (Figure 3). EC_{50}s of the dose-response curves for adrenaline, AII and PDBU were also

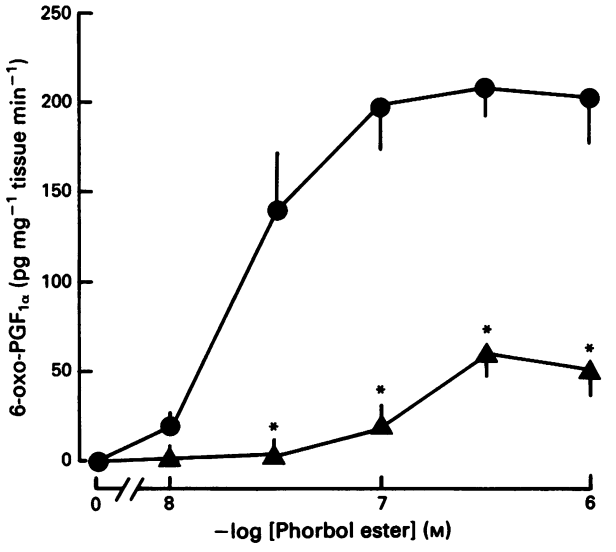


Figure 3 Effect of phorbol ester dibutyrate on prostacyclin (PGI_2 as 6-oxo-$PGF_{1\alpha}$) release by the aortae of diabetic (▲) and age-matched control rats (●). Each point is the mean ± s.d., $n = 7$. *$P < 0.01$. EC_{50}s: control, $1.9 \pm 0.05 \times 10^{-8}$ M; diabetics, $1.4 \pm 0.09 \times 10^{-7}$ M.

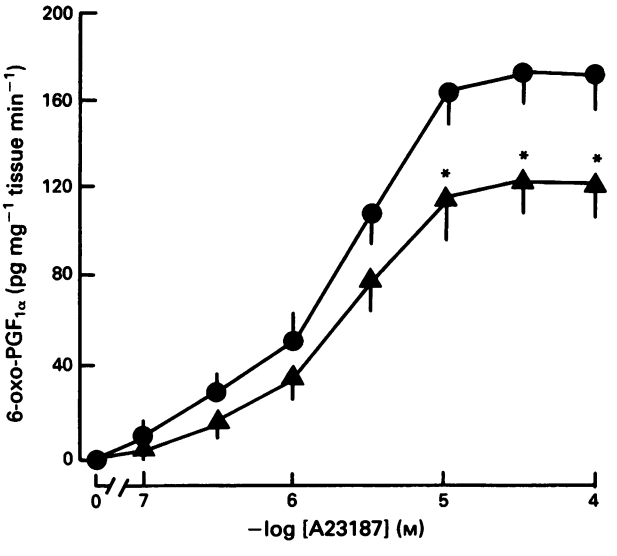


Figure 4 Effect of A23187 on prostacyclin (PGI_2 as 6-oxo-$PGF_{1\alpha}$) release by the aortae of diabetic (▲) and age-matched control rats (●). Each point is the mean ± s.d., $n = 7$. *$P < 0.01$.

markedly increased in aortae from diabetic rats compared to controls (see figure legends for values). Although there was decreased output of PGI_2 in response to A23187 by aortae from diabetic rats compared to controls (Figure 4), there was no marked difference in the EC_{50}s (diabetic, $2.7 \pm 0.2 \times 10^{-6}$ M; controls, $2 \pm 0.18 \times 10^{-6}$ M). There were no differences in PGI_2 release in response to thapsigargin between diabetic and control aortae (Figure 5) and a marginal increase in the EC_{50}s (control, $4.2 \pm 0.38 \times 10^{-7}$ M; diabetics, $6.2 \pm 0.48 \times 10^{-7}$ M). There were no differences in PGI_2 release in response to AA in aortae from control (maximal output, 118 pg mg^{-1} min^{-1}; EC_{50}, $3.4 \pm 0.22 \times 10^{-5}$ M) or diabetic rats (maximal output, 116 pg mg^{-1} min^{-1}; EC_{50}, $3.6 \pm 0.18 \times 10^{-5}$ M).

In contrast to the aorta there was a marked increase in PGI_2 release in response to ACh in the urinary bladder of diabetic rats compared to controls (Figure 6) and a marked decrease in EC_{50}s (diabetic, $5.8 \pm 0.32 \times 10^{-7}$ M; controls, $2.2 \pm 0.15 \times 10^{-6}$ M). Although there was an increase in PGI_2 output in the urinary bladders from diabetic rats in response to A23187 (Figure 7), there were no differences in the EC_{50}s (control, $1.8 \pm 0.2 \times 10^{-6}$ M; diabetic, $1.1 \pm 0.15 \times 10^{-6}$ M). There were no differences in PGI_2 release in response to PDBU in bladders from control (maximal output, 48 pg mg^{-1} min^{-1}; EC_{50}, $1 \pm 0.08 \times 10^{-6}$ M) and diabetic rats (maximal output, 52 pg mg^{-1} min^{-1}; EC_{50}, $1 \pm 0.09 \times 10^{-6}$ M). There were also no differences in PGI_2 release in response to thapsigargin in bladders from control (maximal output, 21 pg mg^{-1} min^{-1}; EC_{50}, $2 \pm 0.14 \times 10^{-6}$ M) or diabetic rats (maximal output, 23 pg mg^{-1} min^{-1}; EC_{50}, $2.1 \pm 0.05 \times 10^{-6}$ M). There were no differences in PGI_2 release in response to AA in bladders from control (maximal output, 62 pg mg^{-1} min^{-1}; EC_{50}, $1 \pm 0.1 \times 10^{-5}$ M) or diabetic rats (maximal output, 66 pg mg^{-1} min^{-1}; EC_{50}, $8.5 \pm 0.92 \times 10^{-6}$ M).

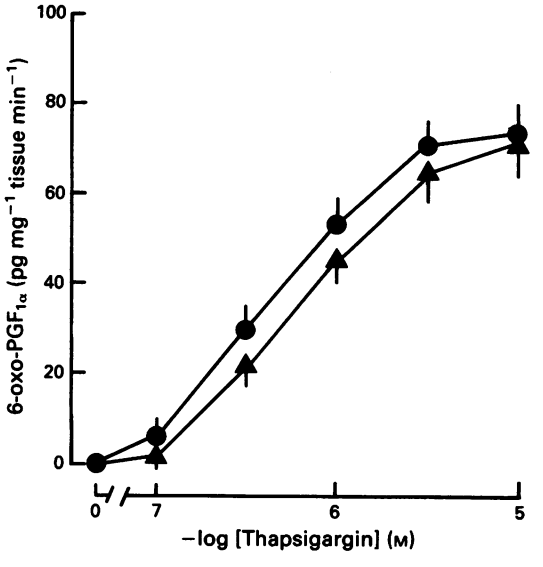


Figure 5 Effect of thapsigargin on prostacyclin (PGI_2 as 6-oxo-$PGF_{1\alpha}$) release by the aortae of diabetic (▲) and age-matched control rats (●). Each point is the mean ± s.d., $n = 7$. *$P < 0.01$.

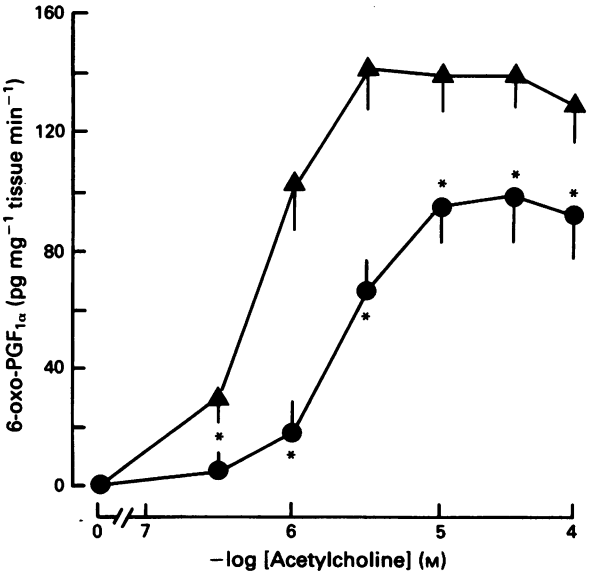


Figure 6 Effect of acetylcholine on prostacyclin (PGI_2 as 6-oxo-$PGF_{1\alpha}$) release by the urinary bladder of diabetic (▲) and age-matched control rats (●). Each point is the mean ± s.d., $n = 7$. *$P < 0.01$.

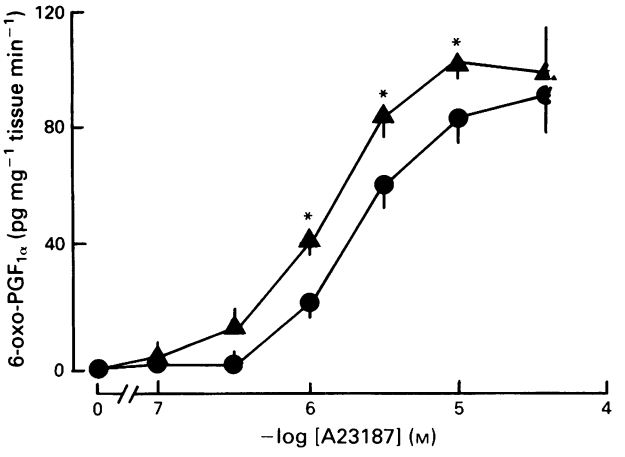


Figure 7 Effect of A23187 on prostacyclin (PGI_2 as 6-oxo-$PGF_{1\alpha}$) release by the urinary bladder of diabetic (▲) and age-matched control rats (●). Each point is the mean ± s.d., $n = 7$. $^*P < 0.01$.

Discussion

Although it is well established that PGI_2 synthesis by the rat aorta is diminished in experimental DM (Harrison *et al.*, 1978; Rogers *et al.*, 1981; Jeremy *et al.*, 1987a), the mechanisms that determine this reduction are not clearly defined. In the present study there was a marked reduction in PGI_2 release in aortae from diabetic rats in response to adrenaline, AII and phorbol ester. Since receptor-linked PGI_2 synthesis in the rat aorta is mediated by PKC (Jeremy *et al.*, 1988a) and the reduction in PGI_2 release stimulated by agonists and phorbol ester were almost identical, these data indicate that the defect in receptor-linked PGI_2 synthesis in the aortae of diabetic rats may be due to alterations of PKC activity rather than to changes of affinity and/or number of receptors linked to PGI_2 synthesis (viz. α-adrenoceptor and AII receptors). It should be noted that in the rat aorta, the endothelium contributes negligible amounts of the total PGI_2 generated by this vessel in response to agonists, phorbol ester and ionophores (Jeremy & Dandona, 1989). The present data therefore reflect PGI_2 released by the vascular smooth muscle component of the aorta rather than that generated by the endothelium.

Recent data consolidate the proposal that vascular PKC activity is altered in DM. Lee *et al.* (1989) demonstrated a marked reduction in cytosolic (but an increase in membrane-associated) PKC in aortae of diabetic rats, an effect mimicked *in vitro* by high concentrations of glucose. Under normal conditions, receptor activation by agonists elicits the activation of cytosolic PKC (Iwamoto *et al.*, 1992). Activated PKC phosphorylates effector proteins and also moves into the membrane where it down-regulates receptor activity (Iwamoto *et al.*, 1992). The data of Lee *et al.* (1989) therefore points to a generalized movement of PKC into the plasma membrane of vascular cells in diabetic rats which in turn would serve to diminish receptor-linked responses, including PGI_2 synthesis. A reduction in cytosolic PKC would also account for the diminished response to phorbol ester reported in this study, since phorbol esters elicit responses by activating cytosolic PKC (Nishizuka, 1984; Iwamoto *et al.*, 1992). In terms of other receptor-linked functions, both attenuation and potentiation of receptor-linked contraction of aortae from diabetic rats has been reported (for review see Tomlinson *et al.*, 1992). How PKC relates to these variable alterations of contractility warrants consideration.

With regard to Ca^{2+} mobilization, no differences were found in the output of PGI_2, in response to thapsigargin, between diabetic and control aortae. Although PGI_2 output by diabetic aortae was diminished when stimulated with A23187, this reduction was far less than that seen with agonists and phorbol ester. Furthermore, the EC_{50}s for A23187 in both the diabetic and control aortae were similar. It has been shown that AA stores in vascular tissue, as well as circulating AA, are reduced in experimental DM (Thomson, 1980; Holman *et al.*, 1983; Dang *et al.*, 1988). This probably accounts for the reduction of PGI_2 release in response to A23187 by aortae from diabetic rats. Since thapsigargin mobilizes intracellular Ca^{2+} (Tharstrup, 1990; Levine & Watanabe, 1991) and A23187 elicits entry of extracellular Ca^{2+} via creation of artificial Ca^{2+} channels (Reed & Lardy, 1972), these data indicate that Ca^{2+} pools and/or PLA_2 activity in the aorta are unaltered by DM in the rat. It is also notable that in response to thapsigargin, the absolute output of PGI_2 was markedly less than that derived from A23187, adrenaline, AII or phorbol ester. This may indicate that intracellular Ca^{2+} elevation liberates AA from distinctly different phospholipid stores from other stimulators.

With AA-stimulated PGI_2 synthesis, no difference in the output of PGI_2 was seen between control and diabetic aortae. This consolidates previous conclusions that there is no alteration of either cyclo-oxygenase or PGI_2 synthase activity in blood vessels from diabetic rats (Jeremy *et al.*, 1987a).

In contrast to the aorta, there was a marked increase in PGI_2 output in response to ACh, as well as a profound decrease in the EC_{50} for ACh, in urinary bladders from diabetic rats when compared with controls. Although there was a significant increase in PGI_2 output in response to A23187 by bladders from diabetic animals there was no marked differences in the EC_{50}s of A23187 in diabetic and control bladders. There were also no differences in the EC_{50}s or output of PGI_2 in response to phorbol ester, thapsigargin, A23187 or AA. Thus, in the case of the bladder, there appears to be a specific increase in muscarine receptor number/activity linked to PGI_2 but no changes in PKC, Ca^{2+} stores, PLA_2, cyclo-oxygenase or synthase enzymes. These conclusions are in agreement with a recent study in which marked left shifts of the dose-response curves for ACh-stimulated contraction were found in urinary bladders from diabetic rats (Latifpour *et al.*, 1992). The diametrically opposite pattern of receptor-linked PGI_2 synthesis by the urinary bladder and the aorta can be ascribed to the particular stresses imposed on the bladder in experimental DM. In diabetic rats, the urinary bladder is markedly enlarged, a result of chronic distension caused by polydypsia and polyuria (Lincoln *et al.*, 1984). Thus, the up-regulation of muscarinic receptors (and the associated increase in PGI_2 [a contractor of urinary bladder smooth muscle; Mikhailidis *et al.*, 1987]) in the urinary bladder of diabetic rats is probably a specific adaptive event relating to increased micturition rate. It is notable that distension (and attendant hyperplasia) elicits an increase in PGI_2 synthesis in other tissues (e.g. human urinary bladder – Jeremy *et al.*, 1987c, human aorta – Tsang *et al.*, 1987; and portal vein – Ritter *et al.*, 1986).

Changes of PGI_2 synthesis in DM appear to be organ-specific. For example, there are no changes in 'spontaneous' (non-stimulated) PGI_2 synthesis in any region of the gastrointestinal tract (also markedly enlarged due to hyperphagia; Schedl *et al.*, 1982), whereas there is a three-fold increase in PGI_2 synthesis by the splanchnic vasculature and hepatic portal vein of the diabetic rat (Jeremy *et al.*, 1988b). There is also a marked output of PGI_2 from perfused mesenteric vessels from diabetic rats (Fujii *et al.*, 1986). Decreases in PGI_2 have been reported in all regions of the brain and the penis (Jeremy *et al.*, 1985b; 1987b). In contrast, Stevens *et al.* (1992) found no significant differences in PGI_2 release stimulated by AII from perfused lungs from diabetic rats. Taken together, these experimental findings indicate that large arteries appear to be more susceptible to DM than microvasculature. This in turn may be of importance from a clinical point of view since vascular complications (viz. atherosclerosis in large arteries) are the predominant cause of death in DM. Thus, apart from mediating PGI_2 release, PKC also controls other vascular functions including contractility

(Danthaluri & Deth, 1984; Rasmussen *et al.*, 1984) and cell proliferation, the pathognomonic lesion of atherosclerosis (Berridge, 1984). PKC also modulates the generation of vascular nitric oxide (NO; Lewis & Henderson, 1987; Smith & Lang, 1990), also an important vasodilator and inhibitor of platelet aggregation (Palmer *et al.*, 1987; Radomski *et al.*, 1987). Thus, the mechanisms and consequences of changes in PKC in vascular tissue from diabetic animals warrants further investigation.

References

BANGA, J.D. & SIXMA, J.J. (1986). Diabetes mellitus, vascular disease and thrombosis. *Clincs Haematol.*, **15**, 465–470.

BERRIDGE, M.J. (1984). Inositol trisphosphate and diacyl glycerol as second messengers. *Biochem. J.*, **220**, 345–360.

DANG, A.Q., FAAS, F.H., LEE, J.A. & CARTER, W.J. (1988). Altered fatty acid composition in the plasma, platelets and aorta of streptozotocin-induced diabetic rat. *Metabolism*, **37**, 1065–1072.

DANTHALURI, N.R. & DETH, R.C. (1984). Phorbol ester-induced contraction of arterial smooth muscle and inhibition of alpha-adrenergic response. *Biochem. Biophys. Res. Commun.*, **125**, 1103–1112.

FUJII, K., SOMA, M., HUANG, Y.-S., MANKU, M.S. & HORROBIN, D.F. (1986). Increased release of prostaglandins from the mesenteric vascular bed of diabetic animals: the effects of glucose and insulin. *Prostagl. Leuk. Med.*, **24**, 151–162.

GRIENDLING, K.K., RITTENHOUSE, S.E., BROCK, T.A., EKSTEIN, L.S., GIMBRONE, M.A. & ALEXANDER, R.W. (1986). Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. *J. Biol. Chem.*, **261**, 5901–5906.

HAJJAR, D.P., WEKSLER, B.B., FALCONE, D.J., HEPTON, J.M., TACK-GOLDMAN, K. & MINICK, C.R. (1982). Prostacyclin modulates cholesteryl ester hydrolytic activity by its effect on cyclic adenosine monophosphate in rabbit aortic smooth muscle. *J. Clin. Invest.*, **70**, 70479–70488.

HALLAM, T.J., PEARSON, J.D. & NEEDHAM, L.A. (1988). Thrombin-stimulated elevation of human endothelial cell cytoplasmic free calcium concentrations causes prostacyclin production. *Biochem. J.*, **251**, 243–249.

HARRISON, H.E., REECE, A.H. & JOHNSON, M. (1978). Decreased vascular prostacyclin in experimental diabetes. *Life Sci.*, **33**, 351–356.

HASSID, A. & WILLIAMS, C. (1983). Vasoconstrictor evoked prostaglandin synthesis in cultured vascular smooth muscle. *Am. J. Physiol.*, **245**, C278–C284.

HOLMAN, R.T., JOHNSON, S.B., GERRARD, J.M., MAUER, S.M., KUPCHO-SANBERG, S. & BROWN, D. (1983). Arachidonic acid deficiency in streptozotocin induced diabetes. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 2375–2379.

HUTTNER, I.I., GWEBE, E.T., PANGANAMALA, R.V., SHARMA, H.M. & GEER, J.C. (1977). Fatty acids and their prostaglandin derivatives: inhibitors of proliferation in aortic smooth muscle cells. *Science*, **197**, 289–291.

IRVINE, R.F. (1982). How are the levels of arachidonic acid controlled in mammalian cells? *Biochem. J.*, **204**, 3–16.

IWAMOTO, Y., KOIDE, H., OGITA, K. & NISHIZUKA, Y. (1992). The protein kinase C family for the regulation of cellular functions. *Bio. Med. Rev.*, **1**, 1–6.

JEREMY, J.Y. & DANDONA, P. (1986). Role of the diacyl glycerol protein kinase C system in mediating adrenoceptor-prostacyclin synthesis coupling in the rat aorta. *Eur. J. Pharmacol.*, **136**, 311–316.

JEREMY, J.Y. & DANDONA, P. (1989). Effect of endothelium removal on stimulatory and inhibitory modulation of rat aortic prostacyclin synthesis. *Br. J. Pharmacol.*, **96**, 243–250.

JEREMY, J.Y. & MIKHAILIDIS, D.P. (1990). Vascular and platelet eicosanoids, smoking and atherosclerosis. In *Tobacco Smoking and Atherosclerosis*. ed. Diana, J. *Adv. Exp. Med. Biol.*, Vol 273, pp. 135–146. New York, London: Plenum Press.

JEREMY, J.Y., MIKHAILIDIS, D.P. & DANDONA, P. (1985a). Adrenergic modulation of vascular prostacyclin synthesis. *Eur. J. Pharmacol.*, **114**, 33–40.

JEREMY, J.Y., MIKHAILIDIS, D.P., THOMPSON, C.S. & DANDONA, P. (1986a). The effect of streptozotocin-induced diabetes on PGI_2 synthesis by the rat urinary bladder. *J. Urol.*, **135**, 1290–1292.

JEREMY, J.Y., MIKHAILIDIS, D.P. & DANDONA, P. (1986b). Prostanoid synthesis by the rat urinary bladder: evidence for stimulation through muscarine receptor-linked calcium channels. *Naunyn Schmiedebergs Arch Pharmacol.*, **334**, 463–467.

JEREMY, J.Y., MIKHAILIDIS, D.P. & DANDONA, P. (1988a). Excitatory receptor linked prostanoid synthesis in mammalian smooth muscle: the role of calcium, protein kinase C and G proteins. *Prostagl. Leuk. Essential Fatty Acids Rev.*, **34**, 215–228.

JEREMY, J.Y., THOMPSON, C.S., MIKHAILIDIS, D.P. & DANDONA, P. (1985b). Experimental diabetes inhibits prostacyclin synthesis by the rat penis: pathological implications. *Diabetologia*, **28**, 365–368.

JEREMY, J.Y., THOMPSON, C.S. & DANDONA, P. (1988b). Prostanoid synthesis by the gastrointestinal tract, mesenteric vasculature and hepatic portal vein of diabetic rat. *Diabetes*, **37**, 196A.

JEREMY, J.Y., THOMPSON, C.S., MIKHAILIDIS, D.P., OWEN, R.H. & DANDONA, P. (1987a). Fasting and diabetes mellitus elicit opposite effects on agonist-stimulated prostacylin synthesis by the rat aorta. *Metabolism*, **36**, 616–620.

JEREMY, J.Y., THOMPSON, C.S., MIKHAILIDIS, D.P. & DANDONA, P. (1987b). Changes in prostanoid synthesis in the cerebrum, cerebellum and brain stem of the diabetic rat. *Diab. Res.*, **6**, 95–98.

JEREMY, J.Y., TSANG, V., MIKHAILIDIS, D.P., ROGERS, H., MORGAN, R.J. & DANDONA, P. (1987c). Eicosanoid synthesis by human urinary bladder mucosa: pathological implications. *Br. J. Urol.*, **59**, 36–39.

LATIFPOUR, J., GOUSSE, A., YOSHIDA, M. & WEISS, R.M. (1992). Effect of insulin and myoinositol on muscarinic receptor: alterations in diabetic rat bladder. *J. Urol.*, **147**, 760–763.

LEE, T.-S., SALTSMAN, K.A., OHASHI, H. & KING, G.L. (1988). Activation of protein kinase C by elevation of glucose concentration: proposal for a mechanism in the development of diabetic vascular complications. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5141–5145.

LEVINE, L. & WATANABE, M. (1991). Amplification of prostaglandin I_2 production by thapsigargin. *Prostaglandins*, **42**, 291–302.

LEWIS, M.J. & HENDERSON, A.H. (1987). A phorbol ester inhibits the release of endothelium-derived relaxing factor. *Eur. J. Pharmacol.*, **137**, 167–171.

LINCOLN, J., HAVEN, A.J., SAWYER, M. & BURNSTOCK, G. (1984). The smooth muscle of rat bladder in the early stages of streptozotocin-induced diabetes. *Br. J. Urol.*, **56**, 24–27.

MARUI, N., SAKAI, T., HOSOKAWA, N., YOSHIDA, M., AOIKE, A., KAWAI, K., NISHINO, H. & FUKUSHIMA. M. (1990). N-myc supression and cell cycle arrest at G_1 phase by prostaglandins. *FEBS Lett.*, **66**, 15–18.

MIKHAILIDIS, D.P., JEREMY, J.Y. & DANDONA, P. (1987). Urinary bladder prostanoids: their synthesis, function and possible role in the pathogenesis and treatment of disease. *J. Urol.*, **137**, 1–6.

MIKHAILIDIS, D.P., JEREMY, J.Y. & DANDONA, P. (1988). The role of prostaglandins, leukotrienes and essential fatty acids in the pathogenis of the complications associated with diabetes mellitus. *Prostagl. Leuk. Essential Fatty Acids Rev.*, **33**, 205–206.

MONCADA, S., & VANE, J.R. (1978). Prostacylin formation and effects. In *Chemistry, Biochemistry and Pharmacology of Prostacyclin*. ed. Roberts, S.M. & Scheinman, F. pp. 258–273. Oxford: Pergamon Press.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature*, **308**, 693–698.

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.

POMERANTZ, K.B. & HAJJAR, D.P. (1984). Eicosanoid effects on cell proliferation, in vitro: relevance to atherosclerosis. *Prostagl Leuk Med.*, **16**, 1–10.

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.

RASMUSSEN, H., FORDER, J., KOJIMA, I. & SCRIABINE, A. (1984). TPA induced contraction of isolated rabbit vascular smooth muscle. *Biochem Biophys. Res. Commun.*, **122**, 776–786.

REED, P.W. & LARDY (1972). A23187: a divalent cation ionophore. *J. Biol. Chem.*, **247**, 6970–6978.

RITTER, J.M., HAMILTON, D.P., BARROW, S.E., HEAVEY, D.J., HICKLING, N.E., TAYLOR, K.M., HOBBS, K.E. & DOLLERY, C.T. (1986). Prostacyclin in the circulation of patients with vascular disorders undergoing surgery. *Clin. Sci.*, **71**, 743–747.

ROGERS, S.P. & LARKINS, R.G. (1981). Production of 6-oxo-$PGF_{1\alpha}$ by rat aorta: influence of diabetes, insulin treatment and caloric deprivation. *Diabetes*, **30**, 935–939.

SCHEDL, H.P., WILSON, H.D., RAMASWAMY, K. & LICHTENBERGER, L. (1982). Gastrin and growth of the alimentary tract in the streptozotocin diabetic rat. *Am. J. Physiol.*, **242**, G460–G463.

SMITH, J.A. & LANG, D. (1990). Release of endothelium-derived relaxing factor from pig cultured aortic endothelial cells, as assessed by changes in endothelial cell cyclic GMP content, is inhibited by phorbol ester. *Br. J. Pharmacol.*, **99**, 565–571.

STEVENS, E.J., WILLARS, G.B. & TOMLINSON, D.R. (1992). Basal and angiotensin II-stimulated prostacyclin release by perfused rat lung in experimental diabetes: effects of insulin and aldose reductase inhibition. *Br. J. Pharmacol.*, **105**, 46P.

THARSTRUP, O. (1990). Role of Ca^{2+} (+)-ATPase in the regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca^{2+} (+)-ATPase inhibitor, thapsigargin. *Agents Actions*, **29**, 8–15.

THOMSON, A.B.R. (1980). Unidirectional flux rate of cholesterol and fatty acids into the intestine of rats with drug-induced diabetes mellitus. *J. Lipid Res.*, **21**, 687–698.

TOMLINSON, K.C., GARDINER, S.M., HEBDEN, R.A. & BENNETT, T. (1992). Functional consequences of streptozotocin-induced diabetes mellitus, with particular reference to the cardiovascular system. *Pharmacol. Rev.*, **44**, 103–149.

TSANG, V., JEREMY, J.Y., MIKHAILIDIS, D.P., WALESBY, R.K., WRIGHT, J.C. & DANDONA, P. (1988). Release of prostacyclin from the human aorta. *Cardiovasc. Res.*, **22**, 489–493.

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Inhibition of spinal opioid antinociception by intrathecal β-endorphin$_{1-27}$ in the rat

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1 The effects of intrathecal (i.t.) administration of β-endorphin and two shorter fragments, human and ovine β-endorphin$_{1-27}$, were examined for antinociceptive activity in the tail-flick and paw-pressure tests in the rat. Additionally, the ability of ovine β-endorphin$_{1-27}$ to influence the action of i.t. β-endorphin, morphine and [D-Pen^2-D-Pen^5]enkephalin (DPDPE) was also examined in these tests.

2 After i.t. injection, β-endorphin produced potent dose-related antinociception in the tail-flick and paw-pressure tests. Shorter endorphins produced much weaker effects. The order of antinociceptive efficacy was β-endorphin > human β-endorphin$_{1-27}$ > ovine β-endorphin$_{1-27}$.

3 Administration of ovine β-endorphin$_{1-27}$ (0.72, 1.44 nmol, i.t.) significantly attenuated the antinociceptive effect of β-endorphin (2.88 nmol, i.t.) in the tail-flick and paw-pressure tests.

4 Both i.t. morphine and DPDPE produced dose-related antinociception in the tail-flick and paw-pressure tests. The potency of DPDPE was lower than that of morphine in both tests; however, the effect of DPDPE was weaker in the paw-pressure test.

5 Administration of ovine β-endorphin$_{1-27}$ (1.44 nmol, i.t.) significantly attenuated the antinociceptive effect of morphine (14.9 nmol, i.t.) in both tests and the effect of DPDPE (38.7 nmol) in the tail-flick test.

6 The results show that β-endorphin$_{1-27}$ acts as an opioid antagonist at the spinal level in the rat. Its ability to inhibit the action of morphine and DPDPE suggests that it may attenuate β-endorphin action by an interaction with μ- and/or δ-opioid receptors.

Keywords: β-Endorphin; β-endorphin$_{1-27}$; antinociception; tail-flick test; paw-pressure test; antagonist; intrathecal administration

Introduction

The presence of β-endorphin, a 31-amino acid endogenous opioid peptide of the proopiomelanocortin family in the pituitary and brain is associated with the existence of shorter carboxy terminal fragments, β-endorphin$_{1-26}$ and β-endorphin$_{1-27}$, in acetyl and non-acetyl forms (Zakarian & Smyth, 1982; Dores *et al.*, 1986). These peptide fragments have also been detected in the external medium following incubation of rat hypothalamic slices under physiological conditions (Hong *et al.*, 1989). Pharmacological studies involving the use of opioid-sensitive isolated tissue preparations have demonstrated that β-endorphin$_{1-27}$ behaves as an agonist and that in certain preparations its potency is comparable to that of the parent peptide, β-endorphin (McKnight *et al.*, 1983). However, several *in vivo* studies have demonstrated that β-endorphin$_{1-27}$ also has the potential to inhibit certain effects of β-endorphin. In mice, intracerebroventricular (i.c.v.) injections of β-endorphin$_{1-27}$ have been reported to inhibit the antinociceptive (Hammonds *et al.*, 1984), hypothermic (Suh *et al.*, 1987) and motivational effects (Bals-Kubik *et al.*, 1988) of β-endorphin administered by the same route. However, i.c.v. β-endorphin$_{1-27}$ was found not to influence the antinociceptive effects of centrally administered morphine or [D-Pen^2-D-Pen^5]enkephalin (DPDPE), agonists which selectively activate μ- and δ-receptors, respectively (Suh *et al.*, 1988). In contrast, i.c.v. β-endorphin$_{1-27}$ was found to block the motivational effect of β-endorphin, DPDPE and D-Ala^2,N-Me-Phe^4,Gly-ol-enkephalin (DAMGO), a μ-receptor selective agonist. These observations on the opioid antagonism by β-endorphin$_{1-27}$ have led to the suggestion that fragments derived from β-endorphin in the brain may act as endogenous inhibitors of endorphin-mediated functions (Hammonds *et al.*, 1984; Nicolas & Li, 1985; Bals-Kubik *et al.*, 1988).

Until recently, the existence of β-endorphin in the spinal cord tissue was not recognized, although spinal injection of this peptide has been shown to produce potent antinociceptive action in the rat (Yaksh & Henry, 1978). Evidence from anatomical studies has revealed the presence of β-endorphin-immunoreactive fibres, but not cell bodies in the rat spinal cord (Tsou *et al.*, 1986). A recent biochemical study demonstrated that β-endorphin immunoreactive material is present in extracts of the rat spinal cord, and chromatographic analysis of these extracts showed the presence of acetyl and non-acetyl forms of β-endorphin, β-endorphin$_{1-27}$ and β-endorphin$_{1-26}$ (Gianoulakis & Angelogianni, 1989). Thus, these anatomical and biochemical findings have raised important questions regarding the role of different β-endorphin-derived peptides in functions of the spinal cord.

Since the spinal actions of short fragments of β-endorphin have not been evaluated, the present investigation was undertaken to study effects of human β-endorphin as well as the effects of ovine and human forms of β-endorphin$_{1-27}$ in tests of spinal nociception. This paper describes the actions of these peptides in the tail-flick and paw-pressure tests after acute intrathecal administration in the rat. It also describes the influence of ovine β-endorphin$_{1-27}$ on the antinociceptive actions of spinally administered β-endorphin, morphine and DPDPE in these tests.

Methods

Surgical procedure

All experiments were performed on male, Sprague-Dawley rats weighing 270–300 g. Animals were housed separately, given full access to food and water, and acclimatized to the environment three to four days before experimentation. Room temperature was maintained at 21–22°C under a 12-h

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light/12-h dark cycle.

The technique of intrathecal drug administration, originally described by Yaksh & Rudy (1976), was used to test the spinal actions of opioids on thermally and mechanically-induced nociceptive reactions. Briefly, the animals were anaesthetized under halothane (4% induction, 2% maintenance) and placed in a rat stereotaxic frame. A PE-10 intramedic polyethylene catheter (i.d. 0.28 mm × o.d. 0.61 mm) (Clay Adams; Becton, Dickinson and Company, Parsippany, NJ, U.S.A.) was inserted into the intrathecal space through a transverse slit made in the cisternal membrane and carefully passed 7.5 cm into the lumbar subarachnoid space. The free end of the catheter was exteriorized through the skin over the skull and the protruding segment of the catheter was plugged with a short piece of stainless steel wire. The skin over the cisternal opening was closed by a suture, anaesthesia discontinued, and the animal returned to its home cage. Animals showing neurological deficits (hind limb flexion, rigidity or paralysis) following recovery from surgery were excluded from experiments.

Experimental protocol

Following a three-day recovery period from surgery, catheter-implanted animals were used only once for antinociception tests (tail-flick and paw-pressure) performed between 09 h 00 min and 12 h 00 min. Saline or drugs were injected via the intrathecal catheter with a 50-μl Hamilton syringe in a 10 μl volume followed by a 10 μl 0.9% saline injection to flush the catheter.

The tail-flick test (D'Amour & Smith, 1941) was used to evaluate the response to thermal nociceptive stimuli. Thermal heat was applied to the base of the tail using an analgesia meter (Owen *et al.*, 1981) with the heat source intensity adjusted to provide a baseline response latency of 2–3 s and the cut-off time set at 10 s.

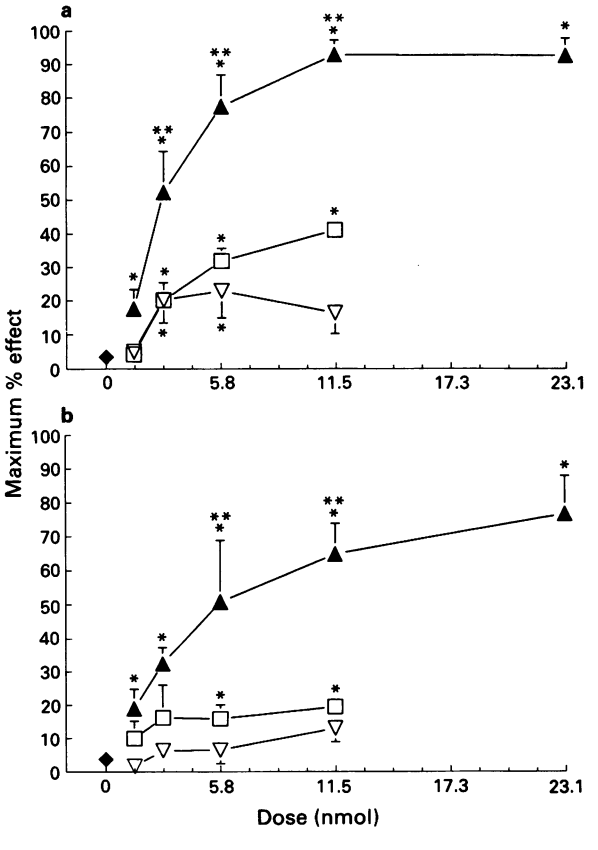


Figure 1 Dose-response curves showing the antinociceptive effects of intrathecally injected saline (◆), human β-endorphin (▲), human β-endorphin$_{1-27}$ (□), and ovine β-endorphin$_{1-27}$ (▽) in the (a) tail-flick test and (b) paw-pressure test. The values shown are the peak responses elicited after injection of each dose. Each point represents the mean ± s.e. of 5–6 animals. *$P<0.05$ versus saline-injected; **$P<0.05$ versus human and ovine β-endorphin$_{1-27}$.

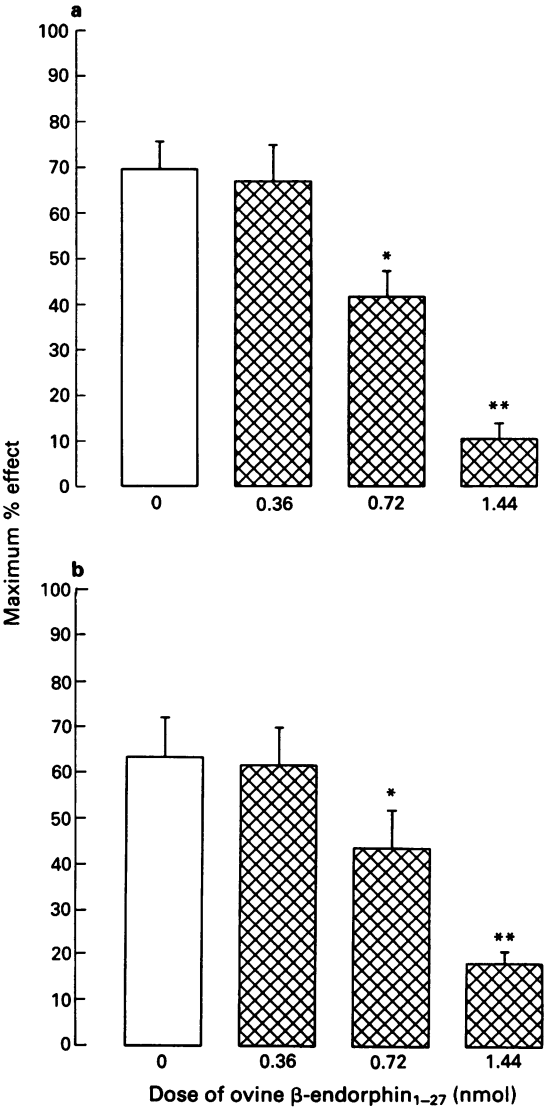


Figure 2 The effect of different intrathecal doses of ovine β-endorphin$_{1-27}$ (0.36, 0.72, 1.44 nmol) (cross-hatched columns) on the antinociceptive response produced by β-endorphin (2.88 nmol, i.t.) (open columns) in the (a) tail-flick test and (b) paw-pressure test. Each column represents the peak response elicited by the treatment. Values shown are a mean ± s.e. of 5–6 animals. *$P<0.05$; **$P<0.01$ (versus β-endorphin alone).

The paw-pressure test, previously described by Loomis *et al.* (1985), was used to evaluate the response to mechanical nociceptive stimuli. Briefly, mechanical pressure was applied to the surface of a non-inflamed hind paw using an air-filled syringe coupled to a pressure gauge until a withdrawal response was observed and the pressure immediately recorded and released. A cut-off pressure of 300 mmHg was used in this test.

Control responses in both tests for each rat were determined before i.t. injections. Following an i.t. injection of saline or drug, the response to each stimulus was determined at 10-min intervals for a period of 60–90 min. The measurement of the paw-pressure withdrawal response was interspersed with tail-flick latency measurements. Previous experiments have demonstrated that no interactions occur between responses in the two tests (Loomis *et al.*, 1985). Results of the tail-flick and paw-pressure tests at each time

point were expressed as the maximum percentage effect (MPE):

$$\frac{\text{Post drug response} - \text{baseline response}}{\text{Cut-off value} - \text{baseline response}} \times 100$$

At the end of each experiment, the placement of the catheter in the lumbar region was verified by dye injections.

Drugs and solutions

β-Endorphin, human and ovine β-endorphin$_{1-27}$, and DPDPE were obtained from Peninsula Laboratories, Inc. (California, U.S.A.). Morphine sulphate was obtained from BDH Pharmaceuticals (Toronto, Canada). All drugs were dissolved in 0.9% saline immediately prior to injection and expressed as quantity injected in nmol. In all experiments, the total volume injected into the intrathecal space did not exceed 20 μl.

Statistical analysis

All data reported represent the mean MPE ± s.e. (standard error). Significant difference at individual time points between two groups was determined by unpaired, two-tailed Student's *t* test where appropriate. Statistical analysis of the time-course effect or dose-response effect between groups was conducted by analysis of variance (ANOVA), followed by Newman-Keuls multiple comparison test. Differences were considered significant at $P \leqslant 0.05$.

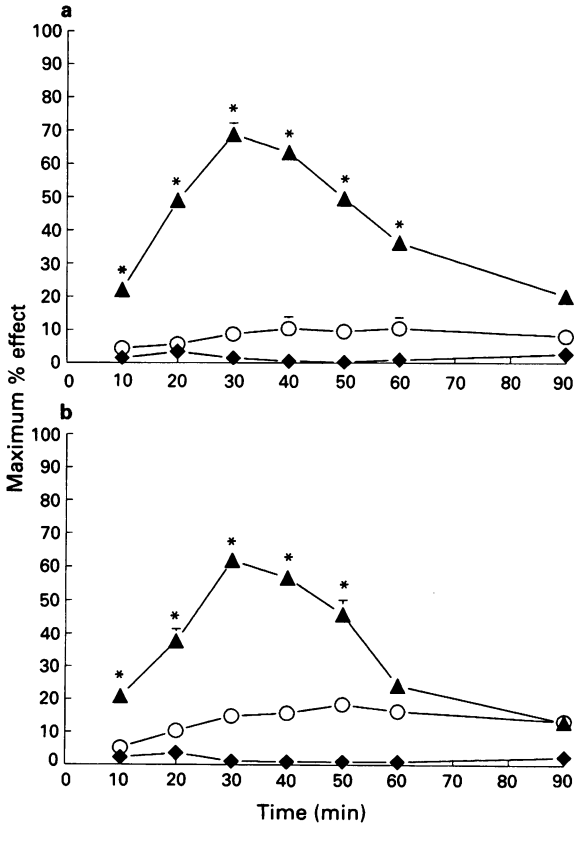


Figure 3 The time-course of antinociceptive effect of intrathecal human β-endorphin (2.88 nmol) alone (▲) and in combination (○) with ovine β-endorphin$_{1-27}$ (1.44 nmol) in the (a) tail-flick test and (b) paw-pressure test; (◆) effect of saline injection. Each point represents mean ± s.e. of 5–6 animals. *$P<0.05$, significantly different from saline-injected animals and drug combination injected animals.

Results

In control experiments ($n = 5$), i.t. administration of saline had no effect in the tail-flick or paw-pressure tests. Ten minutes after i.t. saline, the tail-flick latency was 2.32 ± 0.25 s compared to the pre-injection latency of 2.18 ± 0.17 s, and the threshold pressure for eliciting a hind paw withdrawal response was 99.3 ± 8.2 mmHg compared to the pre-injection threshold pressure of 97.5 ± 7.4 mmHg. These values remained stable over the 60–90 min testing period following the saline injection.

Effect of i.t. β-endorphin and β-endorphin$_{1-27}$

Figure 1 illustrates comparative effects of intrathecally injected β-endorphin, human β-endorphin$_{1-27}$, and ovine β-endorphin$_{1-27}$ in the tail-flick and paw-pressure tests. In both tests, β-endorphin produce a dose-related antinociceptive effect represented by an increased response latency in the tail-flick test and elevated threshold pressure for eliciting a paw withdrawal response in the paw-pressure test. In both tests, the peak effect occurred at 30 min post injection and the response returned to near baseline level 60–90 min post injection (see below). The dose-response curve for the effect of β-endorphin (Figure 1) was characterized by a very steep rise in the response with an increase in the injected dose (1.44, 2.88, 5.8, 11.5, 23.1 nmol, i.t.). The values (MPE) of maximum responses obtained in the tail-flick and paw-pressure tests were 92.9 ± 4.4 and 76.6 ± 11.3, respectively. In contrast, i.t. injections of the two forms of β-endorphin$_{1-27}$ (1.44, 2.88, 5.8, 11.5 nmol, i.t.) produced considerably weaker antinociceptive effects. The maximum responses (MPE) produced by human β-endorphin$_{1-27}$ were 41.2 ± 2.1 (tail-flick test) and 19.5 ± 2.6 (paw-pressure test), and corresponding values produced by the ovine peptide were 23.1 ± 7.9 and 13.0 ± 4.2. Thus, all three peptides tested here produced antinociceptive activity in the tail-flick and paw-pressure tests, the rank order of their apparent efficacy being: β-endorphin > human β-endorphin$_{1-27}$ > ovine β-endorphin$_{1-27}$. None of the three peptides produced signs indicative of an impairment of motor function.

Effect of ovine β-endorphin$_{1-27}$ on the response to i.t. β-endorphin

To examine the interaction between β-endorphin$_{1-27}$ and β-endorphin, i.t. injection of a submaximal dose (2.88 nmol) of β-endorphin was delivered in combination with each of three doses (0.36, 0.72 and 1.44 nmol) of ovine β-endorphin$_{1-27}$. These doses of β-endorphin$_{1-27}$ produced no apparent antinociceptive effect when injected alone, but in combination experiments, two doses (0.72 and 1.44 mol) of the peptide significantly attenuated the effect of β-endorphin in both tests (Figure 2). Administration of 0.72 nmol of β-endorphin$_{1-27}$ reduced the antinociceptive effect of β-endorphin by about 40% in the tail-flick test and 31% in the paw-pressure test. A higher dose (1.44 nmol) reduced this effect by 85% and 71% in the tail-flick and paw-pressure tests, respectively. Thus, spinal administration of the shorter peptide exerted an inhibitory action on the antinociceptive effect elicited by β-endorphin in both tests. The time-course of the antinociceptive effect of β-endorphin, administered alone or in combination with β-endorphin$_{1-27}$, is depicted in Figure 3. As shown, the peak effect in the tail-flick test occurred at 30 min and recovered to near baseline level 90 min post injection. The attenuation of the β-endorphin-induced effect by β-endorphin$_{1-27}$ (1.44 nmol) was apparent 10 min after the injection and this reduction was sustained over the 60–90 min observation period. A similar time-course of effect was seen in the paw-pressure test.

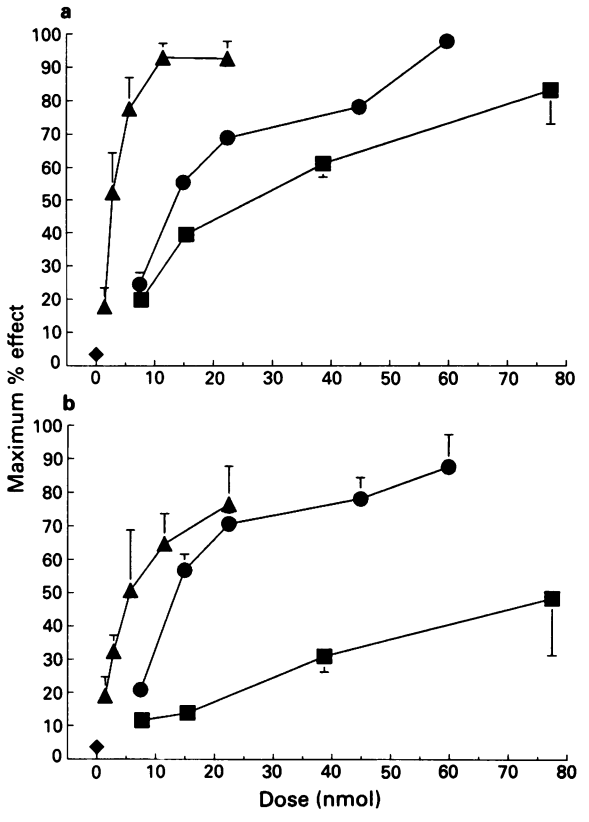


Figure 4 Dose-response curves showing the antinociceptive effects of intrathecal β-endorphin (▲), morphine (●) and [D-Pen^2-D-Pen^5]-enkephalin (DPDPE) (■) in the (a) rat tail-flick test and (b) paw-pressure test; (◆) saline injection (20 μl, i.t.). The values shown are peak responses elicited by each agent. Each point represents the mean ± s.e. of 5–6 animals. The effects shown at all doses, except DPDPE (7.7 nmol) in paw-pressure test, are significantly different ($P<0.05$) from saline-injected.

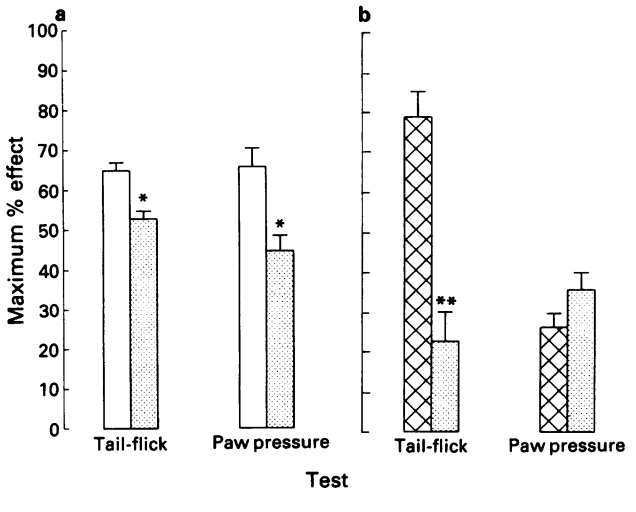


Figure 5 The effect of intrathecal β-endorphin$_{1-27}$ (stippled column, 1.44 nmol) on the antinociceptive effect of (a) morphine (open column, 14.9 nmol, i.t.) and (b) [D-Pen^2-D-Pen^5]enkephalin (DPDPE) (cross hatched column, 38.7 nmol, i.t.) in the tail-flick test and paw-pressure test. Each column represents peak response elicited by the treatment. Values shown are a mean ± s.e. of 5–6 animals. *$P<0.05$ versus morphine alone; **$P<0.01$ versus DPDPE alone.

Effect of ovine β-endorphin$_{1-27}$ on responses to i.t. morphine and DPDPE

In subsequent experiments, the action of ovine β-endorphin$_{1-27}$ on the antinociceptive action of morphine and DPDPE in the two tests was examined. Before evaluating these interactions, the dose-response curves for the spinal antinociceptive effects of morphine and DPDPE were derived with a view to selecting a submaximally effective dose of these agonists for testing in the combination experiments. Figure 4 shows the comparative effects of morphine (7.5, 14.9, 22.4, 44.8, 59.8 nmol, i.t.) and DPDPE (7.7, 15.5, 38.7, 77.4 nmol, i.t.), as well as the effects of β-endorphin (1.44, 2.88, 5.8, 11.5, 23.1 nmol, i.t.) in the two tests. The rank order of apparent agonist potency of the three agents in both tests was: β-endorphin > morphine > DPDPE. The efficacy of morphine and DPDPE in the tail-flick test was comparable to that of β-endorphin in this test; however, DPDPE was considerably less efficacious than morphine or β-endorphin in the paw-pressure test. The maximal response produced by DPDPE (77.4 nmol) in the paw-pressure tests approximated only 50% of that observed in the tail-flick test. Higher doses of DPDPE were not tested owing to their tendency to interfere with motor function.

Figure 5 shows the results of experiments in which ovine β-endorphin$_{1-27}$ (1.44 nmol) was administered in combination with a submaximal dose of morphine (14.9 nmol) or DPDPE (38.7 nmol). In these experiments, β-endorphin$_{1-27}$ attenuated the antinociceptive effect of morphine in both tests and that of DPDPE in the tail-flick test. In the tail-flick and paw-pressure tests, the effect of morphine was significantly reduced by the peptide by 20% and 30%, respectively. In the tail-flick test, the same dose of β-endorphin$_{1-27}$ produced a 72% decrease in the DPDPE-induced response, but failed to modify significantly this response in the paw-pressure test. Thus, a dose of ovine β-endorphin$_{1-27}$ which attenuated the effect of β-endorphin in preceding experiments also inhibited the antinociceptive effect of morphine in both tests and of DPDPE in the tail-flick test.

Discussion

In this study, the intrathecal administration of two shorter carboxy terminal endorphin fragments, ovine and human β-endorphin$_{1-27}$, produced much weaker antinociceptive effects than those of β-endorphin in the tail-flick and paw-pressure tests in the rat. The ovine peptide also effectively attenuated the antinociceptive action produced by intrathecal β-endorphin. The choice of ovine β-endorphin$_{1-27}$ for use in combination experiments was influenced by the observation that it had a very low intrinsic activity, a property which would minimally interfere with the observance of its potential antagonist action. The inhibition of the β-endorphin-induced effect by intrathecal β-endorphin$_{1-27}$ seen here has been reported in earlier antinociception studies which evaluated the action of i.c.v. human β-endorphin$_{1-27}$ on β-endorphin-induced effects in mice (Hammonds *et al.*, 1984; Nicolas & Li, 1985; Suh *et al.*, 1988). However, in contrast to the results of those i.c.v. studies showing that administration of human β-endorphin$_{1-27}$ was ineffective against the antinociceptive effects of morphine or DPDPE, in the present study intrathecal injection of ovine β-endorphin$_{1-27}$ reduced the effect of spinal morphine in both antinociceptive tests and DPDPE in the tail-flick test. This discrepancy between the results obtained with the i.c.v. and those with the i.t. route of administration may be related to differences in the doses and forms of β-endorphin$_{1-27}$, or in the species of animals used in these experiments. However, the discrepancy could also reflect potential differences in the mechanism underlying the actions of β-endorphin, morphine and DPDPE in the brain and spinal cord.

Tseng and coworkers explained the differential action of

β-endorphin$_{1-27}$ against i.c.v. β-endorphin and morphine on the basis that these two agonists produce their antinociceptive effects by activating distinct receptors linked to different descending spinal pathways (Tseng & Fujimoto, 1985). According to their proposal, i.c.v. β-endorphin activates a descending bulbospinal pathway resulting in the release of spinal methionine-enkephalin, an action which is blocked by i.c.v. β-endorphin$_{1-27}$ (Tseng & Li, 1986). On the other hand, i.c.v. morphine activates a similar non-opioid, possibly 5-hydroxytryptaminergic or noradrenergic, pathway to produce antinociceptive effects (Tseng *et al.*, 1985, 1986). Since morphine did not induce a release of spinal methionine-enkephalin, it was suggested that β-endorphin mediates its action through the activation of a brainstem ε-receptor which is not sensitive to morphine (Tseng *et al.*, 1985). Thus, the ability of β-endorphin$_{1-27}$ to antagonize the action of β-endorphin has been attributed to a blockade of the ε-receptor (Suh & Tseng, 1988; Suh *et al.*, 1988). The failure of β-endorphin$_{1-27}$ to influence the action of morphine, a μ-receptor selective agonist, has suggested that this peptide does not interact with the μ-receptor. However, this differential action of β-endorphin$_{1-27}$ is surprising in view of the observation that this shorter peptide fragment does not show selectivity for a specific opioid receptor type in receptor binding studies (Hammonds & Li, 1985). Furthermore, the notion that β-endorphin-induced antinociception involves ε-receptor activation is at variance with the finding that this response is inhibited by antagonists selective for μ- and δ-receptors (Shook *et al.*, 1988). This finding suggests that both μ- and δ-receptors may have a role in β-endorphin-induced effects.

Since evidence for the existence of ε-receptors in the spinal cord is lacking, it is difficult to attribute the antagonist action of intrathecal β-endorphin$_{1-27}$ seen here to a blockade of these receptors. Since β-endorphin is recognized to activate both μ- and δ-receptors (McKnight *et al.*, 1983), its potent spinal antinociceptive effects most probably involve a synergistic activation of these two receptor types. Thus, intrathecal β-endorphin$_{1-27}$ could antagonize the effect of β-endorphin by blocking spinal μ- and/or δ-receptors and thus preventing this synergistic activation. This explanation is supported by the observation that in the present study, β-endorphin$_{1-27}$ attenuated the antinociceptive effects of morphine and DPDPE, agonists acting at μ- and δ-receptors, respectively. Additional support is provided by a previous observation that in certain isolated tissue preparations β-endorphin$_{1-27}$ interacts with μ- and δ-receptors (McKnight *et al.*, 1983). However, in such preparations an inhibition of the β-endorphin effect by the shorter peptide fragment has not been demonstrated (Wuster *et al.*, 1979). In behavioural experiments, i.c.v. human β-endorphin$_{1-27}$ has been found to inhibit motivational effects of β-endorphin, DPDPE, and DAMGO (Bals-Kubik *et al.*, 1988). It is not clear at present, why i.c.v. injections of human β-endorphin$_{1-27}$ influence the motivational but not antinociceptive effects of centrally injected DPDPE and DAMGO.

Although intrathecal β-endorphin$_{1-27}$ significantly reduced the effect of DPDPE in the tail-flick test, it surprisingly failed to influence the antinociceptive effect of this agonist in the paw-pressure test. The effect of DPDPE in the latter test was considerably weaker than that in the tail-flick test, suggesting spinal δ-receptors may be less important in the modulation of mechanically-induced nociception. Alternatively, considering the potential heterogeneity of the δ-receptor (Porreca *et al.*, 1987), the subtypes mediating antinociception in the tail-flick and paw-pressure tests may be different, with β-endorphin$_{1-27}$ preferentially interfering with that involved in thermal antinociception. Additional experiments are required to evaluate the role of different δ-receptors in various antinociception tests.

The presence of β-endorphin$_{1-27}$ in spinal regions (Gianoulakis & Angelogianni, 1989) and the finding that this peptide influences antinociceptive actions of opioids at the spinal level suggests that certain endorphin fragments may have a role in modulating the transmission of nociceptive signals. Its ability to inhibit spinal antinociception mediated via μ- and δ-receptors suggests that this peptide may behave as an inhibitory modulator of endogenous opioids. However, the full significance of β-endorphin$_{1-27}$ action cannot be ascertained until its effects on other opioid receptor-mediated responses including the development of tolerance and dependence have been evaluated.

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References

BALS-KUBIK, R., HERZ, A. & SHIPPENBERG, T.S. (1988). β-Endorphin-(1-27) is a naturally occurring antagonist of the reinforcing effects of opioids. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **338**, 392–396.

D'AMOUR, F.E. & SMITH, D.L. (1941). A method for determining loss of pain sensation. *J. Pharmacol. Exp. Ther.*, **72**, 74–79.

DORES, R.M., JAIN, M. & AKIL, H. (1986). Characterization of the forms of β-endorphin and α-MSH in the caudal medulla of the rat and guinea pig. *Brain Res.*, **377**, 251–260.

GIANOULAKIS, C. & ANGELOGIANNI, P. (1989). Characterization of β-endorphin peptides in the spinal cord of the rat. *Peptides*, **10**, 1049–1054.

HAMMONDS, R.G. Jr. & LI, C.H. (1985). Studies on opioid receptor selectivity of β-endorphin antagonists. *Int. J. Peptide Protein Res.*, **26**, 118–120.

HAMMONDS, R.G. Jr., NICOLAS, P. & LI, C.H. (1984). β-Endorphin-(1-27) is an antagonist of β-endorphin analgesia. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 1389–1390.

HONG, M., JHAMANDAS, K., ANGELOGIANNI, P. & GIANOULAKIS, C. (1989). Hypothalamic release and actions of β-endorphin fragments. *Adv. Biosci.*, **75**, 527–530.

LOOMIS, C.W., CERVENKO, F.W., JHAMANDAS, K., SUTAK, M. & MILNE, B. (1985). Analgesia and autonomic function following intrathecal administration of morphine and norepinephrine to the rat. *Can. J. Physiol. Pharmacol.*, **63**, 656–662.

MCKNIGHT, A.T., CORBETT, A.D. & KOSTERLITZ, H.W. (1983). Increase in potencies of opioid peptides after peptidase inhibition. *Eur. J. Pharmacol.*, **86**, 393–402.

NICHOLAS, P. & LI, C.H. (1985). β-Endorphin-(1-27) is a naturally occurring antagonist to etorphine-induced analgesia. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 3178–3181.

OWEN, J.A., MILNE, B., JHAMANDAS, K. & NAKATSU, K. (1981). Assembly of an inexpensive tail-flick analgesia meter. *J. Pharmacol. Methods*, **6**, 33–37.

PORRECA, F., HEYMAN, J.S., MOSBERG, H.I., OMNAAS, J.R. & VAUGHT, J.L. (1987). Role of mu and delta receptors in the supraspinal and spinal analgesic effects of [D-Pen^2, D-Pen^5]-enkephalin in the mouse. *J. Pharmacol. Exp. Ther.*, **241**, 393–400.

SHOOK, J.E., KAZMIERSKI, W., WIRE, W.S., LEMCKE, P.K., HRUBY, V.J. & BURKS, T.F. (1988). Opioid receptor selectivity of β-endorphin in vitro and in vivo: mu, delta and epsilon receptors. *J. Pharmacol. Exp. Ther.*, **246**, 1018–1025.

SUH, H.H. & TSENG, L.F. (1988). Intrathecal β-funaltrexamine antagonizes intracerebroventricular β-endorphin- but not morphine-induced analgesia in mice. *J. Pharmacol. Exp. Ther.*, **245**, 587–593.

SUH, H.H., TSENG, L.F. & LI, C.H. (1987). Beta-endorphin-(1-27) antagonizes beta-endorphin-induced hypothermia in mice. *Peptides*, **8**, 123–126.

SUH, H.H., TSENG, L.F. & LI, C.H. (1988). β-Endorphin-(1-27) antagonizes β-endorphin- but not morphine-, D-Pen^2-D-Pen^5-enkephalin- and U50,488H-induced analgesia in mice. *Neuropharmacology*, **27**, 957–963.

TSENG, L.F. & FUJIMOTO, J.M. (1985). Differential actions of intrathecal naloxone on blocking the tail-flick inhibition induced by intraventricular β-endorphin and morphine in rats. *J. Pharmacol. Exp. Ther.*, **232,** 74–79.

TSENG, L.F., HIGGINS, M.J., HONG, J.S., HUDSON, P.M. & FUJIMOTO, J.M. (1985). Release of immunoreactive met-enkephalin from the spinal cord by intraventricular β-endorphin but not morphine in anesthetized rats. *Brain Res.*, **343,** 60–69.

TSENG, L.F. & LI, C.H. (1986). β-Endorphin-(1-27) inhibits the spinal beta-endorphin-induced release of Met-enkephalin. *Int. J. Peptide Protein Res.*, **27,** 394–397.

TSENG, L.F., TOWELL, J.F. & FUJIMOTO, J.M. (1986). Spinal release of immunoreactive met-enkephalin by intraventricular beta-endorphin and its analogs in anesthetized rats. *J Pharmacol. Exp. Ther.*, **237,** 65–74.

TSOU, K., KHACHATURIAN, H., AKIL, H. & WATSON, S.J. (1986). Immunocytochemical localization of pro-opiomelanocortin-derived peptides in the adult rat spinal cord. *Brain Res.*, **378,** 28–35.

WUSTER, M., SCHULZ, R. & HERZ, A. (1979). Specificity of opioids towards the μ-, δ- and ε-receptors. *Neurosci. Lett.*, **15,** 193–198.

YAKSH, T.L. & HENRY, J.L. (1978). Antinociceptive effects of intrathecally administered human β-endorphin in the rat and cat. *Can. J. Physiol. Pharmacol.*, **56,** 754–759.

YAKSH, T.L. & RUDY, T.A. (1976). Chronic catheterization of the spinal subarachnoid space. *Physiol. Behav.*, **17,** 1031–1036.

ZAKARIAN, S. & SMYTH, D.G. (1982). Distribution of β-endorphin-related peptides in rat pituitary and brain. *Biochem. J.*, **202,** 561–571.

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Postnatal changes in N-methyl-D-aspartate receptor binding and stimulation by glutamate and glycine of [^{3}H]-MK-801 binding in human temporal cortex

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1 Homogenates of human infant and adult temporal cortex were used to measure [^{3}H]-TCP and [^{3}H]-MK-801 binding to the N-methyl-D-aspartate (NMDA)-coupled ion channel phencyclidine site.

2 Both [^{3}H]-TCP and [^{3}H]-MK-801 binding increased in infant cortex by $>100\%$ between term and 26 weeks suggesting that the numbers of NMDA receptors increase during postnatal brain development.

3 [^{3}H]-MK-801 binding was measured under non-equilibrium conditions in temporal cortex homogenates with the addition of 100 μM of L-glutamate plus a range of concentrations (0.05 μM–100 μM) of glycine. Glutamate and glycine increased [^{3}H]-MK-801 binding by stimulating NMDA receptors and improving [^{3}H]-MK-801 access to ion channel binding sites; maximum stimulation in adult and infant temporal cortex was achieved with 100 μM glutamate plus 5 μM glycine; a higher concentration of glycine (50 μM) reduced [^{3}H]-MK-801 binding to below maximum.

4 The stimulation by 100 μM glutamate plus 5 μM glycine of [^{3}H]-MK-801 binding in infant temporal cortex was affected by postnatal age. For example, although the stimulation of [^{3}H]-MK-801 binding in 5–6 week infant cortex (236% of basal) was similar to adult cortex (230% of basal), in samples taken from infants aged 5– 6 months glycine (plus glutamate) stimulation of [^{3}H]-MK-801 binding (392% of basal) was substantially greater than that measured in adult temporal cortex.

5 The binding of [^{3}H]-glycine to the glycine modulatory site associated with the NMDA receptor in infant cortex also increased with postnatal age by $>100\%$ between term and 26 weeks.

6 It is concluded that NMDA receptors in infant cortex increase to levels greater than those in adult cortex during postnatal development. The results do not exclude the possibility that the transiently increased NMDA receptor-ion channel complex in infant cortex shows enhanced responses to agonists and modulators.

Keywords: N-Methyl-D-aspartate receptor; glycine; [^{3}H]-MK-801 binding; infant brain; development

Introduction

Previous work on experimental animals has found that the excitatory amino acids (EAAs) glutamate (Glu) and aspartate are involved in several physiological processes during brain development (see review, McDonald & Johnston, 1990). In addition to its excitatory signalling role, Glu has trophic functions in which it helps to control neurone survival and synaptogenesis. Glu is an endogenous agonist at the multiple EAA receptors including the N-methyl-D-aspartate (NMDA) subtype (Monaghan *et al.*, 1989). NMDA receptors are closely involved in synaptogenesis (Kleinschmidt *et al.*, 1987) and in several forms of activity-dependent synaptic plasticity including long-term potentiation (LTP): a model of learning and memory in which synaptic efficiency increases after a brief train of stimuli along glutamatergic efferents to the hippocampus (Harris *et al.*, 1984; Morris *et al.*, 1986; Collingridge & Bliss, 1987). NMDA receptor antagonists affected the developing visual system in kittens by preventing the consolidation of ocular dominance and experience-dependent plasticity (Kleinschmidt *et al.*, 1987; Rauschecker & Hahn, 1987; Gu *et al.*, 1989).

The processes that underlie the developmental role of Glu and NMDA receptors are readily transformed into potential neurotoxicity when Glu is present is excess. Both AMPA (quisqualate) and NMDA receptor subtypes may be involved in pathophysiological events in adult and developing human brain including excitotoxic neurone damage after hypoxia-ischaemia, hypoglycaemia, epilepsy and in some neurodegenerative diseases (see review, Meldrum & Garthwaite, 1990). It emerged that electrophysiological responses to NMDA receptor activation as well as NMDA-induced neurotoxicity were enhanced in early postnatal rodent brain (DuPont *et al.*, 1987; McDonald *et al.*, 1988; Siviy *et al.*, 1991) when synaptic plasticity reaches a peak. At a similar period of development pre- and postsynaptic markers of EAA synapses may be overexpressed as compared to adult brain (McDonald & Johnston, 1990).

The NMDA receptor complex has a number of recognition sites which regulate the receptor-coupled cation channel including the Glu (NMDA) receptor site, the strychnine-insensitive glycine co-agonist site (Johnson & Ascher, 1987) and the polyamine site (Ransom & Stec, 1988). Within the cation channel there is a Mg^{2+} site (Coan & Collingridge, 1985) and a high-affinity binding site for phencyclidine (PCP) (Huettner & Bean, 1988). PCP blocks NMDA receptor-mediated responses and similar compounds including [^{3}H]-TCP ([1-(2-thienyl)cyclohexyl)piperidine) and [^{3}H]-MK-801 (10,11-dihydro-5-methyl-5H-dibenzo-[a,d]cyclohepten-5, 10-imine maleate, dizocilpine maleate) are used to measure ion-channel PCP sites in receptor binding studies (Vignon *et al.*, 1983; Bowery & Hudson, 1986; Wong *et al.*, 1986; Foster & Wong, 1987; Wong *et al.*, 1988). L-Glutamic acid and glycine increase [^{3}H]-TCP and [^{3}H]-MK-801 binding by making the ion channel PCP sites more accessible (Johnson & Ascher, 1987; Wong *et al.*, 1987; 1988). This allosteric modulation by glycine and/or L-glutamic acid of [^{3}H]-MK-801 binding *in vitro* has been used to make a functional assessment of the

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NMDA receptor complex in human brain samples (Procter et al., 1989).

This study was undertaken to determine whether NMDA receptors are overproduced and if their response to glycine site stimulation alters in the developing human brain.

Methods

Coronal blocks of forebrain were cut from autopsy brains taken from infants of various postnatal ages, some older children and adults. Every subject had a *postmortem* examination and none of the brains used had any identifiable pathology. Details of the subjects are given in Table 1. The majority of the infants were diagnosed as deaths from Sudden Infant Death Syndrome (SIDS). Protracted terminal coma was not a factor which may have affected ligand binding data.

The tissues were quick-frozen in isopentane at − 45°C within 2 h of the autopsy and stored at − 70°C for up to 2 years. Cortex samples identified as Brodmann's neuroanatomical areas 20, 21 (temporal) were removed from the frozen tissue.

[^{3}H]TCP binding assay

Temporal cortex tissue from 19 infants and 3 adults was homogenized in 50 volumes (about 20 mg tissue ml^{-1}) of 50 mM Tris-acetate buffer, pH 7.4. Aliquots (1.4 ml) of homogenate were centrifuged for 10 min at 10,000 *g* (4°C). The pellets were stored at − 70°C for no more than 1 week before assay.

The tissue pellets were thawed at room temperature, cooled on ice and suspended in 1.4 ml of 5 mM Tris acetate, pH 8.2, containing 10 μM EDTA, 10 μM EGTA, 10 μM glycine and 100 μM L-glutamic acid. Triplicate aliquots of this membrane preparation were used in the binding assays which were performed in plastic multiwell microtitre plates. Each well held 200 μl of solution containing membrane preparation and 10 nM [^{3}H]-TCP ([piperidyl-3,4-^{3}H(N)]-TCP, 47.8 Ci mmol^{-1}, NEN-Dupont). The solutions were incubated for 50 min at 4°C. Ketamine (100 μM) was added to some incubations to measure non-specific binding. Bound ligand was separated by filtration through Whatman GF/C glass fibre filters which were presoaked in 0.1% polyethyleneimine and the filters were washed (10 s) with 5 mM Tris acetate buffer (pH 8.2) and dispersed in scintillant for counting.

[^{3}H]-MK-801 binding assay

Tissue pellets made from temporal cortex taken from 21 infant and 3 adult brains were prepared as described above.

The prepared tissue pellets were thawed at room temperature and were suspended in 50 volumes of 5 mM Tris HCl, pH 7.4, and centrifuged at 4°C and 19,000 *g* for 10 min. The pellets were resuspended in 5 mM Tris HCl containing 100 μM glycine and 100 μM L-glutamic acid. Aliquots (160 μl) of tissue suspension were incubated for 30 min at room temperature (21°C) with 4 nM [^{3}H]-MK-801 (22.5 Ci mmol^{-1}, NEN-Dupont) in a total volume of 200 μl. Non-specific binding was determined by adding 10 μM MK-801 to some incubations. Bound ligand was separated by filtration (as above) and the filters were washed for 10 s with 5 mM Tris HCl at 4°C.

[^{3}H]-MK-801 saturation assays

Temporal cortex tissue membranes were prepared as described above. The pellets were suspended in 25 volumes of 5 mM Tris HCl (pH 7.4) containing 80 μM glycine and 80 μM L-glutamic acid. Triplicate aliquots of tissue suspension were incubated for 2 h at room temperature (23°C) with a range of concentrations (0.5 to 100 nM) of [^{3}H]-MK-801. Non-specific binding was measured by adding 100 μM MK-801 to some incubations. Binding was ended by filtration and washing as described above.

Glycine stimulation of [^{3}H]-MK-801 binding

Temporal cortex tissue from 15 infants and 3 adults was homogenized in 9 volumes of 50 mM Tris HCl, pH 7.4, and centrifuged at 4°C and 19,000 *g* for 10 min. The resultant pellets were resuspended in 75 volumes of buffer and centrifuged once more. This procedure was repeated once with 5 mM Tris HCl (pH 7.4) and a further 3 times with deionized water (75 volumes each time). The final pellets were stored at − 70°C for 18 h. On the day of the assay, each pellet was thawed to room temperature and suspended in 75 volumes of 5 mM Tris HCl (pH 7.4), centrifuged at 19,000 *g* for 10 min (4°C) and the procedure was repeated 3 times. The pellets were finally suspended in 50 volumes of Tris HCl except for samples with low protein from 3 youngest infants which were taken up in 25 volumes of buffer.

Binding assays were performed as described elsewhere (Robinson *et al.*, 1990). Tissue aliquots were incubated for 15 min at room temperature (24°C) with 4 nM [^{3}H]-MK-801 alone and with a mixture of 100 μM L-glutamic acid and glycine in a range of concentrations (0.005–50 μM; 0.5–100 μM in preliminary studies) both with and without strychnine (1 μM) in a final volume of 200 μl. The incubation time (15 min) falls well short of the association time for [^{3}H]-MK-801 binding (Robinson *et al.*, 1990). Non-specific [^{3}H]-MK-801 binding was measured by adding 10 μM MK-801 to some incubations. Incubation was ended by rapid filtration of the samples and washing the filters with 5 mM Tris HCl at 4°C.

[^{3}H]-glycine binding

Temporal cortex samples were homogenized in 50 volumes of 50 mM Tris acetate (pH 7.4) and centrifuged (4°C) at 19,000 *g* for 10 min. This process was repeated a further 4 times. The pellets were stored at − 70°C.

On the day of the assays, the tissue pellets were thawed to

Table 1 Details of the subjects

Age range	*Death to autopsy* (h ± s.e.mean)	*Causes of death*
Premature (8)	18 ± 7	Multiple problems (2), immaturity, Respiratory distress syndrome, heart defects (2), growth retardation, pneumonia
0–8 week (6)	19 ± 7	Burns, SIDS (4), subdural haemorrhage
9–12 week (6)	23 ± 6	SIDS (6)
13–20 week (6)	21 ± 4	Inhaled vomit, SIDS (5)
>20 week (3)	23 ± 5	SIDS (3)
Adult (3)	19 ± 6	Heart failure (3)

room temperature and suspended in 25 volumes of 50 mM Tris acetate (pH 7.4). Triplicate aliquots of the suspensions were incubated in buffer containing 50 nM [^{3}H]-glycine (18.5 Ci mmol^{-1}) at 4°C for 60 min. Non-specific binding was determined by adding 2 mM glycine to some incubations. Incubation was ended by filtering the samples through Whatman GF/C filters presoaked in 10 mM $MgSO_4$ solution and washing the filters for 5 s with ice-cold 50 mM Tris-acetate buffer containing 10 mM Mg^{2+}.

Protein measurements

Aliquots of each tissue sample were used for protein estimation (Lowry *et al.*, 1951).

Analysis of binding data

Age-related ligand binding data were examined by one-way analysis of variance or in some cases by multiple ANOVA with *post-hoc* testing (Tukey test) where appropriate. Saturation data were evaluated by Scatchard analysis using EBDA (McPherson, 1983) and LIGAND programme (Munson & Rodbard, 1980); the infant tissues were grouped according to postnatal ages: 0–8 weeks, 9–12 weeks, 13–20 weeks. Correlation coefficients were determined by linear regression analysis.

Results

[^{3}H]-TCP binding

The results of the binding assays carried out with a single concentration of [^{3}H]-TCP in the presence of excess L-glutamate and glycine to stimulate binding are shown in Figure 1. [^{3}H]-TCP binding in individual brain samples were not correlated with postmortem intervals (death to brain removal). There was a significant correlation between [^{3}H]-TCP binding and age in cortex samples from infants aged pre-term to 6 months. However, statistical analysis of all the data showed that age did not have a significant effect on [^{3}H]-TCP binding (ANOVA, $P = 0.06$).

[^{3}H]-MK-801 binding

Single concentration [^{3}H]-MK-801 binding assays included excess concentrations of L-glutamate and glycine to produce maximum binding and the assays used a different set of brains from the [^{3}H]-TCP binding assays. The amount of specific [^{3}H]-MK-801 bound to adult temporal cortex membranes (3 brains, mean age of subjects 72 ± 2 years) was 50.4 ± 4.3 fmol mg^{-1} protein (mean ± s.e.mean) and averaged 76% of the total binding. The amount of [^{3}H]-MK-801 binding measured in adult cortex was similar to that obtained with rat cortex membranes under identical conditions of L-glutamate and glycine stimulation (Robinson *et al.*, 1990).

The proportion of specific binding of [^{3}H]-MK-801 in infant brain (displaced with excess MK-801) averaged 88 ± 2% of the total (mean ± s.e.mean of triplicate assays on 18 brains). The age-related single concentration [^{3}H]-MK-801 binding data (Figure 2) compared with [^{3}H]-TCP binding (Figure 1) suggests that there was more [^{3}H]-MK-801 bound at similar ages. Although this difference between the two ligands might easily result from using different ligand concentrations in binding assays, it is consistent with the earlier report that there were more [^{3}H]-MK-801 binding sites available in human brain than [^{3}H]-TCP sites (Steele *et al.*, 1989). [^{3}H]-MK-801 binding in temporal cortex from infants aged 6 months exceeded the binding levels in adult brains and overall there was a highly significant effect of age on [^{3}H]-MK-801 binding (ANOVA, $P<0.001$). There was no correlation between individual infant brain [^{3}H]-MK-801 binding and *postmortem* delay time ($P<0.05$).

[^{3}H]-MK-801 saturation binding

Saturation analyses of [^{3}H]-MK-801 binding were performed on temporal cortex samples from brains of adults and infants in a similar age range to those used for the single concentration measurements. The binding conditions, L-glutamate and glycine present in excess and a long incubation time, were designed to achieve [^{3}H]-MK-801 binding equilibrium (Steele *et al.*, 1989; Quarum *et al.*, 1990). K_D and B_{max} values calculated from Scatchard analyses are shown in Table 2. The affinity of [^{3}H]-MK-801 for the binding sites in adult brain (17.8 nM) was lower than that often reported, for example 5 nM (Steele *et al.*, 1989); 1.39 and 116 nM (Kornhuber *et al.*, 1989). However, the K_D values for [^{3}H]-MK-801 binding were consistent across the age range studied (term infants to adults aged 70 years) and did not alter with age (Table 2) whereas maximum binding capacities (B_{max}) increased from 670 fmol mg^{-1} protein in temporal cortex from term infants (similar to

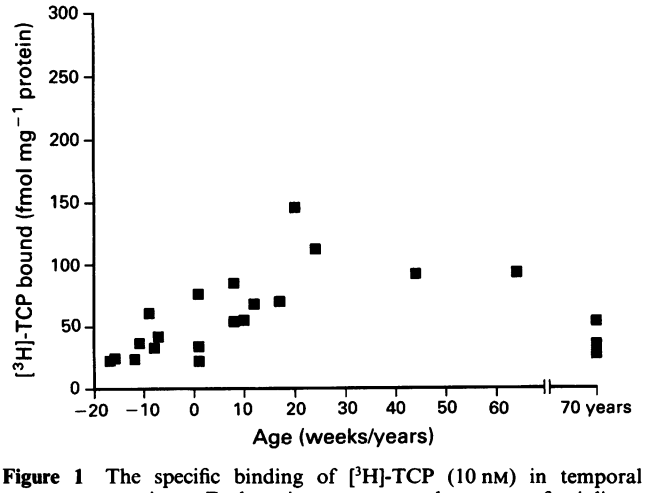


Figure 1 The specific binding of [^{3}H]-TCP (10 nM) in temporal cortex preparations. Each point represents the mean of triplicate assays performed on a single brain. L-Glutamic acid (100 μM) and glycine (10 μM) were added to the binding assays. There was a significant correlation between binding and postnatal age up to 6 months ($r = 0.818$, $P<0.01$).

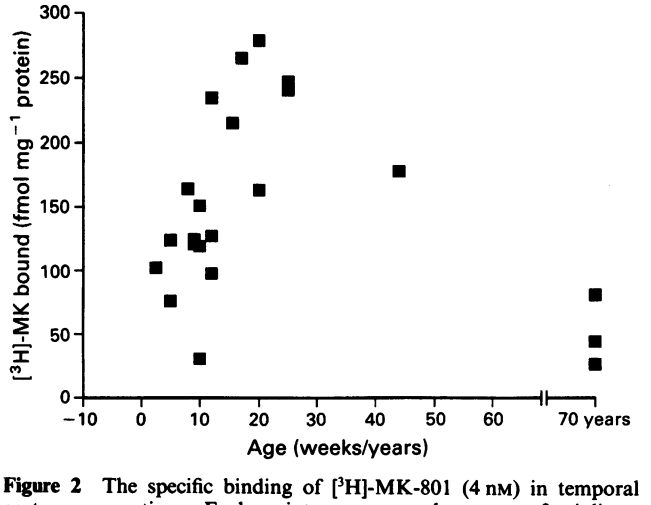


Figure 2 The specific binding of [^{3}H]-MK-801 (4 nM) in temporal cortex preparations. Each point represents the mean of triplicate assays performed on a single brain. Glutamate (100 μM) and glycine (10 μM) were added to the binding assays. There was a significant correlation between binding and postnatal age up to 6 months ($r = 0.886$, $P<0.01$).

Table 2 Binding parameters for specific [^{3}H]-MK-801 binding stimulated with L-glutamate and glycine in temporal cortex membranes

Mean ages of subjects	K_D (nM)	B_{max} (fmol mg^{-1} protein)
<1 week	13.9 ± 1.2	670 ± 40
5.3 weeks	16.5 ± 6.1	540 ± 80
8 weeks	18.9 ± 3.4	640 ± 130
10 weeks	12.7 ± 0.7	880 ± 130
12 weeks	18.9 ± 4.9	1070 ± 170
19.3 weeks	14.6 ± 1.7	1260 ± 160
70 ± 2 years	17.8 ± 0.6	680 ± 110

Mean values (± s.e.mean) were obtained with 3 brains in each group. For the infant brains (<1 to 20 weeks), linear regression analysis showed no correlation between K_D and age but a significant correlation between B_{max} and age ($r = 0.8691$, $P < 0.01$).

the B_{max} recorded in adult cortex) up to the oldest infants from which samples were taken (1260 fmol at age 19 weeks).

Glycine stimulation of [^{3}H]-MK-801 binding

The effects of glycine on the binding of [^{3}H]-MK-801 to temporal cortex membranes were investigated. Every time exogenous glycine was added to membrane preparations an excess of L-glutamate (100 μM) was also present in the assay solutions. Preliminary data (not shown) revealed that single washing of the temporal cortex membrane preparations resulted in a negligible stimulation of [^{3}H]-MK-801 binding by a combination of 100 μM glycine and 100 μM L-glutamate whereas the two amino acids produced substantial stimulation of [^{3}H]-MK-801 binding in well-washed tissue preparations. Hence well-washed tissue preparations (see Methods) were employed throughout the rest of the stimulation studies.

The effects of altering the concentrations of glycine on the single concentration binding of [^{3}H]-MK-801 were measured using temporal cortex membrane preparations made from the brains of 3 infants with a mean postnatal age of 19.3 weeks (individual ages 18 and 20 (2) postnatal weeks). The [^{3}H]-MK-801 binding data are shown in Figure 3. Glycine, at final concentrations ranging from 0.005 to 50 μM and in the presence of 100 μM L-glutamate, stimulated the binding of [^{3}H]-MK-801. Maximum binding was achieved with 100 μM L-glutamate plus 5 μM glycine and the data suggest that glycine dose-response curves would be bell-shape because 50 μM glycine plus L-glutamate caused below-maximum stimulation of [^{3}H]-MK-801 binding (Figure 3).

It was assumed that the effects of glycine were caused by stimulation of the strychnine-insensitive binding sites associated with the NMDA receptor-ion channel complex. Furthermore, strychnine caused a modest blockade of the NMDA channel (Bertolini & Vicini, 1988) and may therefore alter [^{3}H]-MK-801 binding. For these reasons, the effects of strychnine on [^{3}H]-MK-801 binding were tested. Strychnine (1 μM) added to duplicate incubations had no effect on the stimulation of [^{3}H]-MK-801 binding by increasing amounts of glycine (0.005 to 50 μM) in the presence of a fixed concentration of L-glutamate (Figure 3). Strychnine was not included in further glycine stimulation studies.

The investigation into the effect of varying the concentration of glycine on [^{3}H]-MK-801 binding was extended to include a younger infant group (mean postnatal aged 5.3 weeks) and temporal cortex samples from 3 adults (mean aged 77.3 years). In all 3 groups of brain samples the maximum stimulation of [^{3}H]-MK-801 binding was achieved with 5 μM glycine and 100 μM L-glutamate (Figure 4). The stimulation of [^{3}H]-MK-801 binding in the brains from infants aged 19.3 postnatal weeks was greater than in younger infants and adults.

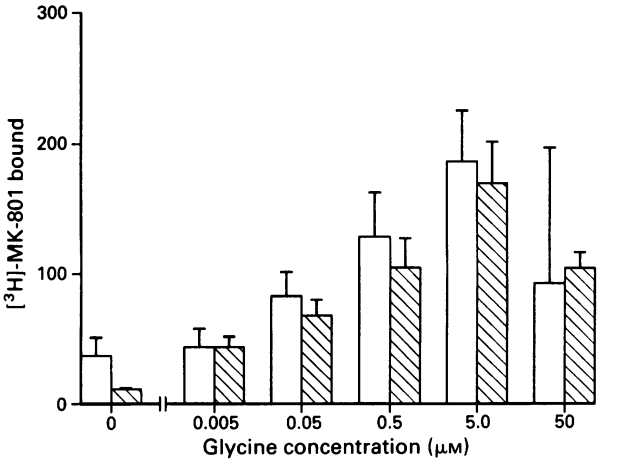


Figure 3 The effect of increasing concentrations of glycine on [^{3}H]-MK-801 binding in well-washed temporal cortex membrane preparations. Each point is the mean ± s.e.mean of the specific binding of [^{3}H]-MK-801 measured on samples from 3 infant brains (mean age 19.3 weeks postnatal). Assays contained 4 nM [^{3}H]-MK-801, 0 or 100 μM L-glutamic acid, 0–50 μM glycine and either no strychnine (open columns) or 1 μM strychnine (hatched columns). Non-specific binding was measured by adding 10 μM MK-801. Glycine had a significant, age-related effect on [^{3}H]-MK-801 binding (ANOVA, $P = 0.002$), whereas strychnine had no effect on the glycine stimulation ($P > 0.05$).

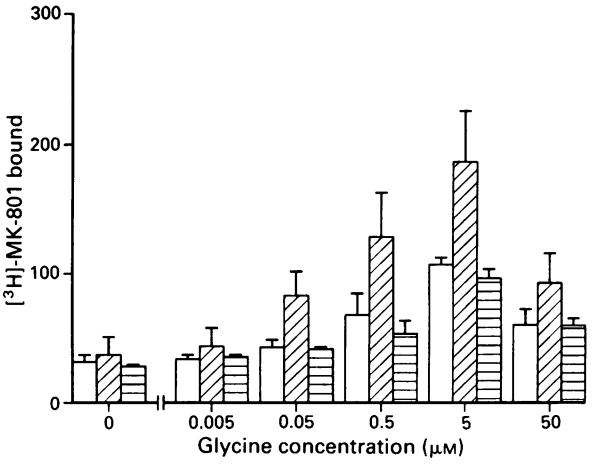


Figure 4 The effects of glycine on [^{3}H]-MK-801 binding in well-washed temporal cortex membrane preparations. Each point is the mean (± s.e.mean) specific binding of [^{3}H]-MK-801 measured on 3 groups of brains, each comprising 3 brains, from infants mean postnatal ages 5.3 weeks (open columns), 19.3 weeks (cross hatched columns) and adults 77.3 years (horizontal hatched columns). Assays contained 4 nM [^{3}H]-MK-801, 0 or 100 μM L-glutamic acid and 0–50 μM glycine. Glycine stimulation was significantly greater in the infant group aged 19.3 weeks ($P < 0.01$).

Age-related stimulation of [^{3}H]-MK-801 binding by glycine

A detailed investigation of the effect of age on the glycine stimulation of [^{3}H]-MK-801 binding was carried out on well-washed temporal cortex membrane preparations from 5 groups of infant brains and one group of adult brains.

[^{3}H]-MK-801 binding (4 nM) was measured with no added L-glutamate/glycine to determine basal binding to the cation channel PCP site and in the presence of 100 μM L-glutamate and one of 5 concentrations (0.5 to 100 μM) of glycine. The

concentration-response curves shown in Figure 5 revealed that maximum [^{3}H]-MK-801 binding was reached with 100 μM L-glutamate and 5 μM glycine. L-Glutamate/glycine stimulated [^{3}H]-MK-801 binding in infant temporal cortex was age-related; binding in tissues from infants aged 19.3 weeks was greater than in the other groups at all glycine concentrations.

Glycine site binding

In order to investigate further the mechanisms behind the glycine-induced increase in [^{3}H]-MK-801 binding in infant cortex, [^{3}H]-glycine binding sites were measured in temporal cortex tissue from infants and adults. The data shown in Figure 6 revealed that [^{3}H]-glycine binding in infant brain cortex increased with age.

Many of the [^{3}H]-glycine binding assays were made on aliquots of the same infant brain tissue samples used for glycine/L-glutamate stimulation of [^{3}H]-MK-801 binding (reported above). For these samples there was a positive correlation between the amounts of [^{3}H]-MK-801 binding and [^{3}H]-glycine binding ($r = 0.596$; $P < 0.05$) which suggests that the PCP and glycine modulatory sites, both part of the NMDA receptor-ion channel complex, develop at similar rates in infant brains.

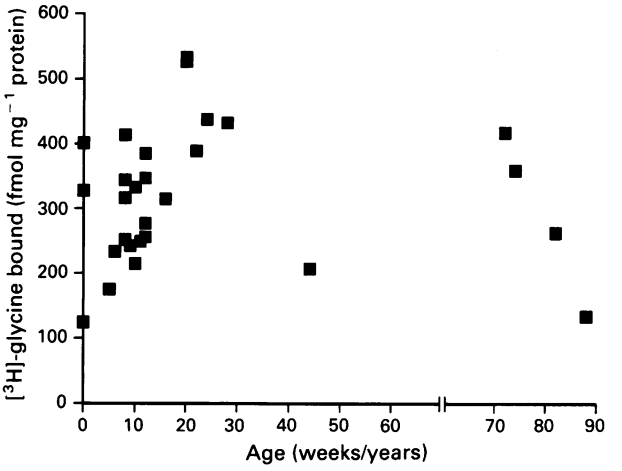


Figure 6 The age-related specific binding of [^{3}H]-glycine (50 nM) in fmol mg^{-1} protein in temporal cortex preparations. Each point represents the mean of triplicate assays performed on a single brain. [^{3}H]-glycine binding in infant brain was influenced by the ages of the subjects (ANOVA, $P < 0.05$).

Discussion

The importance of Glu neurones in human brain is considerable because of the part they may have in a diverse range of physiological actions and pathophysiological events, including learning and memory, anoxia/ischaemia-induced neuronal damage and some neurodegenerative diseases (Greenamyre, 1986; Monaghan *et al.*, 1989). The special roles of Glu neurones in the developing mammalian brain may include regulation of neuronal survival, synaptogenesis and synaptic plasticity in response to experience; the NMDA receptor is especially implicated in all of these events (see review, McDonald & Johnston, 1990).

Some of the evidence that Glu receptor activation helps in controlling neurone development is that receptors may be more abundant at crucial stages of development. A transient overproduction of NMDA as well as some other Glu receptors often occurs so that receptor levels briefly rise above those found in adults of the species. This together with increased sensitivity of NMDA receptors to agonists are recognised phenomena which take place in developing brains of experimental animals (Tsumoto *et al.*, 1987; McDonald *et al.*, 1988; Trembley *et al.*, 1988; Bode-Greuel & Singer, 1989; Bowe & Nadler, 1990; McDonald & Johnston, 1990; Tremblay *et al.*, 1990; Chaudieu *et al.*, 1991; Siviy *et al.*, 1991) which is why the present study measured NMDA receptor binding in the developing infant cortex.

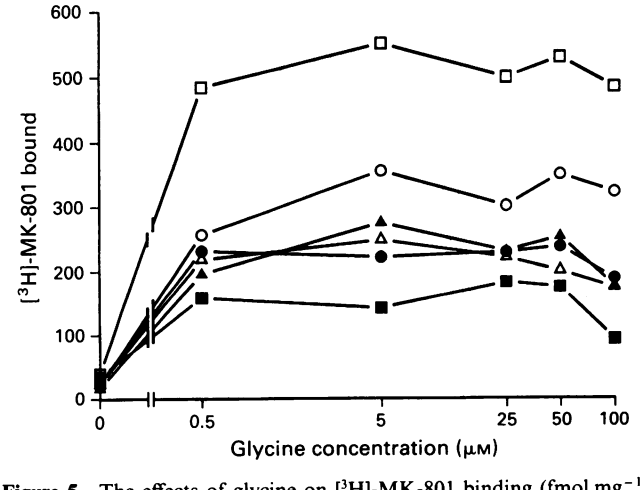


Figure 5 The effects of glycine on [^{3}H]-MK-801 binding (fmol mg^{-1} protein) in temporal cortex membrane preparations. Binding assays included 4 nM [^{3}H]-MK-801, 100 μM L-glutamic acid and the glycine concentrations shown. Where no glycine was added, L-glutamic acid was also absent. Each point is the mean obtained from triplicate assays on tissue from 3 infant or adult brains. The mean age of the subjects comprising each group were 0.33 weeks postnatal (■), 5 weeks (▲), 8 weeks (●), 12 weeks (○), 19.3 weeks (□) and adults aged 70.1 years (△). Stimulated [^{3}H]-MK-801 binding was significantly greater ($P < 0.01$) in the 19.3 week group at all glycine concentrations.

The findings that [^{3}H]-TCP and [^{3}H]-MK-801 binding to the NMDA-associated ion channel site and [^{3}H]-glycine binding to the glycine allosteric modulatory site all demonstrated age-related increases during the first 5 postnatal months are evidence that cortical NMDA receptors proliferate in the developing infant brain. We reported recently that when binding to the glycine site was measured with the selective ligand [^{3}H]-5,7-dichlorokynurenic acid it too showed a postnatal increase indicating that the glycine modulatory site which is part of the NMDA receptor complex is more abundant in temporal cortex of older infants than in neonates (D'Souza *et al.*, 1992). Once infants reached 20 weeks of age, the maximum number of [^{3}H]-MK-801 binding sites (B_{max}) had risen to almost twice the number measured in comparable regions of adult brains. A recent study of brains taken from all ages ranging from infants to elderly adults found that a rapid rise in [^{3}H]-MK-801 binding in infant brain frontal cortex was followed by a protracted age-related decline in [^{3}H]-MK-801 binding levels (Piggott *et al.*, 1992). Apart from these findings, data on Glu receptor binding in the developing human brain are sparse although NMDA receptor binding in frontal cortex taken from a small number of subjects increased sharply during the early postnatal period and peaked transiently between 1 and 2 years of age (Kornhuber *et al.*, 1988).

The high affinity uptake transporter for Glu which is associated with nerve terminals is often used to assess densities of glutamatergic synapses although perisynaptic astrocytes also take up Glu. Previous studies found that binding of the Glu uptake site ligand D-[^{3}H]-aspartate increased sharply in the developing infant cerebral and cerebellar cortices from almost undetectable levels in the newborn to above-adult levels by about 6 months of age (Slater *et al.*, 1992a,b). It is clear that postnatal development in human brain is accompanied by growth of glutamatergic synapses.

Morphological measurements on immature human cerebral cortex have found striking growths of synapses and dendritic spines in infancy and early childhood and revealed that many of the overproduced synapses are eliminated during later childhood and up to adolescence (Purves & Lichtman, 1980; Huttenlocher, 1990). The exuberant synapses that are a feature of early infancy up to 8 months may impart substantial plasticity to the immature cerebral cortex which is eventually lost in the adolescent and adult (Huttenlocher *et al.*, 1982).

In previous work, the binding of [^{3}H]-TCP or [^{3}H]-MK-801 to PCP sites has been used to make *in vitro* functional analyses of the NMDA receptor complex (Hosford *et al.*, 1990; Chessell *et al.*, 1991) based on the principle that compounds acting at the NMDA receptor or the glycine modulatory site increase [^{3}H]-TCP or [^{3}H]-MK-801 binding by moving the cation channel into the open configuration and exposing PCP binding sites. Provided that [^{3}H]-MK-801 binding is measured under non-equilibrium conditions, the amount of binding is a function of the duration and frequency of individual channel openings and the density of channels in any brain area (Hosford *et al.*, 1990). The present study investigated the effect of allosteric interaction between glycine and a fixed concentration of L-glutamate on [^{3}H]-MK-801 binding with the aim of assessing ion channel opening *in vitro*. [^{3}H]-MK-801 binding measured in tissue preparations which were well-washed to remove some endogenous amino acids which would interfere with [^{3}H]-MK-801 binding revealed a substantial postnatal rise in the ability of glycine to stimulate ion channel opening. The possibility that different amounts of endogenous glutamate and glycine remained in the well-washed tissue preparations cannot be ruled out. This appears to be unlikely though because several measurements of stimulated [^{3}H]-MK-801 binding were made on independent sets of samples and produced similar age-related data.

The present data suggest that some of the enhanced [^{3}H]-MK-801 binding that occurred in the brains of infants as they reached 20 weeks of age was caused by a rise in the numbers of NMDA receptors as evidenced by the postnatal rise in [^{3}H]-MK-801 B_{max} but this may not account for the whole of the postnatal rise in stimulated [^{3}H]-MK-801 binding. Work on animal brains has raised the possibility that the multiple recognition and regulatory sites of the NMDA receptor complex may not develop together. NMDA receptor numbers rose in parts of neonate rat brain in the first postnatal days (Tremblay *et al.*, 1988; Insel *et al.*, 1990) but [^{3}H]-glycine binding peaked later at around 10 days (Shinohara *et al.*, 1990). In the hippocampus of neonatal rats, NMDA receptors became transiently hypersensitive to glycine around 1–2 weeks of age (Kleckner & Dingledine, 1991). Furthermore, the developmental curve of NMDA receptor binding was different from that of [^{3}H]-TCP binding (Tremblay *et al.*, 1988; Shinohara *et al.*, 1989). Whilst noting that the binding characteristics of [^{3}H]-MK-801 were similar in rat brain and human neocortex (Procter *et al.*, 1991), it is not possible to extrapolate the animal brain findings to human brain in which postnatal brain development has greatly different timing but the present findings seem to suggest that ion channel PCP sites and glycine sites increased postnatally at similar rates. New work is in progress to chart the ontogeny of NMDA recognition sites and glycine sites in brains from infants and older children which will give some answers to the question of whether the NMDA receptor complex in developing human brain is not only overexpressed but also becomes more efficient in terms of its response to agonists which act at the multiple modulatory sites.

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References

BERTOLINI. M. & VINCINI, S. (1988). Voltage-dependent block by strychnine of N-methyl-D-aspartic acid-activated cationic channels in rat cortical neurons in culture. *Mol. Pharmacol.*, **34**, 98–103.

BODE-GREUEL, K.M. & SINGER, W. (1989). The development of N-methyl-D-aspartate receptors in cat visual cortex. *Dev. Brain Res.*, **46**, 197–204.

BOWE, M.A. & NADLER, J.V. (1990). Developmental increase in the sensitivity to magnesium of NMDA receptors on CA1 hippocampal pyramidal cells. *Dev. Brain Res.*, **56**, 55–61.

BOWERY, N.G. & HUDSON, A.L. (1986). The distribution of [^{3}H]-MK-801 binding sites in rat brain determined by *in vitro* receptor autoradiography. *Br. J. Pharmacol.*, **89**, 775P.

CHAUDIEU, I., OUNT, H., QUIRION, R. & BOKSA, P. (1991). Transient postnatal increased in excitatory amino acid binding sites in rat ventral mesencephalon. *Neurosci. Lett.*, **133**, 267–270.

CHESSELL, I.P., PROCTER, A.W., FRANCIS, P.T. & BOWEN, D.M. (1991). D-Cycloserine, a putative cognitive enhancer, facilitates activation of the N-methyl-D-aspartate receptor-ionophore complex in Alzheimer brain. *Brain Res.*, **565**, 345–348.

COAN, E.J. & COLLINGRIDGE, G.L. (1985). Magnesium ions block an N-methyl-D-aspartate receptor-mediated component of synaptic transmission in rat hippocampus. *Neurosci. Lett.*, **53**, 21–26.

COLLINGRIDGE, G.L. & BLISS, T.V.P. (1987). NMDA receptors–their role in long term potentiation. *Trends Neurosci.*, **7**, 288–293.

D'SOUZA, S.W., McCONNELL, S.E., SLATER, P. & BARSON, A.J. (1992). N-Methyl-D-aspartate binding sites in neonatal and adult brain. *Lancet*, **339**, 1240.

DUPONT, L., GARDETTE, R. & CREPEL, F. (1987). Postnatal development of the chemosensitivity of rat cerebellar Purkinje cells to excitatory amino acids: an in vitro study. *Dev. Brain Res.*, **34**, 59–68.

FOSTER, A.C. & WONG, E.F.H. (1987). The novel anticonvulsant MK-801 binds to the activated state of the N-methyl-D-aspartate receptor. *Br. J. Pharmacol.*, **91**, 403–409.

GREENAMYRE, J.T. (1986). The role of glutamate in neurotransmission and in neurologic disease. *Arch. Neurol.*, **43**, 1058–1063.

GU, Q., BEAR, M.F. & SINGER, W. (1989). Blockade of NMDA receptors prevents ocularity changes in kitten visual cortex after reversed monocular deprivation. *Dev. Brain Res.*, **47**, 281–288.

HARRIS, E.W., GANONG, A.H. & COTMAN, C.W. (1984). Long-term potentiation in the hippocampus involves activation of N-methyl-D-aspartate receptors. *Brain Res.*, **323**, 132–137.

HOSFORD, D.A., BONHAUS, D.W. & McNAMARA, J.O. (1990). A radiohistochemical measure of [^{3}H]-TCP binding to the activated NMDA-receptor-gated ion channel in rat brain. *Brain Res.*, **516**, 192–200.

HUETTNER, J.E. & BEAN, B.P. (1988). Block of N-methyl-D-aspartate activated current by the anti-convulsant MK-801: selective binding to open channels. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 1307–1311.

HUTTENLOCHER, P.R. (1990). Morphometric study of human cerebral cortex development. *Neuropsychologia*, **28**, 517–527.

HUTTENLOCHER, P.R., DE COURTEN, C., GAREY, L.J. & VAN DER LOOS, H. (1982). Synaptogenesis in human visual cortex–evidence for synapse elimination during normal development. *Neurosci. Lett.*, **33**, 247–252.

INSEL, T.R., MILLER, L.P. & GELHARD, R.E. (1990). The ontogeny of excitatory amino acid receptors in the rat forebrain–I. N-Methyl-D-aspartate and quisqualate receptors. *Neuroscience*, **35**, 31–43.

JOHNSON, J.W. & ASCHER, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, **325**, 529–531.

KLECKNER, N.W. & DINGLEDINE, R. (1971). Regulation of hippocampal NMDA receptors by magnesium and glycine during development. *Mol. Brain Res.*, **11**, 151–159.

KLEINSCHMIDT, A., BEAR, M.F. & SINGER, W. (1987). Blockade of NMDA receptors disrupts experience-dependent plasticity of kitten striate cortex. *Science*, **238**, 355–358.

KORNHUBER, J., MACK-BURKHARDT, F., KORNHUBER, M.E. & RIEDERER, P. (1989). [^{3}H]-MK-801 binding sites in post-mortem human frontal cortex. *Eur. J. Pharmacol.*, **162**, 483–490.

KORNHUBER, J., RETZ, W., RIEDERER, P., HEINSEN, H. & FRITZE, J. (1988). Effect of antemortem and postmortem factors on [^{3}H]-glutamate binding in the human brain. *Neurosci. Lett.*, **93**, 312–317.

LOWRY, L.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 355–358.

MCDONALD, J.W. & JOHNSTON, M.V. (1990). Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. *Brain Res. Rev.*, **15**, 41–70.

MCDONALD, J.W., SILVERSTEIN, F.S. & JOHNSTON, M.V. (1988). Neurotoxicity of N-methyl-D-aspartate is markedly enhanced in developing rat central nervous system. *Brain Res.*, **459**, 200–203.

MCPHERSON, G.A. (1983). A practical computer based approach to the analysis of radioligand binding experiments. *Comput. Prog. Biomed.*, **17**, 107–114.

MELDRUM, B. & GARTHWAITE, J. (1990). Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.*, **11**, 379–387.

MONAGHAN, D.T., BRIDGES, R.J. & COTMAN, C.W. (1989). The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.*, **29**, 365–402.

MORRIS, R.G.M., ANDERSON, E., LYNCH, G.S. & BAUDRY, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature*, **319**, 774–776.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.*, **107**, 220–239.

PIGGOTT, M.A., PERRY, E.K., PERRY, R.H. & COURT, J.A. (1992). [^{3}H]-MK-801 binding to the NMDA receptor complex, and its modulation in human frontal cortex during development and aging. *Brain Res.*, **588**, 277–286.

PROCTER, A.W., STRATMANN, G.C., FRANCIS, P.T., LOWE, S.L., BERTOLUCCI, P.H.F. & BOWEN, D.M. (1991). Characterization of the glycine modulatory site of the N-methyl-D-aspartate receptor ionophore complex in human brain. *J. Neurochem.*, **56**, 299–310.

PROCTER, A.W., WONG, E.H.F., STRATMANN, G.C., LOWE, S.L. & BOWEN, D.M. (1989). Reduce glycine stimulation of [^{3}H]-MK-801 binding in Alzheimer's disease. *J. Neurochem.*, **53**, 698–704.

PURVES, D. & LICHTMAN, J.W. (1980). Elimination of synapses in the developing nervous system. *Science*, **210**, 153–157.

QUARUM, M.L., PARKER, J.D., KEANA, J.F.W. & WEBER, E. (1990). (+)-[^{3}H]-MK-801 binding sites in postmortem human brain. *J. Neurochem.*, **54**, 1163–1168.

RANSOM, R.W. & STEC, N.L. (1988). Cooperative modulation of [^{3}H]-MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine and polyamines. *J. Neurochem.*, **51**, 830–836.

RAUSCHECKER, J.P. & HAHN, S. (1987). Ketamine-xylazine anaesthesia blocks consolidation of ocular dominance changes in kitten visual cortex. *Nature*, **326**, 183–185.

ROBINSON, T.N., ROBERTSON, C., CROSS, A.J. & GREEN, A.R. (1990). Modulation of [^{3}H]-dizocilpine ([^{3}H]-MK-801) binding to rat cortical N-methyl-D-aspartate receptors by polyamines. *Mol. Neuropharmacol.*, **1**, 31–35.

SHINOHARA, K., NISHIKAWA, T., ISHII, S., YAMAZAKI, K. & TAKAHASHI, K. (1989). Embryonic and postnatal development of N-(1-[2-thienyl]cyclohexyl) [^{3}H]piperidine binding sites in rat forebrain homogenates and slices. *Neurosci. Lett.*, **107**, 307–312.

SHINOHARA, D., NISHIKAWA, T., YAMAZAKI, K. & TAKAHASHI, K. (1990). Ontogeny of strychnine-insensitive [^{3}H]glycine binding sites in rat forebrain. *Neurosci. Lett.*, **105**, 307–311.

SIVIY, S.M., BUCHWALD, N.A. & LEVINE, M.S. (1991). Enhanced responses to NMDA receptor activation in the developing cat caudate nucleus. *Neurosci. Lett.*, **132**, 77–81.

SLATER, P., MCCONNELL, S., D'SOUZA, S.W., BARSON, A.J., SIMPSON, M.D.C. & GILCHRIST, A.C. (1992a). Age-related changes in binding to excitatory amino acid uptake site in temporal cortex of human brain. *Dev. Brain. Res.*, **65**, 157–160.

SLATER, P., MCCONNELL, S.E., D'SOUZA, S.W. & BARSON, A.J. (1992b). Age-related changes in binding to excitatory amino acid uptake sites in human cerebellum. *Brain Res.*, **579**, 219–226.

STEELE, J.E., ROBINSON, T.N., CROSS, A.J., BOWEN, D.M. & GREEN, A.R. (1989). [^{3}H]-MK-801 labels more NMDA receptor sites than [^{3}H]-TCP in human cortical membranes. *Br. J. Pharmacol.*, **98**, 616P.

TREMBLAY, E., ROISIN-LALLEMAND, M.-P. & BEN-ARI, Y. (1990). Developmental study of [^{3}H]TCP and [^{3}H]glycine binding sites in the rat hippocampus. *Dev. Brain. Res.*, **57**, 21–28.

TREMBLAY, E., ROISIN, M.P., REPRESA, A., CHARRIAUT-MARLANGUE, C. & BEN-ARI, Y. (1988). Transient increased density of NMDA binding sites in the developing rat hippocampus. *Brain Res.*, **461**, 393–396.

TSUMOTO, T., HAGIHARA, K., SATO, H. & HATA, Y. (1987). NMDA receptors in the visual cortex of young kittens are more effective than those of adult cats. *Nature*, **327**, 513–514.

VIGNON, J., CHICHEPORTICHE, R., CHICHEPORTICHE, M., KAMENKA, J.M., GENESTE, P. & LAZDUNSKI, M. (1983). [^{3}H]TCP: a new tool with high affinity for the PCP receptor in rat brain. *Brain Res.*, **280**, 194–197.

WONG, E.H.F., KEMP, J.A., PRIESTLEY, T., KNIGHT, A.R., WOODRUFF, G.N. & IVERSEN, L.L. (1986). The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7104–7108.

WONG, E.H.F., KNIGHT, A.R. & RANSOM, R. (1987). Glycine modulates [^{3}H]-MK-801 binding to the NMDA receptor in rat brain. *Eur. J. Pharmacol.*, **142**, 487–488.

WONG, E.H.F., KNIGHT, A.R. & WOODRUFF, G.N. (1988). [^{3}H]-MK-801 labels a site on the N-methyl-D-aspartate receptor channel complex in rat brain membranes. *J. Neurochem.*, **50**, 274–281.

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Pharmacological characterization of the contractile responses to angiotensin analogues in guinea-pig isolated longitudinal muscle of small intestine

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1 The contractile responses to angiotensin II, angiotensin III and two synthetic analogues, [Lys^2] angiotensin II and [Sar^1]angiotensin II, in the guinea-pig isolated longitudinal muscle preparation of small intestine have been characterized *in vitro*.

2 Tachyphylaxis to the angiotensin analogues was reduced by use of a Krebs-Henseleit solution containing a raised (sub-contractile) concentration of potassium (11.2 mM). Under these conditions, reproducible cumulative concentration-response curves to all agonists were established. The pD_2 estimates for angiotensin II, [Lys^2]angiotensin II, angiotensin III and [Sar^1]angiotensin II were 9.15 ± 0.14, 7.42 ± 0.06, 7.69 ± 0.18 and 9.50 ± 0.15 respectively and the maximum responses achieved were not significantly different.

3 The contractile responses to angiotensin II, angiotensin III and [Sar^1]angiotensin II were reduced by greater than 80% by tetrodotoxin (TTX; 0.1 μM). However, the responses to [Lys^2]angiotensin II were reduced by only $63 \pm 5\%$. Atropine (0.1 μM) also reduced the responses to angiotensin II, angiotensin III and [Lys^2]angiotensin II, although its effect was less than that produced by TTX. Furthermore, while responses to these agonists were not significantly modified by the NK_1 receptor antagonist (±)-CP-96,345 (30 nM) alone, the combined pre-incubation with both atropine and (±)-CP-96,345 reduced maximum agonist responses to a level not significantly different from those produced by TTX.

4 Indirect and direct contractile responses to angiotensin II and [Lys^2]angiotensin II (in the presence of TTX) respectively were characterized by use of the selective AT_1 receptor antagonist, losartan and the AT_2 receptor antagonist, PD123177. Losartan produced parallel rightward displacement of the concentration-response curve to angiotensin II and [Lys^2]angiotensin II, with an estimated pK_B of 8.56 (8.42–8.68) and 9.18 (8.63–9.50) respectively. The AT_2 receptor antagonist, PD123177 (3 μM) failed to modify the contractile responses to either angiotensin II or [Lys^2]angiotensin II.

5 We conclude that two populations of angiotensin II receptors exist in the guinea-pig longitudinal muscle of small intestine, one located neuronally mediating the release of both acetylcholine and substance P and the other located on the smooth muscle mediating direct contractile responses. The neuronal component provides the major contribution to the agonist responses. Both receptor populations are of the AT_1 receptor subtype.

Keywords: Angiotensin; guinea-pig small intestine; losartan; angiotensin II receptor subtypes

Introduction

Early studies have shown that angiotensin II is a potent spasmogen of guinea-pig isolated ileum, acting via the release of acetylcholine and via direct stimulation of the smooth muscle (Robertson & Rubin, 1962; Godfraind *et al.*, 1966). However, these early observations have not been substantiated by others. Paiva *et al.* (1976) demonstrated that acetylcholine was not involved in the response to angiotensin II. In addition, while it has been shown that some indirectly acting spasmogens contract the guinea-pig ileum via the release of both acetylcholine and substance P (Buchheit *et al.*, 1985; Guard & Watson, 1987; Lucaites *et al.*, 1991), no detailed study has investigated the involvement of substance P in the response to angiotensin. Thus, further studies to characterize the nature of the contractile response to angiotensin II in this tissue are warranted.

Studies investigating the actions of angiotensin II in various isolated tissues are additionally complicated because of the susceptibility to tachyphylaxis (Paiva *et al.*, 1974; Shimuta *et al.*, 1989). As a consequence, antagonist studies have produced very limited quantitative data (Wong *et al.*, 1990). However, Miasiro *et al.* (1983) demonstrated that a synthetic analogue of angiotensin II, [Lys^2]angiotensin II does not induce tachyphylaxis, compared to angiotensin II itself or [Sar^1]angiotensin II. Thus, [Lys^2]angiotensin II may provide a useful tool for characterization studies in tissues in which tachyphylaxis occurs readily. An additional way to reduce tachyphylaxis to angiotensin II may be to increase the potassium concentration in the bathing solution. This method has successfully prevented tachyphylaxis to the angiotensin II response in rat isolated femoral arteries (Juul *et al.*, 1987) and in the rat isolated uterus (Freer, 1975).

The subdivision of angiotensin II receptors into two subtypes, AT_1 and AT_2 is now well established (Bumpus *et al.*, 1991). Losartan (DuP 753) is a selective antagonist at the AT_1 subtype, while PD123177 is selective for the AT_2 subtype (Chiu *et al.*, 1989). Thus, the purpose of the present study was to characterize the contractile responses to a range of angiotensin analogues, angiotensin II, angiotensin III, [Lys^2] angiotensin II and [Sar^1]angiotensin II in the guinea-pig isolated longitudinal muscle of small intestine (ileum and jejunum), a high potassium Krebs-Henseleit solution being used to reduce tachyphylaxis. To investigate whether the agonist responses are mediated by receptors on the smooth muscle or via the release of neurotransmitter, we have evaluated the actions of tetrodotoxin (TTX), atropine and the NK_1 receptor antagonist, (±)-CP-96,345 (Snider *et al.*, 1991). Also, using the non-peptide angiotensin II receptor antagonists, losartan and PD123177, we have characterized the angiotensin II receptor subtype involved.

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Methods

Male Dunkin-Hartley guinea-pigs weighing 200–250 g were killed by cervical dislocation. A 12–15 cm length of small intestine was removed proximal to the terminal 10 cm, and divided into 6 equal lengths. Each piece was placed on a 1 ml glass pipette and the longitudinal muscle layer with adhering myenteric plexus was separated from the circular muscle and mucosa by rubbing with a cotton bud. The muscle preparations were then mounted in 5 ml organ baths under 0.5 g tension.

Experiments were carried out in modified Krebs-Henseleit solution of composition (mM): NaCl 112.7, KCl 10, $MgSO_4$ 1.6, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 5.6 and $CaCl_2$ 1.2 aerated with 5% CO_2 in oxygen, maintained at 37°C. Responses were recorded isotonically on Bioscience T3 transducers. At the start of each experiment, increasing concentrations of [Lys^2]angiotensin II were applied in a cumulative manner, ensuring that each concentration had achieved a maximal response before the addition of the next. Repeated cumulative concentration-response curves were constructed at 60 min intervals. The use of [Lys^2]angiotensin II as a 'priming' agonist helped to stabilize angiotensin responses throughout the course of each experiment. With this procedure, up to 6 cumulative agonist concentration-response curves could be constructed reproducibly on any one tissue.

In the first series of experiments, responses to [Lys^2]angiotensin II, angiotensin II, angiotensin III and [Sar^1]angiotensin II were compared. After stable responses to [Lys^2]angiotensin II were established, cumulative concentration-response curves to angiotensin II, angiotensin III and [Sar^1]angiotensin II were constructed. In each experiment, one tissue receiving only [Lys^2]angiotensin II served as a control. Agonist potency was expressed as pD_2, the $-$ log of the effective concentration inducing 50% of the maximum response (EC_{50}). EC_{50} values were measured graphically. Maximum responses (relative to [Lys^2]angiotensin II) were also measured.

The effects of TTX, atropine and (±)-CP-96,345 against concentration-response curves to all agonists were then investigated. In these experiments, [Lys^2]angiotensin II was again used as the 'priming' agonist until stable responses were achieved. Concentration-response curves to the test agonist without and 45 min following TTX (0.1 μM), atropine (0.1 μM), (±)-CP-96,345 (30 nM) or atropine plus (±)-CP-96,345 were then constructed. A control tissue receiving the test agonist alone was also included in each experiment.

In view of the residual response to [Lys^2]angiotensin II (but not angiotensin II) remaining in the presence of TTX, the antagonist actions of losartan (1–100 nM) and PD123177 (3 μM) were investigated against both [Lys^2]angiotensin II (in the presence of TTX, 0.1 μM) and angiotensin II. The antagonist preincubation time was 45 min for all experiments. To measure antagonist potency, parallel lateral displacements of the concentration-response curves were estimated by eye at the control EC_{50} level, to give 'dose ratios' between drug-treated and control curves. Potency was expressed as the negative logarithm of the apparent dissociation constant (pK_B) obtained from the equation, $pK_B = \log(\text{dose ratio} - 1) - \log(\text{antagonist concentration})$. Where three or more concentrations of antagonist were used, Schild analysis (Arunlakshana & Schild, 1959) was applied and if the slope of the linear regression was not significantly different from unity, a pK_B estimate was obtained using a slope constrained to 1. Statistical analyses used Student's t test, with a criterion for statistical significance of $P<0.05$.

The drugs used were supplied from the following sources: angiotensin II, angiotensin III, [Sar^1]angiotensin II (Peninsula laboratories), [Lys^2]angiotensin II (Peptide & Protein Research consultants, Washington Singer Laboratories, University of Exeter, Perry Road, Exeter), losartan potassium (Du Pont Merck Pharmaceutical Co.), atropine sulphate, tetrodotoxin, (Sigma), PD123177 (1- (4-amino-3-methylphenyl) methyl-5- (diphenyl-acetyl)-4,5,6,7- tetrahydro-1H-imidazol [4,5-C] pyridine-6-carboxylic acid) and (±)-CP-96,345 (*cis*-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo [2.2.2] octan-3-amine) (synthesized in our laboratories). All drugs were dissolved as the base in distilled water and diluted in Krebs-Henseleit solution.

Results

Effect of agonists

Cumulative concentration-response curves to angiotensin II, angiotensin III and [Sar^1]angiotensin II, compared to [Lys^2] angiotensin II are shown in Figure 1. The curves of all agonists were parallel and maximum responses achieved were not significantly different. E_{max} values relative to [Lys^2]angiotensin II (100%) were 99 ± 5% (angiotensin II), 99 ± 2% (angiotensin III) and 95 ± 5% ([Sar^1]angiotensin II). The mean (± s.e.mean) pD_2 estimates for angiotensin II, [Lys^2] angiotensin II, angiotensin III and [Sar^1]angiotensin II were 9.15 ± 0.14, 7.42 ± 0.06, 7.69 ± 0.18 and 9.50 ± 0.15 respectively.

Effect of tetrodotoxin, atropine and (±)-CP-96,345

Table 1 shows the effect of TTX, atropine and (±)-CP-96,345 on responses to angiotensin II, [Lys^2]angiotensin II, angiotensin III and [Sar^1]angiotensin II. The effect of TTX, atropine and (±)-CP-96,345 on the responses to angiotensin II are also expressed graphically in Figure 2. Preincubation with TTX (0.1 μM) markedly attenuated the responses to all agonists. Maximum responses to angiotensin II, angiotensin III and [Sar^1]angiotensin II were reduced by greater than 80%. However, there was a much greater residual response remaining to [Lys^2]angiotensin II in the presence of TTX, and the maximum response was only reduced by 63 ± 5% (Table 1). TTX (0.1 μM) abolished responses to the indirectly acting nicotine receptor agonist, dimethylphenylpiperazinium (DMPP) (data not shown). Atropine also substantially reduced the maximum responses to angiotensin II, angiotensin III and [Lys^2]angiotensin II, although less effectively than TTX (Table 1, Figure 2b). The concentration of atropine used in these experiments (0.1 μM) also abolished responses to DMPP (data not shown). The effects of the NK_1 receptor antagonist, (±)-CP-96,345 against responses to angiotensin II, angiotensin III and [Lys^2]angiotensin II were evaluated alone or in combination with atropine. The concentration of (±)-CP-96,345 used was 30 nM, which has previously been shown to displace the concentration-response curve to the NK_1 receptor agonist substance P methylester by approximately 30 fold in the guinea-pig ileum (Beresford *et al.*, 1991). Concentrations of (±)-CP-96,345 greater than 30 nM could not be used because of antagonist activity at L-type calcium channels occurring at higher concentrations (Schmidt *et al.*, 1992). Preincubation of (±)-CP-96,345 alone reduced the maximum agonist responses by less than 10% (Table 1, Figure 2c). However, in combination with atropine (0.1 μM), maximum agonist responses achieved were much less than following preincubation with either (±)-CP-96,345 or atropine alone. Furthermore, maximum responses achieved under these conditions were not significantly different from those achieved in the presence of TTX (Table 1, Figure 2d).

Effects of antagonists

To characterize the angiotensin receptor mediating the indirect (TTX-sensitive) contractile responses, angiotensin II was used as the agonist, as responses to angiotensin II were almost abolished by TTX (see above). The effect of losartan on responses to angiotensin II are shown in Figure 3. Losar-

tan produced parallel, concentration-dependent rightward displacements of the angiotensin II concentration-response curve (Figure 3a). A total of 4 concentrations of losartan was used for Schild analysis and the Schild plot constructed from these data had a gradient of 1.03 (not significantly different from unity) (Figure 3b). The pK_B estimate (95% confidence limits) was 8.56 (8.42–8.68).

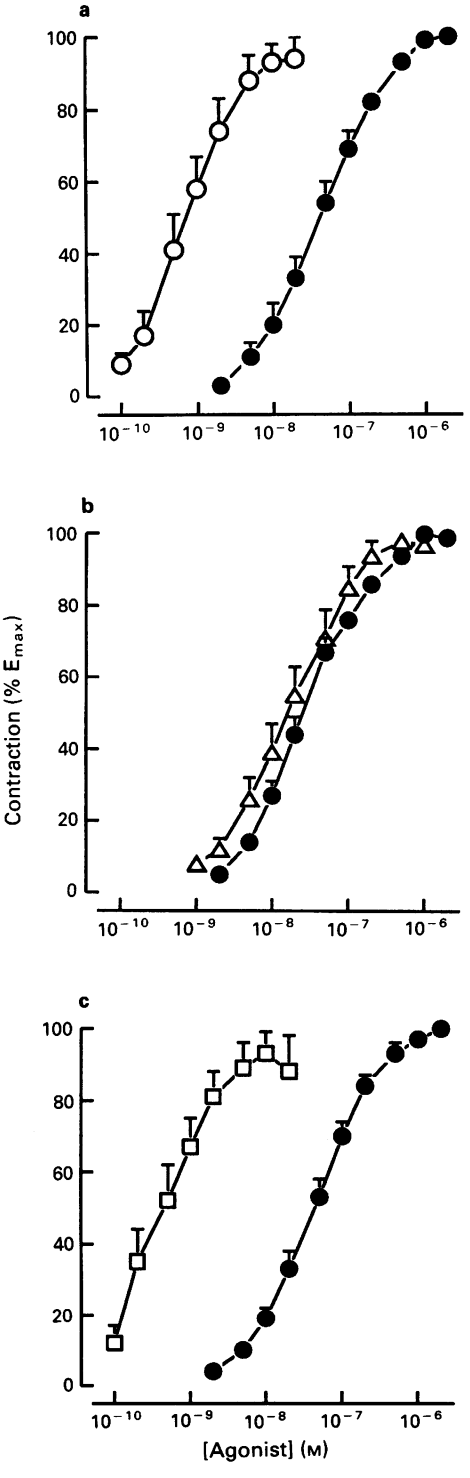


Figure 1 Cumulative concentration – response curves to [Lys^2]angiotensin II, angiotensin II, angiotensin III and [Sar^1]angiotensin II in the guinea-pig isolated longitudinal muscle preparation of small intestine. Agonist responses were compared to [Lys^2]angiotensin II (●) on the same tissue. Open symbols represent responses to (a) angiotensin II, (b) angiotensin III and (c) [Sar^1]angiotensin II. All responses are expressed as mean (± s.e.mean) values, as percentage of the maximum control response (E_{max}) to [Lys^2]angiotensin II ($n = 4-6$).

To characterize the angiotensin II receptor mediating the direct contractile responses, [Lys^2]angiotensin II was used as the agonist and all experiments were conducted in the presence of TTX (0.1 μM). Under these conditions, losartan displaced the concentration-response curve to [Lys^2]angiotensin II to the right in a parallel and concentration-dependent manner (Figure 4). However, we encountered difficulties obtaining dose-ratio shifts greater than 10, because of the low potency of [Lys^2]angiotensin II and the limitation of not being able to increase the agonist concentration high enough to re-establish agonist responses. Despite this, concentrations of 1 and 3 nM yielded an apparent pK_B estimate (95% confidence limits) of 9.16 (8.63–9.50) ($n = 7$). There was a significant difference ($P < 0.05$) between the pK_B estimate of losartan against [Lys^2]angiotensin II and that obtained with angiotensin II as the agonist.

The AT_2 receptor antagonist, PD123177 (3 μM) had no significant effect on the concentration-response curve to angiotensin II or [Lys^2]angiotensin II in the presence of TTX (data not shown).

Discussion

The occurrence of tachyphylaxis to angiotensin II in many isolated tissues has often limited detailed pharmacological investigations of the actions of this peptide *in vitro*. In the present study, using guinea-pig small intestine, we have shown that, in the presence of a raised concentration of potassium, just below that required to increase baseline contractile tone, cumulative concentration-response curves to [Lys^2]angiotensin II, angiotensin II, angiotensin III and [Sar^1] angiotensin II can be established. Comparison of the contractile responses to [Lys^2]angiotensin II, angiotensin II, angiotensin III and [Sar^1]angiotensin II revealed that the slopes of the concentration-response curves were not significantly different. Furthermore, there was no difference in the maximal contractile responses achieved. These observations suggest that there was little evidence for any marked and progressive desensitization to the agonists upon repeated application. Previous studies have shown that [Sar^1]angiotensin II and angiotensin II in particular, induce tachyphylaxis in guinea-pig ileum very readily and near-maximal responses to these agonists have failed to induce consistent contractile responses upon subsequent application (Miasiro *et al.*, 1983; Shimuta *et al.*, 1989). Although the precise mechanisms by which increased potassium concentration can reduce tachyphylaxis in this and in other preparations (Freer, 1975; Juul *et al.*, 1987) are not fully understood, this method has allowed us to characterise successfully the contractile responses to different angiotensin analogues.

Angiotensin II and [Sar^1]angiotensin II were the most potent agonists, angiotensin III and [Lys^2]angiotensin II being approximately 30–100 fold weaker. While some of these data are comparable with previous studies in guinea-pig ileum (Shimuta *et al.*, 1989), other studies have shown angiotensin II and angiotensin III to be less potent (Robertson *et al.*, 1992; Schinke *et al.*, 1991). Furthermore, differences in the relative potencies of angiotensin II and angiotensin III in different tissues are also evident. In some tissues, angiotensin II and angiotensin III have been shown to be equipotent (Peach & Chiu, 1974; Jackson *et al.*, 1988). While differences in agonist orders of potency led to early speculation about the possible heterogeneity of angiotensin receptors (Peach 1977), it is now well established that potencies of some angiotensin peptide agonists can be markedly increased by inhibitors of peptidase enzymes (Robertson *et al.*, 1992; Hawcock *et al.*, 1992). Thus, from these observations as well as differences in the tachyphylactic properties of different peptides in different tissues, the use of agonist orders of potency to characterize angiotensin II receptors is limited.

Table I Effect of tetrodotoxin (TTX), atropine and (±)-CP-96,345 on the maximum responses to [Lys^2]angiotensin II, angiotensin II, angiotensin III and [Sar^1]angiotensin II in guinea-pig isolated longitudinal muscle preparation of small intestine

Agonist	*TTX* (0.1 μM)	*Atropine* (30 nM)	*(±)-CP-96,345* (30 nM)	*Atropine plus (±)-CP-96,345*
[Lys^2]angiotensin II	63 ± 5	33 ± 4	9 ± 1	72 ± 6
Angiotensin II	88 ± 3	60 ± 7	6 ± 4	84 ± 2
Angiotensin III	80 ± 4	49 ± 13	10 ± 6	84 ± 4
[Sar^1]angiotensin II	81 ± 5	ND	ND	ND

Values are expressed as mean (± s.e.mean) % reduction of control E_{max}. ($n = 4-6$). ND, not determined.

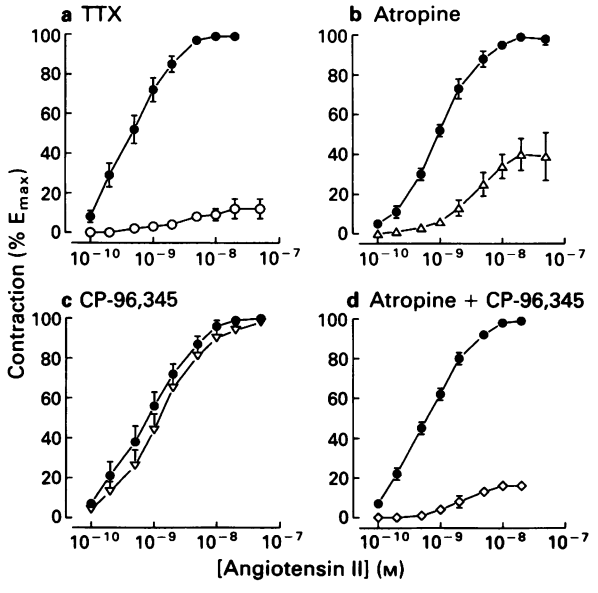


Figure 2 Effect of tetrodotoxin (TTX), atropine, (±)-CP-96,345 and atropine plus (±)-CP-96,345 on contractile responses to angiotensin II in the guinea-pig isolated longitudinal muscle preparation of small intestine. The filled symbols represent control responses to angiotensin II. The open symbols represent responses to angiotensin II in the presence of (a) TTX (0.1 μM), (b) atropine (0.1 μM), (c) (±)-CP-96,345 (30 nM) and (d) atropine plus (±)-CP-96,345. All responses are expressed as mean (± s.e.mean) values, as a percentage of the maximum control response (E_{max}) to angiotensin II ($n = 4-6$).

A more important observation revealed by the use of different agonists in the present studies is the possibility that different mechanisms of action may exist. Experiments using TTX, atropine and the tachykinin NK_1 receptor antagonist (±)-CP-96,345 have shown that the contractile responses to all angiotensin agonists are mediated in part via the release both of acetylcholine and a tachykinin, probably substance P, from the myenteric plexus. The ability of atropine and (±)-CP-96,345, applied in combination, to reduce all agonist responses to a level not significantly different from that measured in the presence of TTX indicates that acetylcholine and substance P are the two major neurotransmitters which mediate the indirect responses in this tissue. Furthermore, in contrast to angiotensin II, angiotensin III and [Sar^1]angiotensin II, a significant proportion of the response to [Lys^2] angiotensin II was insensitive to the actions of TTX. As this residual response could not be inhibited further by atropine or (±)-CP-96,345 (data not shown), it is unlikely that [Lys^2]angiotensin II acts to release acetylcholine or substance P by direct depolarization of nerve terminals. Thus, the most likely explanation is a direct effect via receptors on the smooth muscle. With regard to the indirect responses, it is interesting that (±)-CP-96,345, in the absence of atropine, produced very little reduction of the contractions produced

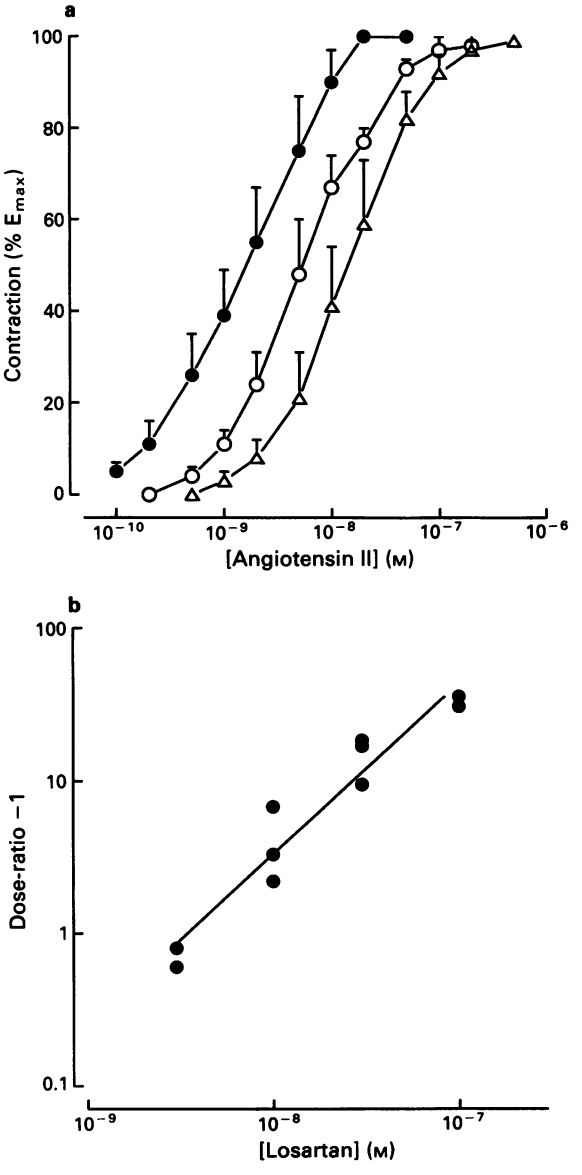


Figure 3 Effect of losartan on contractile responses to angiotensin II in the guinea-pig isolated longitudinal muscle preparation of small intestine. In (a) the filled symbols represent control responses to angiotensin II. The open symbols represent responses to angiotensin II in the presence of losartan 10 nM (○) or 30 nM (△). All responses are expressed as mean (± s.e.mean) values, as a percentage of the maximum control response (E_{max}) to angiotensin II ($n = 3$). (b) Shows the Schild plot, using 4 concentrations of losartan (2 determinations at 3 or 100 nM and 3 determinations at 10 or 30 nM).

by all agonists. As the concentration of (±)-CP-96,345 used has been shown to produce a 30 fold displacement of the concentration-response curve to an NK_1 receptor agonist in

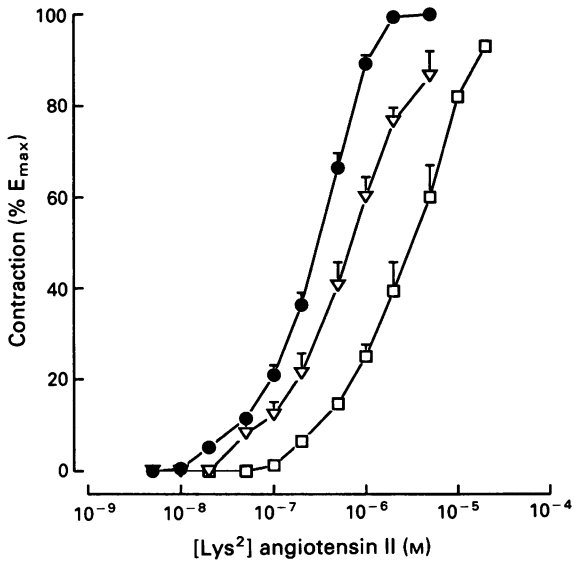


Figure 4 Effect of losartan on contractile responses to [Lys^2]angiotensin II (in the presence of tetrodotoxin 0.1 μM) in the guinea-pig isolated longitudinal muscle preparation of small intestine. The filled symbols represent control responses to angiotensin II. The open symbols represent responses to angiotensin II in the presence of losartan 1 nM (▽), or 3 nM (□). All responses are expressed as mean (± s.e.mean) values, as a percentage of the maximum control response (E_{max}) to [Lys^2]angiotensin II ($n = 3-4$).

the guinea-pig isolated ileum preparation (Beresford *et al.*, 1991), it is unlikely that the lack of effect of this non-peptide antagonist alone is due to insufficient antagonism at NK_1 receptors. We would prefer to conclude that the lack of effect of (±)-CP-96,345 alone more probably relates to the relatively minor role of substance P compared to acetylcholine in mediating contractile responses to these angiotensin analogues under normal conditions and only when the actions of acetylcholine are inhibited can substance P exert its contractile effect. However, while the inhibition of L-type calcium channels by higher concentrations of (±)-CP-96,345 (Schmidt *et al.*, 1992) limits extensive use of this antagonist, further studies using a wider concentration range of more specific non-peptide NK_1 receptor antagonists such as CP-99,994 (McLean *et al.*, 1992) could be used to substantiate these findings.

Assuming that the TTX-sensitive and insensitive components of the agonist responses are due to activation of angiotensin receptors located neuronally and on the smooth muscle respectively, we attempted to characterize both populations of receptors using the AT_1 selective antagonist, losartan and the AT_2 selective antagonist, PD123177. Angiotensin II was the agonist used to characterize the neuronal receptors, and [Lys^2]angiotensin II, in the presence of TTX was used to study the receptors on the smooth muscle. The AT_1 receptor antagonist losartan produced concentration-related parallel displacements of angiotensin II curves, yielding a pK_B estimate of 8.56. The potency of losartan is close to that previously measured against responses to angiotensin II in other tissues (Barnes *et al.*, 1991; Hawcock *et al.*, 1992; Robertson *et al.*, 1992). Furthermore, these findings agree with previous studies showing AT_1 receptor-mediated contractile responses to angiotensin II in guinea-pig (Wong *et al.*, 1990) and rat ileum (Schinke *et al.*, 1991). The lack of effect of the AT_2 receptor selective antagonist, PD123177 confirms that the neuronal responses to angiotensin II are mediated by the AT_1 receptor. Experiments conducted in the presence of TTX, using [Lys^2]angiotensin II as the agonist confirmed that receptors on the smooth muscle were also of the AT_1 subtype. Losartan, but not PD123177, antagonized responses to [Lys^2]angiotensin II. It is interesting that losartan was approximately 4 fold more potent in antagonizing responses to [Lys^2]angiotensin II on the smooth muscle than those mediated neuronally by angiotensin II. The significance of this is not yet clear. However, in view of the recent evidence from cloning studies highlighting the existence of more than one losartan-sensitive angiotensin receptor (Iwai *et al.*, 1992; Sandberg *et al.*, 1992), it is tempting to speculate that this difference may relate to agonist actions at different angiotensin receptor subtypes. Sandberg *et al.* (1992) have shown a similar difference in the affinity of losartan for displacement of angiotensin binding to the two cloned receptor subtypes. Furthermore, a distinction between neuronal and smooth muscle receptors of other neuropeptides in the guinea-pig ileum has been previously established (see Laufer *et al.*, 1988 and references cited therein). However, further work is required to substantiate this possibility.

In conclusion, we have demonstrated that in the presence of raised potassium concentration in the bathing medium, it is possible to construct cumulative concentration-response curves to angiotensin II and related agonist analogues. Under these conditions, two populations of angiotensin receptors have been shown to exist. One receptor population is located neuronally and mediates release of acetylcholine and substance P. The other is located on the smooth muscle and mediates direct contraction. Both of these receptor populations appear to be of the AT_1 receptor subtype.

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References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol,*. **14,** 48–58.

BARNES, J.C., BROWN, J.D., HAWCOCK, A.B. & MICHEL, A.D. (1991). Angiotensin II-induced contractions of the isolated preparation of rat uterus are mediated through the AT_1 receptor subtype. *Br. J. Pharmacol.*, **104,** 43P.

BERESFORD, I.J.M., BIRCH, P.J., HAGAN, R.M. & IRELAND, S.J. (1991). Investigation into species variants in tachykinin NK_1 receptors by use of the non-peptide antagonist, (±)-CP-96,345. *Br. J. Pharmacol.*, **104,** 292–293.

BUCHHEIT, K.H., ENGEL, G., MUTSCHLER, E. & RICHARDSON, B. (1985). A study of the contractile effects of 5-hydroxytryptamine (5-HT) in the isolated longitudinal muscle strip from guinea-pig ileum. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **329,** 36–41.

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. *Hypertension*, **17,** 720–721.

CHIU, A.T., HERBLIN, W.F., MCCALL, D.E., ARDECKY, R.J., CARINI, D.J., DUNCIA, J.V., PEASE, L.J., WONG, P.C., WEXLER, R.R., JOHNSON, A.L. & TIMMERMANS, P.B.M.W.M. (1989). Identification of angiotensin II receptor subtypes. *Biochem. Biophys. Res. Commun.*, **165,** 196–203.

FREER, R.J. (1975). Calcium and angiotensin tachyphylaxis in rat uterine smooth muscle. *Am. J. Physiol.*, **228,** 1423–1430.

GODFRAIND, T., KABA, A. & POLSTER, P. (1966). Specific antagonism to the direct and indirect action of angiotensin on isolated guinea-pig ileum. *Br. J. Pharmacol. Chemother.*, **28,** 93–104.

GUARD, S. & WATSON, S.P. (1987). Evidence for neurokinin-3 receptor-mediated tachykinin release in the guinea-pig ileum. *Eur. J. Pharmacol.*, **144,** 409–412.

HAWCOCK, A.B., BARNES, J.C. & MICHEL, A.D. (1992). Pharmacological characterisation of angiotensin-induced depolarisations of rat isolated superior cervical ganglion *in vitro*. *Br. J. Pharmacol.*, **105,** 686–690.

IWAI, N. & INAGAMI, T. (1992). Identification of 2 subtypes in the rat type 1 angiotensin II receptor. *FEBS Lett.*, **298**, 257–260.

JACKSON, T.R., BLAIR, L.A.C., MARSHALL, J., GOEDERT, M. & HANLEY, M.R. (1988). The mas oncogene encodes an angiotensin receptor. *Nature*, **335**, 437–440.

JUUL, B., AALKJAER, C. & MULVANY, M.J. (1987). Responses of femoral resistance vessels to angiotensin *in vitro*. *Eur. J. Pharmacol.*, **135**, 61–68.

LAUFER, R., GILON, C., CHOREV, M. & SELINGER, Z. (1988). Desensitisation with a selective agonist discriminates between multiple tachykinin receptors. *J. Pharmacol. Exp. Ther.*, **245**, 639–643.

LUCAITES, V.L., MENDELSOHN, L.G., MASON, N.R. & COHEN, M.L. (1991). CCK-8, CCK-4 and gastrin-induced contractions in guinea-pig ileum: evidence for differential release of acetylcholine and substance P by CCK-A and CCK-B receptors. *J. Pharmacol. Exp. Ther.*, **256**, 695–703.

McLEAN, S., GANONG, A., SEYMOUR, P.A., SNIDER, R.M., DESAI, M.C., ROSEN, T., BRYCE, D.K., LONGO, K.P., REYNOLDS, L.S., ROBINSON, G., SCHMIDT, A.W., SIOK, C. & HEYM, J. (1992). Pharmacology of CP-99,994; a nonpeptide antagonist of the tachykinin NK_1 receptor. *Regul. Pept.*, (Suppl 1), S120.

MIASIRO, N., OSHIRO, M.E.M., PAIVA, T.B. & PAIVA, A.C.M. (1983). Role of the two N-terminal residues of angiotensin II in the production of tachyphylaxis. *Eur. J. Pharmacol.*, **87**, 397–406.

PAIVA, T.B., JULIANO, L., NOUAILHETAS, V.L.A. & PAIVA, A.C.M. (1974). The effect of pH on tachyphylaxis to angiotensin peptides in the isolated guinea-pig ileum and rat uterus. *Eur. J. Pharmacol.*, **25**, 191–196.

PAIVA, T.B., MENDES, G.B., ABOULAFIA, J. & PAIVA, A.C.M. (1976). Evidence against cholinergic mediation of the effect of angiotensin II on the guinea-pig ileum. *Pflügers Arch.*, **365**, 129–133.

PEACH, M.J. (1977). Renin angiotensin system: biochemistry and mechanism of action. *Physiol. Rev.*, **57**, 313–353.

PEACH, M.J. & CHIU, A.T. (1974). Stimulation and inhibition of aldosterone biosynthesis *in vitro* by angiotensin II and analogues. *Circ. Res.*, **34–35**, (suppl 1), 17–113.

ROBERTSON, M.J., CUNOOSAMY, M.P. & CLARK, K.L. (1992). Effect of peptidase inhibition on angiotensin receptor agonist and antagonist potency in rabbit isolated thoracic aorta. *Br. J. Pharmacol.*, **106**, 166–172.

ROBERTSON, P.A. & RUBIN, D. (1962). Stimulation of intestinal nervous elements by angiotensin. *Br. J. Pharmacol. Chemother.*, **19**, 5–12.

SANDBERG, K., JI. H., CLARK, A.J.L., SHAPIRA, H. & CATT, K.J. (1992). Cloning and expression of a novel angiotensin II receptor subtype. *J. Biol. Chem.*, **267**, 9455–9458.

SCHINKE, M., DOODS, H.N., GANTEN, D., WIENEN, W. & ENTZEROTH, M. (1991). Characterisation of rat intestinal angiotensin II receptors. *Eur. J. Pharmacol.*, **204**, 165–170.

SCHMIDT, A.W., McLEAN, S. & HEYM, J. (1992). The substance P receptor antagonist CP-96,345 interacts with Ca^{2+} channels. *Eur. J. Pharmacol.*, **215**, 351–352.

SHIMUTA, S.I., NAKAIE, C.R., PAIVA, A.C.M., CORDOPATIS, P. & THEODOROPOULOS, D. (1989). Changes in residues 1 and 4 of angiotensin II affect differently its agonist and tachyphylactic properties. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **340**, 309–313.

SNIDER, R.M., CONSTANTINE, J.W., LOWE, J.A., LONGO, K.P., LEBEL, W.S., WOODY, H.A., DROZDA, S.E., DESAI, M.C., VINICK, F.J., SPENCER, R.W. & HESS, H.-J. (1991). A potent nonpeptide antagonist of the substance P (NK_1) receptor. *Science*, **251**, 435–437.

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. (1990). Nonpeptide angiotensin II receptor antagonists. Studies with EXP9270 and DuP 753. *Hypertension*, **15**, 823–834.

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The glycine/NMDA receptor antagonist, R-(+)-HA-966, blocks activation of the mesolimbic dopaminergic system induced by phencyclidine and dizocilpine (MK-801) in rodents

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1 The effects of the glycine/N-methyl-D-aspartate (NMDA) receptor antagonist, R-(+)-HA-966 on the neurochemical and behavioural responses to phencyclidine (PCP) and dizocilpine (MK-801) have been determined in rodents.

2 In rats, pretreatment with PCP (5 and 10 mg kg^{-1}) or MK-801 (0.25 and 0.5 mg kg^{-1}) dose-dependently stimulated dopamine turnover in nucleus accumbens, amygdala and medial prefrontal cortex, but had no effect in striatum. In contrast, pretreatment with (+)-HA-966 (10 and 30 mg kg^{-1}) did not affect dopamine turnover in any brain region investigated.

3 Pretreatment with (+)-HA-966 (10 and 30 mg kg^{-1}) significantly antagonized the stimulation of dopamine turnover induced by both PCP (10 mg kg^{-1}) and MK-801 (0.5 mg kg^{-1}) in rat nucleus accumbens, amygdala and medial prefrontal cortex.

4 Intracerebral dialysis studies in conscious rats demonstrated that systemic injection of PCP (10 mg kg^{-1}) markedly stimulated dopamine release from the nucleus accumbens, an effect that was abolished by pretreatment with (+)-HA-966 (30 mg kg^{-1}).

5 Pretreatment with PCP (3–30 mg kg^{-1}) or MK-801 (0.1–1.6 mg kg^{-1}) significantly increased locomotor activity in mice. In contrast, subcutaneous injection of (+)-HA-966 (10–100 mg kg^{-1}) failed to stimulate activity.

6 Pretreatment with (+)-HA-966 (10 and 30 mg kg^{-1}) dose-dependently antagonized both PCP (10 mg kg^{-1}) and MK-801 (0.4 mg kg^{-1}) induced hyperactivity in mice.

7 Blockade of PCP-induced hyperactivity by (+)-HA-966 is unlikely to be explained by the induction or potentiation of sedation/ataxia since PCP-induced rotarod deficits were not significantly different in mice pretreated with (+)-HA-966 (30 mg kg^{-1}) or saline.

8 The results demonstrate that (+)-HA-966 antagonizes both the neurochemical and behavioural effects of PCP and MK-801, possibly through interactions at the glycine/NMDA receptor.

Keywords: (+)-HA-966; glycine site; NMDA receptor complex; dizocilpine (MK-801); phencyclidine; hyperactivity; dopamine turnover

Introduction

Several studies have demonstrated that phencyclidine (PCP) facilitates dopaminergic transmission in brain (for review see Johnson, 1983). However, whilst stimulation of basal dopamine release from striatal slices by PCP has been reported *in vitro* (Vickroy & Johnson, 1982; Snell *et al.*, 1984), *ex vivo* studies have shown little effect in intact striatum, in contrast to the marked stimulation of release that has been demonstrated from mesocorticolimbic dopaminergic terminals (Bowers & Hoffman, 1984; Deutch *et al.*, 1987; Rao *et al.*, 1989; 1990). Increased dopamine release from limbic brain is thought to mediate PCP-induced hyperactivity since the behaviour is blocked by dopamine receptor antagonists and by 6-hydroxydopamine lesions of the ventral tegmental area or nucleus accumbens (French & Vantini, 1984; French *et al.*, 1985). Given the psychotomimetic effect of PCP in man (Snyder, 1980; Javitt & Zukin, 1991), blockade of PCP-stimulated dopamine release or hyperactivity may be predictive of antipsychotic potential.

Radioligand binding studies have identified three recognition sites in brain membranes which bind PCP with submicromolar affinity: the PCP binding site associated with the ion channel of the N-methyl-D-aspartate (NMDA) receptor complex, the sigma binding site (Largent *et al.*, 1986) and the dopamine uptake site (Vignon *et al.*, 1988). All are pharmacologically distinct: dizocilpine (MK-801) selectively blocks NMDA receptor ion channels (Wong *et al.*, 1986; 1988) whilst haloperidol, 1,3,di-(2-tolyl)guanidine (DTG) and (+)-3-PPP potently bind to sigma sites (Su, 1982; Tam & Cook, 1984; Largent *et al.*, 1984; Weber *et al.*, 1986). PCP and cocaine, but not MK-801 or ketamine, bind to the dopamine uptake site labelled by [^{3}H]-N-[2-benzo(b)thiophenyl)cyclohexyl]piperidine (Vignon *et al.*, 1988). Although recent attention has focused on the involvement of the sigma recognition site in the psychotomimetic effects of PCP (Largent *et al.*, 1988), behavioural data suggest that blockade of NMDA receptors is responsible for the major pharmacological properties of the compound. Thus, in anticonvulsant tests and drug discrimination paradigms, the potency of many PCP-like compounds correlates with their potency in binding assays using [^{3}H]-PCP, [^{3}H]-TCP and [^{3}H]-MK-801 as radioligands (Quirion *et al.*, 1981; Mendelsohn *et al.*, 1984; Hayes & Balster, 1985; Singh *et al.*, 1990c). Furthermore, the highly selective non-competitive NMDA receptor antagonist, MK-801 generalizes to the PCP discriminative stimulus (Tricklebank *et al.*, 1987), is anticonvulsant in mice and induces PCP-like motor stimulation (Tricklebank *et al.*, 1989). Whilst stimulation of mesolimbic dopamine release by PCP could result from inhibition of dopamine uptake (Vignon *et al.*, 1988; Rothman *et al.*, 1989; Maurice *et al.*, 1991), MK-801 has little effect on uptake mechanisms at pharmacologically relevant concentrations (Maurice *et al.*, 1991),

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yet increases dopamine turnover in a manner identical to PCP (Rao *et al.*, 1990).

In addition to compounds blocking the NMDA receptor ion channel, at least three other distinct classes of NMDA receptor antagonists exist: viz, those acting (i) competitively at the neurotransmitter recognition site, (ii) by antagonizing the potentiating effects of polyamines and (iii) by antagonizing the potentiating effects of glycine (for review, see Lodge & Johnson, 1990). R-(+)-HA-966 (R-(+)-3-amino-1-hydroxypyrrolid-2-one) is an antagonist at the glycine/NMDA modulatory site (Singh *et al.*, 1990a) and exhibits a behavioural profile distinct from PCP and other NMDA receptor ion channel blockers: in rodents, (+)-HA-966 does not generalize to the PCP discriminative stimulus, stimulate motor activity or enhance dopamine turnover (Singh *et al.*, 1990a,b; Tricklebank & Saywell, 1990). Previous studies have shown that (+)-HA-966 can selectively modulate the mesolimbic dopaminergic system (Hutson *et al.*, 1991): pretreatment with (+)-HA-966 antagonized amphetamine-induced dopamine synthesis in the nucleus accumbens, but not in the striatum. (+)-HA-966 also antagonized the hyperactivity, but not the stereotypy, induced by infusion of amphetamine into the rat nucleus accumbens and striatum respectively. Thus, since PCP and MK-801 are able to activate selectively mesolimbic dopamine systems, the present studies have examined the effects of (+)-HA-966 on the stimulation of dopamine turnover and motor activity induced in rodents by these compounds.

Methods

Animals

Male Sprague Dawley rats (250–300 g) and BKTO mice (20–30 g) (Bantin and Kingman, Hull) were housed in groups of 5 on a 12 h light/dark cycle (lights on at 06 h 00 min) and allowed standard laboratory diet and tap water *ad libitum*.

Neurochemical studies

Rats were injected with either saline (0.9% NaCl, 1 ml kg^{-1}, i.p.), PCP (5 and 10 mg kg^{-1}, i.p.) or MK-801 (0.25 and 0.5 mg kg^{-1}, i.p.) and killed by stunning and decapitation 60 min later. Brains were removed and the striatum, nucleus accumbens, amygdala and prefrontal cortex dissected and stored at − 70°C.

In a separate study, rats were pretreated with either saline (1 ml kg^{-1}, i.p.) or (+)-HA-966 (10 and 30 mg kg^{-1}, i.p.) followed 20 min later by either saline (1 ml kg^{-1}, i.p.), PCP (10 mg kg^{-1}, i.p.) or MK-801 (0.5 mg kg^{-1}, i.p.). All animals were killed after a further 60 min, brain regions dissected and stored as previously described.

Tissue samples were homogenized in 10 vol 0.4 M perchloric acid containing 0.1% sodium metabisulphite, 0.01% EDTA and 0.1% cysteine and centrifuged at 3000 *g* for 20 min. Aliquots of the supernatant were analysed by high performance liquid chromatography (h.p.l.c.) with electrochemical detection essentially as described by Hutson *et al.* (1991).

Intracerebral dialysis in conscious rats

Rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.; Sagatal, RMB) and implanted with a concentric dialysis probe in the nucleus accumbens according to coordinates derived from Paxinos & Watson, 1982 (A + 1.7 mm; L 1.5 mm from bregma; V − 8 mm from dura). The probe was essentially as described by Hutson & Suman-Chauhan (1990) except that a 2 mm length of membrane (Filtral 16, Hospal, France) was used. Eighteen h later, the probe was perfused with a physiological salt solution (composition in mM: NaCl 125, KCl 2.5, $MgCl_2$ 1.18, $CaCl_2$ 1.26) at a rate of 2 µl min^{-1}. Samples were collected at 20 min intervals and analysed immediately by h.p.l.c. with electrochemical detection for dopamine content (Hutson *et al.*, 1991). Values were not corrected for *in vitro* recovery of dopamine through the probe. Two h after beginning perfusion, rats were injected with either saline (1 ml kg^{-1}, i.p.) or (+)-HA-966 (30 mg kg^{-1}, i.p.) followed 20 min later by either saline (1 ml kg^{-1}, i.p.) or PCP at a dose (10 mg kg^{-1}, i.p.) that was found in preliminary experiments to give a robust, but submaximal increase in dopamine efflux. Probe placements were confirmed by flushing the probe with indian ink and histological examination at the end of each experiment.

Mouse locomotor activity

Although both rats and mice show marked hyperlocomotion to PCP, mice were used for studying the antagonist properties of (+)-HA-966 because in preliminary studies the dose-response relationship to PCP in this species appeared broader and less likely to be compromised by the onset of ataxia.

Mouse activity was quantified in automated cages (230 × 280 × 210 mm) lined with wire grids and equipped with two parallel infra red photocell beams at the top and base of the cage with input to a BBC Acorn Microcomputer. Whilst a number of behavioural parameters can be measured, cage crossings (i.e. consecutive beam breaks across the base of the cage) have been selected as the measure of locomotion.

Mice were habituated to individual cages for 2 h and then pretreated with either saline (10 ml kg^{-1}, s.c.) or (+)-HA-966 (10 and 30 mg kg^{-1}, s.c.) followed 30 min later by either saline (10 ml kg^{-1}, s.c.), PCP (10 mg kg^{-1}, s.c.) or MK-801 (0.4 mg kg^{-1}, s.c.). The animals were immediately returned to the photocell cages and the number of beam breaks occurring in 10 min intervals recorded for 2 h.

Rotarod performance

The sedative/ataxic effects of (+)-HA-966 and PCP were examined in mice by use of a rotarod apparatus. Animals were trained to remain on a rotarod revolving at 15 r.p.m. for 2 min and then pretreated with either saline (10 ml kg^{-1}, s.c.) or (+)-HA-966 (30 mg kg^{-1}, s.c.). Thirty min later, mice received either saline (10 ml kg^{-1}, s.c.) or PCP (3–30 mg kg^{-1}, s.c.) and the latency to fall off the rotarod determined 30 min later. Animals not falling off within 2 min were given a maximum score of 120 s.

Statistical analysis

Mouse motor activity data were analysed by analysis of variance for repeated measures followed by one way analysis of variance with Tukey's *t* test for pair-wise comparisons at individual time points. Neurochemical data were also analysed by analysis of variance followed by Tukey's test.

Drugs

Phencyclidine HCl (Ultrafine Chemicals), MK-801 (dizocilpine, Merck Sharp and Dohme Research Laboratories) and (+)-HA-966 (R-(+)-3-amino-1-hydroxypyrrolid-2-one, Merck, Sharp and Dohme Research Laboratories) were dissolved in 0.9% NaCl.

Results

Effect of PCP and MK-801 on regional brain dopamine turnover in the rat

Pretreatment with PCP (5 and 10 mg kg^{-1}, i.p.; Figure 1a) dose-dependently increased dopamine turnover, as defined by the ratio of [DOPAC + HVA]/[DA], in the medial prefrontal

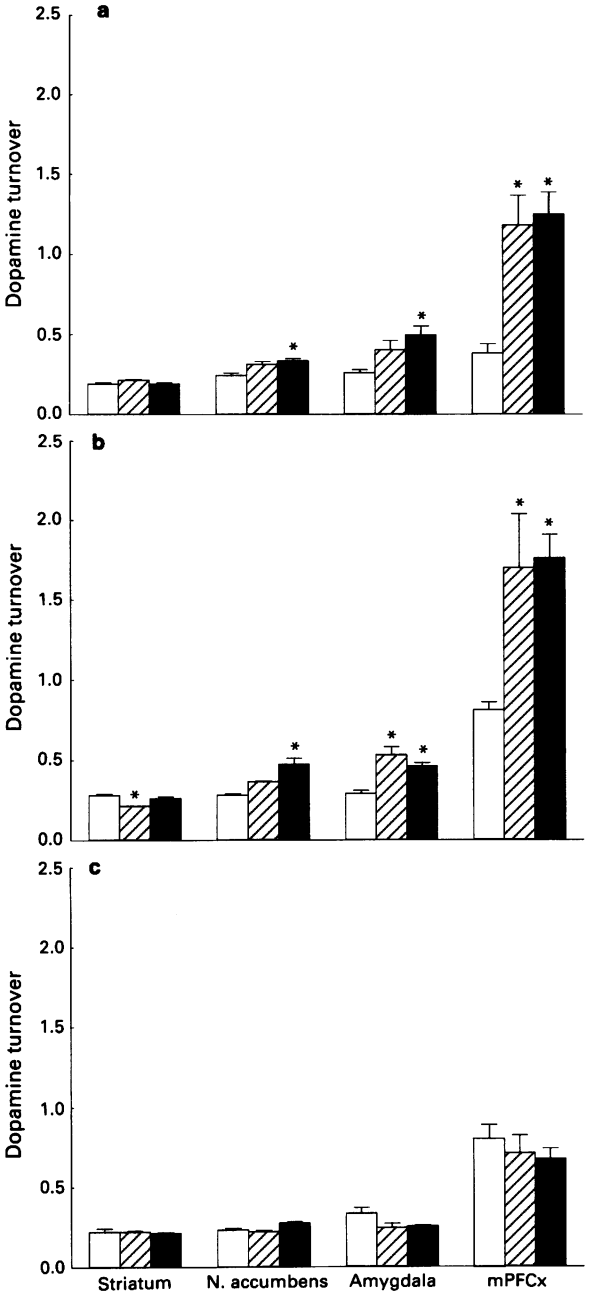


Figure 1 Effect of (a) phencyclidine (PCP, 5 mg kg^{-1}, i.p., hatched columns and 10 mg kg^{-1}, i.p., solid columns), (b) dizocilpine (MK-801, 0.25 mg kg^{-1}, i.p., hatched columns and 0.5 mg kg^{-1}, i.p., solid columns) and (c) (+)-HA-966 (10 mg kg^{-1}, i.p., hatched columns and 30 mg kg^{-1}, i.p., solid columns) on dopamine turnover in rat striatum, nucleus accumbens (N. accumbens), amygdala and medial prefrontal cortex (mPFCx). Open columns show the effects of pretreatment with saline (1 ml kg^{-1}, i.p.). Each column shows the mean for 4–12 rats (± s.e.mean). Dopamine turnover is expressed as the ratio of the levels of [DOPAC + HVA]/[dopamine] measured by h.p.l.c. with electrochemical detection. Data were analysed by one way analysis of variance followed by Tukey's t test; $^*P<0.05$ compared with saline-treated rats.

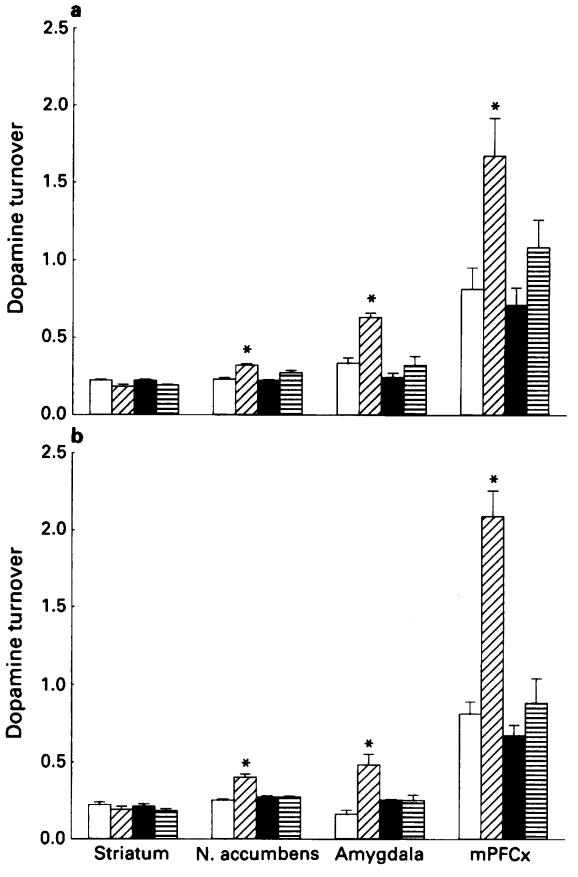


Figure 2 Effect of (a) (+)-HA-966 (10 mg kg^{-1}, i.p.) and (b) (+)-HA-966 (30 mg kg^{-1}, i.p.) on dopamine turnover stimulated by phencyclidine (PCP, 10 mg kg^{-1}, i.p.) in rat striatum, nucleus accumbens (N. accumbens), amygdala and medial prefrontal cortex (mPFCx). Each column shows the mean for 6–12 rats (± s.e.mean); open columns: saline (1 ml kg^{-1}, i.p.) + saline (1 ml kg^{-1}, i.p.); columns with diagonal hatching: saline (1 ml kg^{-1}, i.p.) + PCP (10 mg kg^{-1}, i.p.); solid columns: (+)-HA-966 (10 mg kg^{-1}, i.p (a), 30 mg kg^{-1}, i.p. (b)) + saline (1 ml kg^{-1}, i.p.); columns with horizontal hatching: (+)-HA-966 (10 mg kg^{-1}, i.p. (a) or 30 mg kg^{-1}, i.p. (b)) + PCP (10 mg kg^{-1}, i.p.). Dopamine turnover is expressed in individual rats as the ratio of [DOPAC + HVA]/[DA] measured by h.p.l.c. with electrochemical detection. Data were analysed by two way analysis of variance followed by Tukey's t test. $^*P<0.01$ compared with appropriate control group.

cortex (210% and 229% of controls), amygdala (54% and 88%) and nucleus accumbens (29% and 38% respectively). In contrast, PCP (5 and 10 mg kg^{-1}) did not significantly alter dopamine turnover in rat striatum (Figure 1a). Stimulation of dopamine turnover was also observed in the medial prefrontal cortex (110 and 106%), amygdala (83% and 59%) and nucleus accumbens (28% and 68%) following the intraperitoneal injection of MK-801 (0.25 and 0.5 mg kg^{-1} respectively; Figure 1b). A small, but significant reduction in dopamine turnover was demonstrated in the striatum following the lowest dose of MK-801 (0.25 mg kg^{-1}, Figure 1b). The stimulation of dopamine turnover by PCP and MK-801 reflected an increase in metabolite concentrations since in all experiments, dopamine concentration was not significantly altered (Table 1). Pretreatment with (+)-HA-966 (10 and 30 mg kg^{-1}, i.p.) was without effect on dopamine turnover in any brain region investigated (Figure 1c).

Effect of (+)-HA-966 on the stimulation of dopamine turnover by PCP and MK-801 in corticolimbic brain regions

Pretreatment with (+)-HA-966 (10 and 30 mg kg^{-1}, i.p.) 20 min prior to injection of PCP (10 mg kg^{-1}, i.p.) significantly and dose-dependently attenuated the increase in dopamine turnover induced by PCP in rat nucleus accumbens (reduced by 55% and 87%), amygdala (reduced by 100% and 72%) and medial prefrontal cortex (reduced by 69% and 94% respectively, Figure 2a,b). Similar effects were also observed in rats treated with MK-801 (0.5 mg kg^{-1}, i.p.; Figure 3). Thus, MK-801-stimulated dopamine turnover was

Table 1 Effect of phencyclidine (PCP), dizocilpine (MK- 801) and (+)-HA-966 on dopamine concentration (ng g^{-1}; mean ± s.e.mean, $n = 4-12$) in rat brain regions

Dose (mg kg^{-1})	*Striatum*	*N. accumbens*	*Amygdala*	*mPFCx**
PCP				
0	8569 ± 350	5877 ± 350	475 ± 22	126 ± 12
5	8766 ± 222	6452 ± 445	621 ± 55	97 ± 20
10	8508 ± 496	5562 ± 186	397 ± 27	73 ± 10
MK-801				
0	7354 ± 354	5573 ± 273	447 ± 18	101 ± 7
0.25	9339 ± 654	5407 ± 361	376 ± 55	92 ± 6
0.5	8962 ± 623	5872 ± 143	343 ± 40	102 ± 9
(+)-HA-966				
0	8075 ± 291	6933 ± 227	469 ± 38	76 ± 8
10	8070 ± 508	7192 ± 475	525 ± 46	88 ± 5
30	9404 ± 342	5796 ± 489	577 ± 74	242 ± 35

*Medial prefrontal cortex.

significantly attenuated in the nucleus accumbens (59%), amygdala (87%) and medial prefrontal cortex (76%) following pretreatment with (+)-HA-966 (30 mg kg^{-1}, i.p.; Figure 3). The effect of (+)-HA-966 on dopamine turnover was mediated solely via attenuation of the increase in dopamine metabolites induced by PCP or MK-801 (data not shown).

Effect of (+)-HA-966 on PCP-stimulated dopamine efflux from the nucleus accumbens in conscious rats

Intraperitoneal injection of PCP (10 mg kg^{-1}) markedly stimulated dopamine efflux from the nucleus accumbens of conscious rats which reached a maximum value of 480 fmol 40 μl^{-1}, 20 min after injection and declined over the next 4 h (Figure 4). (+)-HA-966 (30 mg kg^{-1}, i.p.) did not alter basal dopamine release, but when given 20 min prior to PCP (10 mg kg^{-1}, i.p.) markedly attenuated PCP-stimulated dopamine efflux (Figure 4).

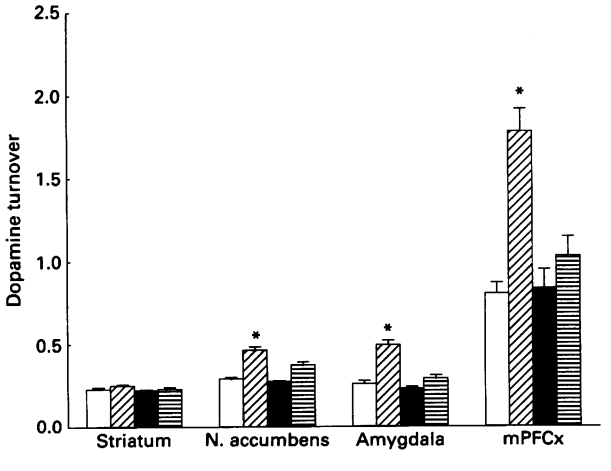


Figure 3 Effect of (+)-HA-966 (30 mg kg^{-1}, i.p.) on the stimulation of dopamine turnover induced by dizocilpine (MK-801, 0.5 mg kg^{-1}, i.p.) in rat striatum, nucleus accumbens (N. accumbens), amygdala and medial prefrontal cortex (mPFCx). Each column shows the mean for 6–12 rats (± s.e.mean); open columns: saline (1 ml kg^{-1}, i.p.) + saline (1 ml kg^{-1}, i.p.); columns with diagonal hatching: saline (1 ml kg^{-1}, i.p.) + MK-801 (0.5 mg kg^{-1}, i.p.); solid columns: (+)-HA-966 (30 mg kg^{-1}, i.p.) + saline (1 ml kg^{-1}, i.p.); columns with horizontal hatching: (+)-HA-966 (30 mg kg^{-1}, i.p.) + MK-801 (0.5 mg kg^{-1}, i.p.). Dopamine turnover is expressed as the ratio of [DOPAC + HVA]/[DA] measured by h.p.l.c. with electrochemical detection. Data were analysed by two way analysis of variance followed by Tukey's t test. *$P < 0.05$ compared with appropriate control group.

Effect of PCP, MK-801 and (+)-HA-966 on spontaneous activity in mice

Administration of PCP (1–30 mg kg^{-1}, s.c.) or MK-801 (0.1–1.6 mg kg^{-1}, s.c.) to mice previously habituated for 2 h to individual photocell activity cages significantly and dose-dependently increased the frequency of cage crossings compared to saline-pretreated animals (Figure 5). Peak hyperactivity responses occurred following 0.4 mg kg^{-1} MK-801 and 10 mg kg^{-1} PCP, the biphasic dose-response curves probably resulting from the marked ataxia induced by the higher doses (Figure 5).

Pretreatment with (+)-HA-966 (10–100 mg kg^{-1}, s.c.) did not alter motor activity in mice (Figure 5).

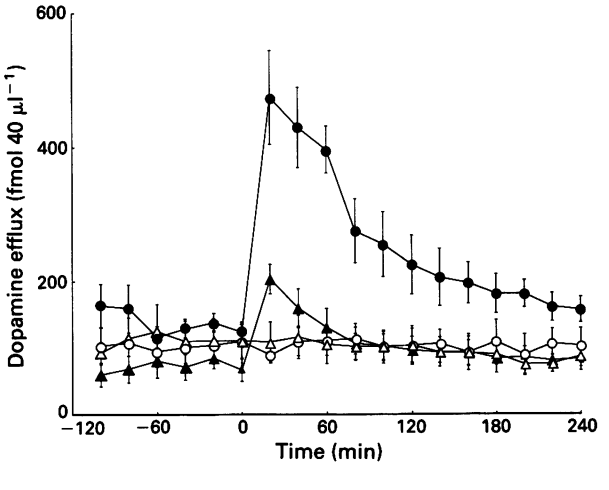


Figure 4 Effect of (+)-HA-966 (30 mg kg^{-1}, i.p.) on phencyclidine (PCP, 10 mg kg^{-1}, i.p.)-stimulated dopamine efflux from nucleus accumbens in conscious rats. Results are expressed as the mean concentration of dopamine (fmol) per 40 µl aliquot collected every 20 min (± s.e.mean, $n = 5-6$); (○) saline (1 ml kg^{-1}, i.p.) + saline (1 ml kg^{-1}, i.p.); (●) saline (1 ml kg^{-1}, i.p.) + PCP (10 mg kg^{-1}, i.p.); (△) (+)-HA-966 (30 mg kg^{-1}, i.p.) + saline (1 ml kg^{-1}, i.p.); (▲) (+)-HA-966 (30 mg kg^{-1}, i.p.) + PCP (10 mg kg^{-1}, i.p.). Areas under the curves were analysed by 2-way analysis of variance followed by Tukey's t test. Basal efflux (−120 min–0 min) did not differ significantly between groups. The area under the curve for animals given only PCP during the time period 0 min–240 min (262 ± 29 fmol per 40 µl per min) was significantly different from animals given saline (105 ± 27 fmol per 40 µl per min), (+)-HA-966 (96 ± 20 fmol per 40 µl per min), or (+)-HA-966 + PCP (110 ± 20 fmol per 40 µl per min), $P < 0.05$.

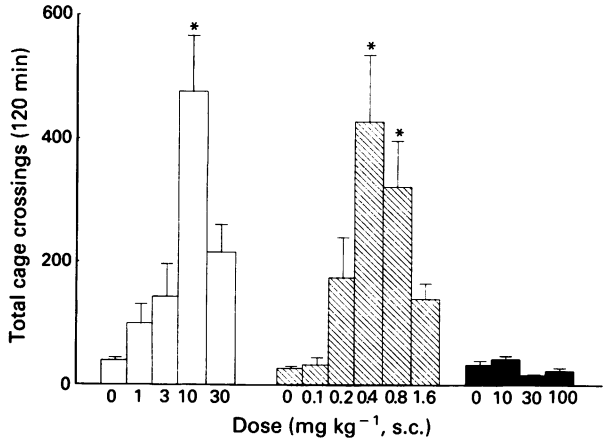


Figure 5 Effect of phencyclidine (PCP, 1–30 mg kg^{-1}, s.c., open columns), dizocilpine (MK-801, 0.1–1.6 mg kg^{-1}, s.c., hatched columns) and (+)-HA-966 (1–100 mg kg^{-1}, s.c., solid columns) on cage crossings recorded from 0–120 min following drug administration in mice. Results are expressed as mean activity counts (± s.e.mean, $n = 7$–13). Data were analysed by one way analysis of variance followed by Tukey's t test. *$P < 0.05$ compared to saline-pretreated mice.

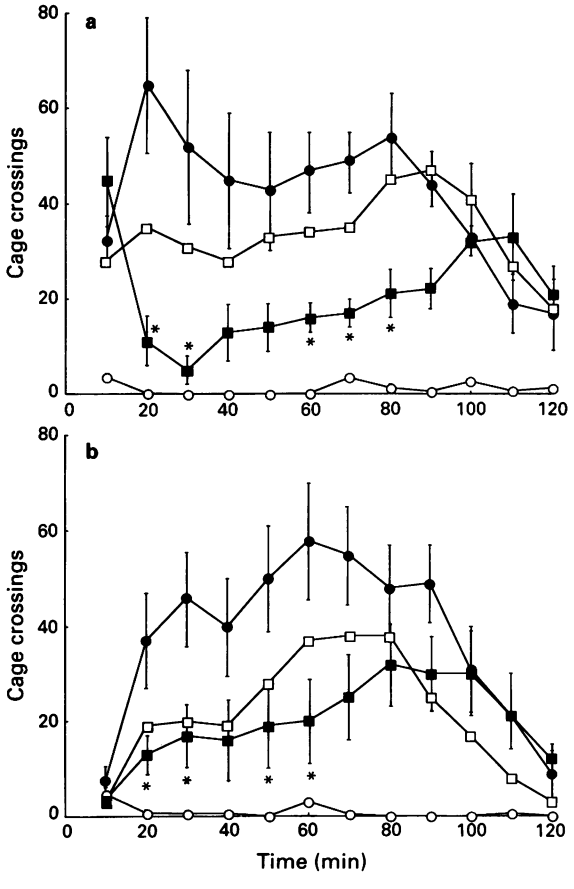


Figure 6 Effect of 30 min pretreatment with (+)-HA-966 (10 or 30 mg kg^{-1}, s.c.) on the stimulation of cage crossing induced by (a) phencyclidine (PCP, 10 mg kg^{-1}, s.c.) and (b) dizocilpine (MK-801, 0.4 mg kg^{-1}, s.c.) in mice. Results are expressed as mean activity counts (± s.e.mean, $n = 8$–11); (a) (○) saline (10 ml kg^{-1}, s.c.) + saline (10 ml kg^{-1}, s.c.); (●) saline (10 ml kg^{-1}, s.c.) + PCP (10 mg kg^{-1}, s.c.); (□) (+)-HA-966 (10 mg kg^{-1}, s.c.) + PCP (10 mg kg^{-1}, s.c.); (■) (+)-HA-966 (30 mg kg^{-1}, s.c.) + PCP (10 mg kg^{-1}, s.c.); (b) (○) saline (10 ml kg^{-1}, s.c.) + saline (10 ml kg^{-1}, s.c.); (●) saline (10 ml kg^{-1}, s.c.) + MK-801 (0.4 mg kg^{-1}, s.c.); (□) (+)-HA-966 (10 mg kg^{-1}, s.c.) + MK-801 (0.4 mg kg^{-1}, s.c.); (■) (+)-HA-966 (30 mg kg^{-1}, s.c.) + MK-801 (0.4 mg kg^{-1}, s.c.). Data were analysed by analysis of variance for repeated measures followed by Tukey's t test. *$P < 0.05$ compared to saline/PCP or saline/MK-801-treated mice.

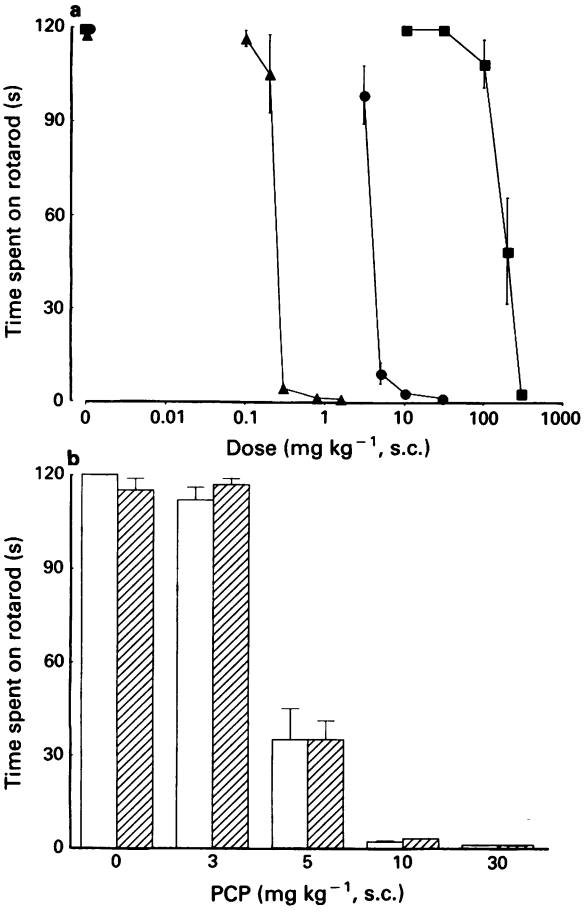


Figure 7 (a) Effect of (▲) dizocilpine (MK-801, 0.1–1.6 mg kg^{-1}, s.c.), (●) phencyclidine (PCP, 3–30 mg kg^{-1}, s.c.) and (■) (+)-HA-966 (10–300 mg kg^{-1}, s.c.) on rotarod performance tested 30 min after injection in mice. Results are expressed as the mean latency to fall from a rotarod revolving at 15 revolutions per min (± s.e.mean, $n = 8$). (b) Effect of 30 min pretreatment with (+)-HA-966 (30 mg kg^{-1}, s.c., hatched columns) or saline (10 ml kg^{-1}, s.c., open columns) on PCP (3–30 mg kg^{-1}, s.c.)-induced rotarod impairment in mice. Results are expressed as the mean latency to remain on a rotarod (15 r.p.m.); data were analysed by analysis of variance followed by Tukey's t test and were not significantly different.

Effects of (+)-HA-966 on PCP and MK-801-induced hyperactivity in mice

The pretreatment of mice with (+)-HA-966 (10 and 30 mg kg^{-1}, s.c.) dose-dependently antagonized the increase in locomotor counts recorded following the injection of PCP (10 mg kg^{-1}, s.c., Figure 6a) and MK-801 (0.4 mg kg^{-1}, s.c., Figure 6).

Effect of (+)-HA-966 on PCP-induced impairments in rotarod performance in mice

Both PCP and MK-801 significantly reduced the ability of mice to remain on a rotarod revolving at 15 r.p.m. at doses similar to those also stimulating motor activity in mice (Figure 7a). Pretreatment with (+)-HA-966 also induced a rotarod deficit, although the minimum effective dose was considerably greater than those attenuating PCP or MK-801 induced hyperactivity (200 mg kg^{-1}, s.c., Figure 7a cf. 10 and 30 mg kg^{-1}, s.c.). Administration of (+)-HA-966 (30 mg kg^{-1}, s.c.) 30 min prior to injection of PCP did not alter the PCP-induced motor deficit (Figure 7b).

Discussion

The present study extends our previous observations (Hutson *et al.*, 1991) that the glycine/NMDA receptor antagonist, (+)-HA-966 is able to modulate the activity of mesolimbic dopamine neurones in rodent brain. Thus, in the absence of any direct effect on brain dopamine metabolism or release, (+)-HA-966 significantly attenuated the increase in dopamine turnover induced in rat nucleus accumbens, amygdala and medial prefrontal cortex by the systemic injection of PCP and MK-801. Furthermore, the PCP-induced increase in dopamine outflow in the nucleus accumbens of the conscious rat, measured by intracerebral dialysis, was almost completely abolished by pretreatment with (+)-HA-966, suggesting an action on presynaptic dopaminergic systems.

In behavioural studies, (+)-HA-966 was also able to attenuate the actions of PCP and MK-801: whilst (+)-HA-966 was without effect on motor activity, it significantly and dose-dependently attenuated the increase in cage crossings induced by both stimulants. It is unlikely that this behavioural antagonism results from a sedative action of (+)-HA-966 since neither spontaneous activity (Hutson *et al.*, 1991) nor rotarod performance (present study) were impaired by (+)-HA-966 at doses inhibiting PCP or MK-801-induced hyperlocomotion. Since the motor hyperactivity response to both compounds declines at high doses as ataxia becomes predominant (Tricklebank *et al.*, 1989), the failure of (+)-HA-966 to enhance the rotarod deficit induced by PCP demonstrates that the blockade of hyperactivity by (+)-HA-966 is mediated by a true antagonism rather than by a leftward shift in the dose-response relationship to the stimulants.

The molecular mechanism by which (+)-HA-966 inhibits the activation of dopamine systems by PCP and MK-801 is clearly difficult to understand if it is assumed that all three compounds are acting as NMDA receptor antagonists (Wong *et al.*, 1986; 1988; Singh *et al.*, 1990a). Although PCP has appreciable affinity for the sigma recognition site (Largent *et al.*, 1986) and inhibits dopamine re-uptake (Vignon *et al.*, 1988), in addition to its ability to block the ion channel on the NMDA receptor, MK-801 shares only the latter property, consistent with other evidence indicating that blockade of NMDA receptors is responsible for the neurochemical and behavioural effects of PCP (Bowers & Hoffman, 1984; Hayes & Balster, 1985; Deutch *et al.*, 1987; Willetts & Balster, 1988; Tricklebank *et al.*, 1987; 1989; Rao *et al.*, 1989; 1990; Singh *et al.*, 1990c). (+)-HA-966 also antagonizes the actions of NMDA, but via blockade of the glycine modulatory site on the NMDA receptor (Singh *et al.*, 1990a), yet, paradoxically, attenuates the behavioural and neurochemical actions of the non-competitive NMDA receptor antagonists. It is, of course, possible that (+)-HA-966 does not attenuate drug-induced dopaminergic hyperactivity via glycine receptors, but via some other as yet unidentified pharmacological property. However, the structurally distinct glycine/NMDA receptor antagonist, 5,7-dichlorokynurenic acid, has also been found to attenuate the stimulation of dopamine synthesis induced by amphetamine (Hutson *et al.*, 1991), suggesting that anti-dopaminergic effects may be a general property of glycine/NMDA receptor antagonists. Alternatively, the results could indicate that the activation of dopamine systems by PCP and MK-801 does not involve the NMDA receptor, although as noted above, there is, at present, little other evidence consistent with this hypothesis.

In support of the ability of NMDA receptor blockade to attenuate dopaminergic hyperactivity, competitive NMDA receptor antagonists have been reported to antagonize the locomotor response to the intra-accumbens injection of amphetamine (Pulvirenti *et al.*, 1991) and to attenuate the increase in firing rate of dopaminergic neurones in the ventral tegmentum induced by PCP (French, 1992). Taken together, these results suggest the presence of a glutamatergic system that enhances mesolimbic dopaminergic functioning. Indeed, NMDA itself stimulates [^{3}H]-dopamine efflux from accumbens and striatal slices and stimulates locomotor activity following infusion into rat nucleus accumbens, ventral tegmental area or substantia nigra (Pycock & Dawbarn, 1980; Donzanti & Uretsky, 1983; 1984; Hamilton *et al.*, 1986; Jones *et al.*, 1987; O'Neill *et al.*, 1989; Ransom & Deschenes, 1989). It is possible that stimulation and blockade of dopaminergic systems by NMDA receptor-mediated events can occur at different loci in the brain. The paradox may not, therefore, lie in why NMDA/glycine receptor antagonists are able to inhibit the effects of NMDA receptor ion channel blockers, but in why the different classes of NMDA receptor antagonists differ in their abilities to stimulate dopamine systems (Kabuto *et al.*, 1989; French *et al.*, 1991; Hutson *et al.*, 1991).

It is pertinent to note that others have shown that the glycine receptor agonists, D-serine and D-alanine, also antagonize PCP-induced stereotypy (Contreras, 1990; Tanii *et al.*, 1991). Whilst functional assays *in vitro* have demonstrated that (+)-HA-966 predominantly antagonizes responses to NMDA, blockade is not complete, consistent with the compound being a partial agonist with a level of efficacy of about 10% of that of glycine (Singh *et al.*, 1990a). Thus, it is possible that the antagonism by (+)-HA-966 of the behavioural and neurochemical effects of non-competitive NMDA receptor antagonists reflects an agonist action at the glycine modulatory site.

In conclusion, it is clear that (+)-HA-966 is able to modulate both the neurochemical and behavioural response to the non-competitive NMDA receptor antagonists, PCP and MK-801. The involvement of the glycine modulatory site in mediating these effects, though likely, will remain difficult to prove until other, structurally unrelated glycine receptor antagonists, or partial agonists have been identified as having similar properties. Nevertheless, the mesolimbic selectivity of non-competitive NMDA receptor antagonists and of the antagonism by (+)-HA-966 of dopaminergic hyperlocomotion, but not stereotypy induced by amphetamine (Hutson *et al.*, 1991) lends the compound aspects of the preclinical pharmacological profile of atypical neuroleptic compounds. Given the strong resemblance of PCP intoxication in man to paranoid schizophrenia (Javitt & Zukin, 1991), the anti-psychotic potential of glycine/NMDA receptor antagonists seems worthy of investigation.

References

BOWERS, M.B. & HOFFMAN, F.J. (1984). Homovanillic acid in rat caudate and prefrontal cortex following phencyclidine and amphetamine. *Psychopharmacol.*, **84**, 136–137.

CONTRERAS, P.C. (1990). D-serine antagonised phencyclidine and MK-801-induced stereotyped behaviour and ataxia. *Neuropharmacol.*, **29**, 291–293.

DEUTCH, A.Y., TAM, S.Y., FREEMAN, A.S., BOWERS, M.B. & ROTH, R.H. (1987). Mesolimbic and mesocortical dopamine activation induced by phencyclidine: contrasting pattern to striatal response. *Eur. J. Pharmacol.*, **134**, 257–264.

DONZANTI, B.A. & URETSKY, N.J. (1983). Effects of excitatory amino acids on locomotor activity after bilateral microinjection into the rat nucleus accumbens: possible dependence on dopaminergic mechanisms. *Neuropharmacol.*, **22**, 971–981.

DONZANTI, B.A. & URETSKY, N.J. (1984). Antagonism of the hypermotility response induced by excitatory amino acids in the rat nucleus accumbens. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325**, 1–7.

FRENCH, E.D. (1992). Competitive NMDA receptor antagonists attenuate phencyclidine-induced excitation of A10 dopamine neurons. *Eur. J. Pharmacol.*, **271**, 1–7.

FRENCH, E.D., FERKANY, J., ABREU, M. & LEVENSON, S. (1991). Effects of competitive N-methyl-D-aspartate antagonists on midbrain dopamine neurones: an electrophysiological and behavioural comparison to phencyclidine. *Neuropharmacol.*, **30**, 1039–1046.

FRENCH, E.D., PILAPIL, C. & QUIRION, R. (1985). Phencyclidine binding sites in the nucleus accumbens and phencyclidine-induced hyperactivity are decreased following lesions of the mesolimbic dopamine system. *Eur. J. Pharmacol.*, **116**, 1–9.

FRENCH, E.D. & VANTINI, G. (1984). Phencyclidine-induced locomotor activity in the rat is blocked by 6-hydroxydopamine lesion of the nucleus accumbens: comparisons to other psychomotor stimulants. *Psychopharmacol.*, **82**, 83–88.

HAMILTON, M.B., DE BELLEROCHE, J.S., GARDINER, I.M. & HERBERG, L.J. (1986). Stimulatory effect of N-methyl aspartate on locomotor activity and transmitter release from rat nucleus accumbens. *Pharmacol. Biochem. Behav.*, **25**, 943–948.

HAYES, B.A. & BALSTER, R.L. (1985). Anticonvulsant properties of phencyclidine-like drugs in mice. *Eur. J. Pharmacol.*, **117**, 121–125.

HUTSON, P.H., BRISTOW, L.J., THORN, L. & TRICKLEBANK, M.D. (1991). R-(+)-HA-966, a glycine/NMDA receptor antagonist, selectively blocks the activation of the mesolimbic dopamine system by amphetamine. *Br. J. Pharmacol.*, **103**, 2037–2044.

HUTSON, P.H. & SUMAN-CHAUHAN, N. (1990). Activation of postsynaptic striatal dopamine receptors, monitored by efflux of cAMP in vivo. *Neuropharmacol.*, **29**, 1011–1016.

JAVITT, D.C. & ZUKIN, S.R. (1991). Recent advances in the phencyclidine model of schizophrenia. *Am. J. Psychiat.*, **148**, 1301–1308.

JOHNSON, K.M. (1983). Phencyclidine: behavioural and biochemical evidence supporting a role for dopamine. *Fed. Proc.*, **42**, 2579–2583.

JONES, S.M., SNELL, L.D. & JOHNSON, K.M. (1987). Inhibition by phencyclidine of excitatory amino acid-stimulated release of neurotransmitters in the nucleus accumbens. *Neuropharmacol.*, **26**, 173–179.

KABUTO, H., YOKOI, I., MIZUKAWA, K. & MORI, A. (1989). Effects of an N-methyl-D-aspartate receptor agonist and its antagonist CPP on the levels of dopamine and serotonin metabolites in rat brain striatum collected in vivo by using a brain dialysis technique. *Neurochem. Res.*, **14**, 1075–1080.

LARGENT, B.L., GUNDLACH, A.L. & SNYDER, S.H. (1984). Psychotomimetic opiate receptors labelled and vizualized with (+)-[^{3}H]-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 4983–4987.

LARGENT, B.L., GUNDLACH, A.L. & SNYDER, S.H. (1986). Pharmacological and autoradiographic discrimination of sigma and phencyclidine receptor binding sites in brain with (+)-[^{3}H]-SKF-10,047, (+)-[^{3}H]-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine and [^{3}H]-1-[1-(2-thienyl)cyclohexyl]piperdine. *J. Pharmacol. Exp. Ther.*, **238**, 739–748.

LARGENT, B.L., WIKSTROM, H., SNOWMAN, A.M. & SNYDER, S.H. (1988). Novel antipsychotic drugs share high affinity for σ receptors. *Eur. J. Pharmacol.*, **155**, 345–347.

LODGE, D. & JOHNSON, K.M. (1990). Non-competitive excitatory amino acid receptor antagonists. *Trends Pharmacol. Sci.*, **11**, 81–86.

MAURICE, T., VIGNON, J., KAMENKA, J.M. & CHICHEPORTICHE, R. (1991). Differential interaction of phencyclidine-like drugs with the dopamine uptake complex in vivo. *J. Neurochem.*, **56**, 553–559.

MENDELSOHN, L.G., KERCHNER, G.A., KALRA, V., ZIMMERMAN, D.M. & LEANDER, J.D. (1984). Phencyclidine receptors in rat brain cortex. *Biochem. Pharmacol.*, **33**, 3529–3535.

O'NEILL, K.A., CARELLI, R.M., JARVIS, M.F. & LIEBMAN, J.M. (1989). Hyperactivity induced by N-methyl-D-aspartate injections into nucleus accumbens: lack of evidence for mediation by dopaminergic neurones. *Pharmacol. Biochem. Behav.*, **34**, 739–745.

PAXINOS, G. & WATSON, C. (1982). *The Rat Brain in Stereotaxic Coordinates*. Sydney: Academic Press.

PULVIRENTI, L., SWERDLOW, N.R. & KOOB, G.F. (1991). Nucleus accumbens NMDA antagonist decreases locomotor activity produced by cocaine, heroin or accumbens dopamine, but not caffeine. *Pharmacol. Biochem. Behav.*, **40**, 841–845.

PYCOCK, C.J. & DAWBARN, D. (1980). Acute motor effects of N-methyl-D-aspartic acid and kainic acid applied focally to mesencephalic dopamine cell body regions in the rat. *Neurosci. Letts.*, **18**, 85–90.

QUIRION, R., HAMMER, R.P., HERKENHAM, M. & PERT, C.B. (1981). Phencyclidine (angel dust)/sigma opiate receptor: vizualisation by tritium sensitive film. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 5881–5885.

RANSOM, R.W. & DESCHENES, N.L. (1989). Glycine modulation of NMDA-evoked release of [^{3}H]-acetylcholine and [^{3}H]-dopamine from rat striatal slices. *Neurosci. Letts.*, **96**, 323–328.

RAO, T.S., KIM, H.S., LEHMANN, J., MARTIN, L.L. & WOOD, P.L. (1989). Differential effects of phencyclidine (PCP) and ketamine on mesocortical and mesostriatal dopamine release in vivo. *Life Sci.*, **45**, 1065–1072.

RAO, T.S., KIM, H.S., LEHMANN, J., MARTIN, L.L. & WOOD, P.L. (1990). Selective activation of dopaminergic pathways in the mesocortex by compounds that act at the phencyclidine (PCP) binding site: tentative evidence for PCP recognition sites not coupled to N-methyl-D-Aspartate (NMDA) receptors. *Neuropharmacol.*, **29**, 225–230.

ROTHMAN, R.B., REID, A.A., MONN, J.A., JACOBSON, A.E. & RICE, K.C. (1989). The psychotomimetic drug phencyclidine labels two high affinity binding sites in guinea pig brain: evidence for N-methyl-D-aspartate coupled and dopamine reuptake carrier-associated phencyclidine binding sites. *Mol. Pharmacol.*, **36**, 887–896.

SINGH, L., DONALD, A.E., FOSTER, A.C., HUTSON, P.H., IVERSEN, L.L., IVERSEN, S.D., KEMP, J.A., LEESON, P.D., MARSHALL, G.R., OLES, R.J., PRIESTLEY, T., THORN, L., TRICKLEBANK, M.D., VASS, C.A. & WILLIAMS, B.J. (1990a). Enantiomers of HA-966 (3-amino-1-hydroxypyrrolid-2-one) exhibit distinct central nervous system effects: (+)-HA-966 is a selective glycine/N-methyl-D-aspartate receptor antagonist, but (−)-HA-966 is a potent γ-butyrolactone-like sedative. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 347–351.

SINGH, L., MENZIES, R. & TRICKLEBANK, M.D. (1990b). The discriminative stimulus properties of (+)-HA-966, an antagonist at the glycine/N-methyl-D-aspartate receptor. *Eur. J. Pharmacol.*, **186**, 129–132.

SINGH, L., WONG, E.H.F., KESINGLAND, A.C. & TRICKLEBANK, M.D. (1990c). Evidence against involvement of the haloperidol-sensitive sigma recognition site in the discriminative stimulus properties of (+)-N-allylnormetazocine ((+)-SKF 10,047). *Br. J. Pharmacol.*, **99**, 145–151.

SNELL, L.D., MUELLER, Z.L., GANNON, R.L., SILVERMAN, P.B. & JOHNSON, K.M. (1984). A comparison between classes of drugs having phencyclidine-like behavioural properties on dopaminergic efflux in vitro and dopamine metabolism in vivo. *J. Pharmacol. Exp. Ther.*, **231**, 261–269.

SNYDER, S.H. (1980). Phencyclidine. *Nature*, **285**, 355–356.

SU, T.P. (1982). Evidence for sigma opioid receptor: binding of [^{3}H]SKF-10,047 to etorphine-inaccessible sites in guinea pig brain. *J. Pharmacol. Exp. Ther.*, **223**, 284–290.

TAM, S.W. & COOK, L. (1984). Sigma opiates and certain antipsychotic drugs mutually inhibit (+)-[^{3}H]-SKF 10,047 and [^{3}H]-haloperidol binding in guinea pig brain membranes. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 5618–5621.

TANII, Y., NISHIKAWA, T., HASHIMOTO, A. & TAKAHASHI, K. (1991). Stereoselective inhibition by D- and L-alanine of phencyclidine-induced locomotor stimulation in the rat. *Brain. Res.*, **563**, 281–284.

TRICKLEBANK, M.D., SINGH, L., OLES, R.J., WONG, E.H.F. & IVERSEN, S.D. (1987). A role for receptors of N-methyl-D-aspartic acid in the discriminative stimulus properties of phencyclidine. *Eur. J. Pharmacol.*, **141**, 497–501.

TRICKLEBANK, M.D., SINGH, L., OLES, R.J., PRESTON, C. & IVERSEN, S.D. (1989). The behavioural effects of MK-801: a comparison with antagonists acting non-competitively and competitively at the NMDA receptor. *Eur. J. Pharmacol.*, **167**, 127–135.

TRICKLEBANK, M.D. & SAYWELL, K. (1990). Behavioural properties of antagonists acting at the glycine modulatory site on the NMDA receptor/ion channel complex. *Neurosci. Abst.*, **16**, 462.

VICKROY, T.W. & JOHNSON, K.M. (1982). Similar dopamine-releasing effects of phencyclidine and non-amphetamine stimulants in striatal slices. *J. Pharmacol. Exp. Ther.*, **223**, 669–674.

VIGNON, J., PINEK, V., CERRUTI, C., KAMENKA, J.M. & CHICHEPORTICHE, R. (1988). [^{3}H]-N-[1-(2-benzo(b)thiophenyl-cyclohexyl]piperidine ([^{3}H]BTCP): a new phencyclidine analog selective for the dopamine uptake complex. *Eur. J. Pharmacol.*, **148,** 427–436.

WEBER, E., SONDERS, M., QUARUM, M., MCLEAN, S., POU, S., & KEANA, J.F.W. (1986). 1,3-Di(2-[5-^{3}H]tolyl)guanidine: a selective ligand that labels sigma-type receptors for psychotomimetic opiates and antipsychotic drugs. *Proc. Natl. Acad. Sci. U.S.A.*, **83,** 8784–8788.

WILLETTS, J. & BALSTER, R.L. (1988). The discriminative stimulus effects of N-methyl-D-aspartate antagonists in phencyclidine-trained rats. *Neuropharmacol.*, **27,** 1249–1256.

WONG, E.H.F., KEMP, J.A., PRIESTLY, T., KNIGHT, A.R., WOODRUFF, G.N. & IVERSEN, L.L. (1986). The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **83,** 7104–7108.

WONG, E.H.F., KNIGHT, A.R. & WOODRUFF, G.N. (1988). [^{3}H]-MK-801 labels a site on the N-methyl-D-aspartate receptor channel complex in rat brain membranes. *J. Neurochem.*, **50,** 274–281.

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Analysis of the CCK_B receptor antagonism of virginiamycin in guinea-pig ileum longitudinal myenteric plexus

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1 Virginiamycin, a macrolide reported to bind selectively to CCK_B/gastrin receptors has been studied in a functional test, namely cholecystokinin-induced contraction of guinea-pig ileum myenteric plexus (LMMP).

2 Virginiamycin (1–10 μM) antagonized the selective CCK_B agonist cholecystokinin tetrapeptide (CCK-4). The antagonism appeared not to be competitive as the highest concentration (10 μM) caused a reduction of its maximal effect. An apparent pA_2 of 6.64 ± 0.06 (s.e.) could be estimated if this depression was ignored. The selective CCK_B antagonist, L-365,260 (0.01–0.3 μM) antagonized competitively the CCK-4 induced contraction and a pK_B of 8.60 ± 0.16 (s.e.) was estimated.

3 The combined dose-ratio analysis for virginiamycin, tested at 3 and 10 μM in association with 0.03 and 0.1 μM L-365,260, respectively, resulted in observed log dose-ratios of 1.39 and 1.53. That was consistent with both antagonists acting on the same receptor in LMMP.

4 These data, represent the first evidence of the antagonism of virginiamycin in a functional assay and they support the hypothesis of homogeneity between CCK_B receptors in the CNS and in peripheral tissues.

Keywords: Guinea-pig ileum; cholecystokinin; L-365,260; virginiamycin; CCK_B receptor

Introduction

Cholecystokinin (CCK) causes marked peripheral and central effects by interacting with two different types of receptor, CCK_A and CCK_B (Bock, 1991). The former is largely distributed in the periphery and the latter is found in most abundance in the CNS (Bock *et al.*, 1989; Woodruff & Hughes, 1991). The two receptors have been distinguished by use of both selective agonists (e.g. CCK-4 for CCK_B receptors) and selective antagonists (e.g. L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3yl)-1H-indole-2-carboxamide) for CCK_A receptors and L-365,260 ((3R(+)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3yl)-N-(3-methylphenyl)-urea) for CCK_B receptors, Chang & Lotti, 1986; Lotti & Chang, 1989). Differences between CCK_B receptors and gastrin receptors, although suggested (Patel & Spraggs, 1992; Botella *et al.*, 1992), have, as yet, not been clearly demonstrated. Recently, the guinea-pig ileum longitudinal muscle myenteric plexus (LMMP) has been shown to contain neuronal receptors for both the CCK_A and CCK_B sub-types (Lucaites *et al.*, 1991; Dal Forno *et al.*, 1992). Thus, in the presence of a selective CCK_A receptor antagonist such as L-364,718, LMMP could be considered as a functional assay for CCK_B receptors.

Selective antagonists for the CCK_B receptor, so far described, tend to fall into a number of discrete chemical classes. These include the benzodiazepines, peptoids and the pyrazolidinones (Kervin, 1991). In addition, virginiamycin, a macrolide antibiotic produced by fermentation of a strain of *Streptomyces olivaceous*, has been reported to bind to CCK_B/gastrin receptors (Lam *et al.*, 1991). Thus, in radiolabelled binding studies virginiamycin has an IC_{50} (nM) of 571 and 710 for CCK_B (guinea-pig brain) and gastrin (guinea-pig gastric glands) receptors, respectively, with no apparent affinity for CCK_A (rat pancreas) receptors (> 100,000 nM). To date it has not been reported whether virginiamycin acts as an agonist or an antagonist at CCK_B receptors. Thus the objective of the study described below was to determine and to quantify the nature of the interaction of virginiamycin with CCK_B receptors in a functional assay, namely LMMP.

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Methods

Male Dunkin-Hartley guinea-pigs weighing approximately 300–400 g were allowed free access to food and water until just before use. The animals were killed by cervical dislocation and 10 cm of terminal ileum was rapidly removed and placed in a modified Krebs solution (composition, mM: NaCl 118.5, $CaCl_2$ 1.2, KCl 4.7, $MgSO_4$ 0.6, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11.1). A 4 cm length of ileum was placed over a glass-pipette and the longitudinal muscle with adherent myenteric plexus was removed with a cotton tip. The preparations were set-up in 2 ml sylanized (5% dimethylchloroxylane 95% toluene) baths at 37°C containing the Krebs solution gassed with a 5% CO_2:95% O_2 mixture. The muscle was attached to an isotonic transducer (Ugo Basile 7006) coupled to a polygraph (Ugo Basile Gemini 7070 and Unirecord 7050). The tissues were washed and adjusted to a tension of 0.5 g. After a resting phase of 60 min the tissues were challenged several times with 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) 30 μM at a cycle time of 10 min until a constant response was achieved.

Experimental protocol

All the experiments described here were performed with Krebs solution containing L-364,718 at 10 nM. Each tissue received only one treatment. Serial concentration-response curves (CRC) for the agonist CCK-4 were run at intervals of 10 min. When evaluating the effect of the antagonist virginiamycin, L-365,260 or the mixture of both, a 60 min contact time was allowed before the first administration of the agonist. The antagonist or the mixture of antagonists was left in contact with the tissue throughout the duration of the experiment. The contractions measured were expressed as a percentage of the effect obtained with 30 μM DMPP.

Analysis of data

Curve fitting The CRC data were routinely fitted by a four parameter logistic equation (De Lean *et al.*, 1978):

$$\text{response} = a + \frac{R_{max}\,[A]^n}{[A]^n + [A_{50}]^n} \quad (1)$$

where a and R_{max} are the minimum and maximal asymptotes respectively, [A] the agonist concentration, n the slope factor and $[A_{50}]$ the concentration of the agonist that induces 50% of the maximal effect (ED_{50}). Antagonism were tested for significant differences in parallelism and of maximal responses in comparison with the control. If no significant difference was found, the analysis of the antagonist effect was performed.

Analysis of individual antagonist effects Curve displacements by antagonists applied singly were analysed fitting CRC data to the equation:

$$\text{response} = \frac{R_{max}\,[A]^n}{(([A_{50}]^n)(1 + ([B]^m/K_B))^n + [A]^n)} \quad (2)$$

where R_{max}, [A], n and $[A_{50}]$ are as above defined, B is the antagonist concentration, m is the equivalent to the Schild slope parameter and K_B is the antagonist dissociation constant when m is not significantly different from one.

Combined dose-ratio analysis This analysis was performed according to the procedure developed by Shankley *et al.* (1988). The theory predicts that when two antagonists (A and C) act through an interaction at the same receptor to produce their effect (additive model), then the measured dose-ratio should be:

$$DR_{A+C} = DR_A + DR_C - 1 \quad (3)$$

where DR_A and DR_C are the dose ratio obtained independently in the presence of the antagonist A and C. The model was tested by comparing the discrepancy between observed and expected statistics for the models with estimates of their standard errors. Thus, for the additive model:

$$S_A = \log ED_{50AC} - \log(ED_{50A} + ED_{50C} - ED_{50}) \quad (4)$$

where ED_{50AC}, ED_{50A}, ED_{50C} and ED_{50} are the concentrations of agonist that induce 50% of response in the presence of the mixture of the antagonists AC, only A or only C and in the absence of both (control) respectively. The term S_A is the test statistic of the model which will have a value of zero when the data comply with the model.

Similarly, when the two antagonists act independently (multiplicative model) the measured dose-ratio should be:

$$DR_{A\cdot C} = DR_A * DR_C \quad (5)$$

For the multiplicative model the test statistic of the model is:

$$S_M = \log ED_{50AC} + \log ED_{50} - \log ED_{50A} - \log ED_{50C} \quad (6)$$

so that S_M, the test statistic will be zero when the data comply with the model.

From the combined dose ratio analysis the dissociation constant (K_Bv) for virginiamycin was estimated using the equation (Black *et al.*, 1986; Leff & Morse, 1987; Trist *et al.*, 1987):

$$DR_{AC} = 1 + \frac{[A]}{K_B I(1 + [C]/K_B v)} \quad (7)$$

where DR_{AC} in this case is the dose-ratio obtained with both antagonists (A and C) together, measured from the curve produced in the presence of C alone, [A] is the concentration of the standard (L-365,260) and K_BI is its dissociation constant.

Values are given ± s.e.mean.

Drugs used

The following drugs were used: cholecystokinin tetrapeptide (CCK-4) and DMPP, both from Sigma. L-364,718, L-365,260 and virginiamycin were synthesized by the chemistry department of Glaxo Italy. DMPP was dissolved in water, whereas CCK-4 (at 30 mM) and L-364,718, L-365,260 and virginiamycin (all three at 100 μM) were dissolved in dimethylsulphoxide. Further dilutions were made in distilled water. Vehicles were found not to produce significant effects on the CCK-4-induced contraction.

Results

CCK-4 (0.01–300 μM), as has already been reported (Dal Forno *et al.*, 1992), contracted the LMMP showing a biphasic CRC. In the same study it was demonstrated that this effect was due to the activation of both types of CCK receptors, with a higher potency of CCK-4 for the CCK_B type. In the presence of L-364,718 at 10 nM, the CCK-4 CRC changes into a mono-phasic curve with a suppression of the upper part, the CCK_A-activated region (Figure 1). To avoid possible interaction with CCK_A receptors, all further experiments described in the present study were carried out in the presence of 10 nM L-364,718.

Analysis of individual antagonistic effects

L-365,260 (0.01–0.3 μM) antagonized CCK-4-induced contractions in a competitive manner (Figure 2). The Schild slope parameter was 1.25 ± 0.15 (95% CL 0.93–1.56) which was not significantly different from one ($P = 0.10$). The analysis of the antagonism resulted in a pK_B of 8.60 ± 0.16 (Figure 2). Virginiamycin (1–10 μM) antagonized in a dose-related manner the CCK-4 CRC. However, at the highest concentration, virginiamycin caused a significant ($P = 0.001$) reduction of the maximal response to the agonist (Figure 3). An apparent dissociation constant (pA_2) was, however, estimated without any assumption of the mechanism of action. It was 6.64 ± 0.06. Comparing the two antagonists, virginiamycin is about 100 fold less potent than L-365,260. Virginiamycin over the range 0.3 to 10 μM did not contract the LMMP (data not shown).

Combined dose-ratio analysis

The effect of the two antagonists acting alone and in combination are shown in Figures 4 and 5. The concentrations were chosen to give a similar individual displacement. Figure 4 demonstrates the effect of L-365,260 0.03 μM ($n = 4$) and virginiamycin 3 μM ($n = 4$) when given alone or in combination ($n = 4$). Thus, if addition of the dose-ratio occurred then

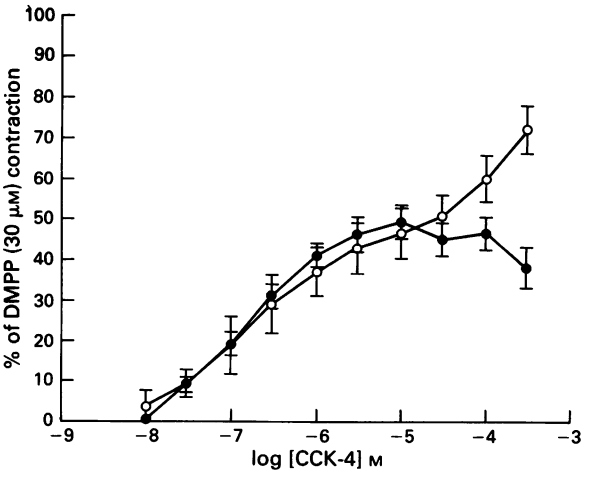


Figure 1 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of 10 nM L-364,718 (●). Each point represents the mean ± s.e. of 7 replications.

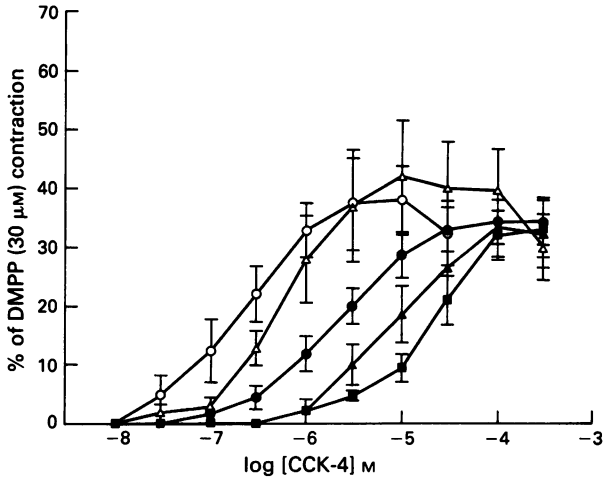


Figure 2 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of L365,260: 0.01 μM (△), 0.03 μM (●), 0.1 μM (▲) and 0.3 μM (■). All the experiments were carried out in the presence of 10 nM L-364,718 and each point is the mean ± s.e. of 5–10 replications.

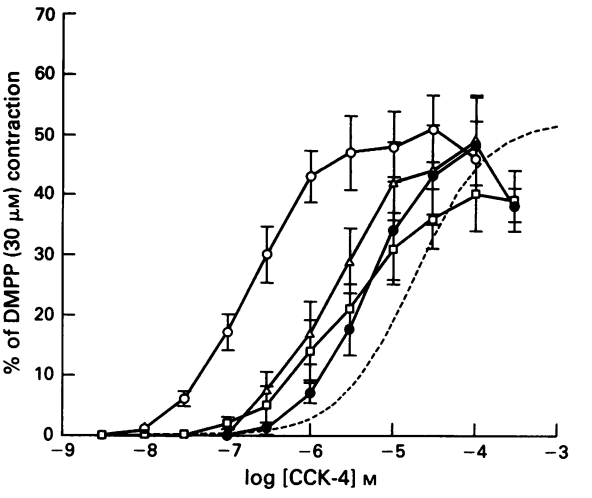


Figure 4 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of 3 μM virginiamycin (△), 0.03 μM L-365,260 (□) and a combination of 3 μM virginiamycin and 0.03 μM L-365,260 (●). All the experiments were carried out in the presence of 10 nM L-364,718 and each point is the mean ± s.e. of 4 replications. The dashed line shows the location of the CCK-4 concentration-response curve which was predicted by assuming that the antagonists acted independently.

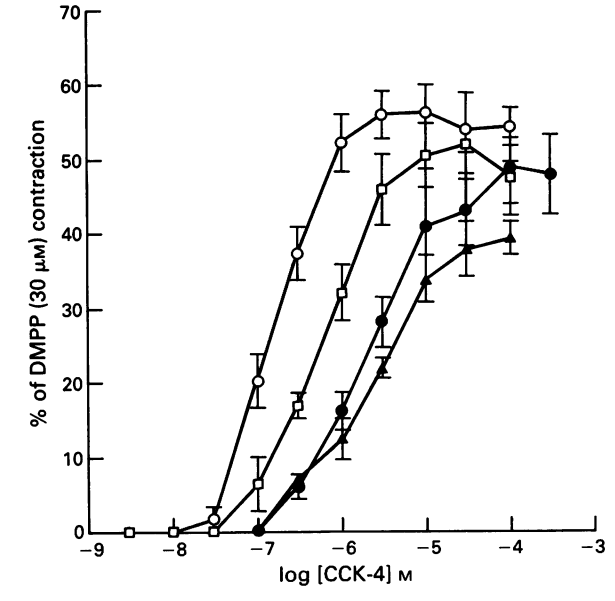


Figure 3 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of virginiamycin: 1 μM (□), 3 μM (●) and 10 μM (▲). All the experiments were carried out in the presence of 10 nM L-364,718 and each point is the mean ± s.e. of 4 replications.

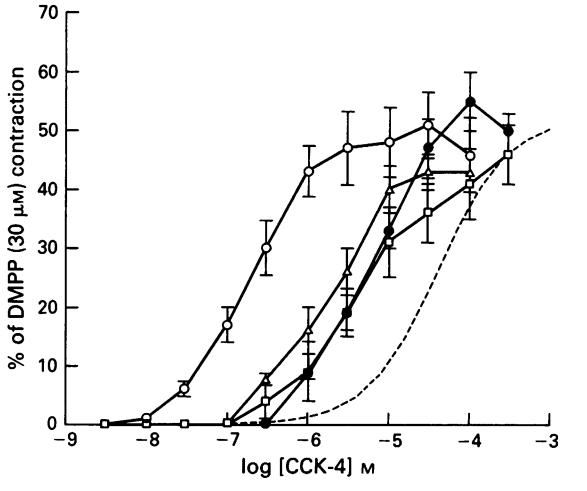


Figure 5 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of 10 μM virginiamycin (△), 0.1 μM L-365,260 (□) and a combination of 10 μM virginiamycin and 0.1 μM L-365,260 (●). All the experiments were carried out in the presence of 10 nM L-364,718 and each point is the mean ± s.e. of 4 replications. The dashed line shows the location of the CCK-4 concentration-response curve which was predicted by assuming that the antagonists acted independently.

the predicted combined log dose-ratio would have been 1.32. On the other hand if the dose-ratios were multipled, then the log dose-ratio would have been 2.06. Figure 5 shows the effect of higher concentrations of the two antagonists, L-365,260 0.1 μM ($n = 4$) and virginiamycin 10 μM ($n = 4$) when given alone or in combination ($n = 4$). With these concentrations, if addition of the dose-ratios occurred then the predicted log dose-ratio would have been 1.49. For the multiplicative model the predicted log dose-ratio would have been 2.37. The data obtained in both experiments were consistent with the additive model and rejected the multiplicative model. In fact the observed log dose-ratios were 1.39 and 1.53 (Table 1). A simulation of the multiplicative model for both experiments have been included in both Figures 4 and 5. From the combined dose-ratio analysis the pK_B for virginiamycin estimated from equation (7) was 6.20 and 6.14 when virginiamycin was tested at 3 and 10 μM respectively.

Discussion

To date, little has been reported on CCK_B antagonists in *in vitro* functional assays. Of those antagonists so far described, L-365,260, the first selective CCK_B antagonist discovered, has become somewhat of a standard, particularly in biochemical and *in vivo* assay (Bock *et al.*, 1989). Also, L-365,260 has

Table 1 Combined dose-ratio analysis of virginiamycin with L-365,260 used as a standard CCK_B antagonist

	A	L-365,260 (0.03 μM)	L-365,260 (0.1 μM)
	C	VGM (3 μM)	VGM (10 μM)
Observed	$\log R_A$	1.11	1.33
	$\log R_C$	0.94	1.06
Multiplicative expected	$\log R_{A\cdot C}$	2.06	2.37
Additive expected	$\log R_{A+C}$	1.32	1.49
observed	$\log R_{AC}$	1.39	1.53
$S_A \pm$ s.e.		0.075 ± 0.167	0.027 ± 0.155
$S_M \pm$ s.e.		0.660 ± 0.292*	0.810 ± 0.076*

*Significantly different from zero ($P<0.05$).
VGM = virginiamycin
S_A and S_M were derived as described in the methods.

been analysed functionally *in vitro* in the guinea-pig ileum (Lucaites *et al.*, 1991; Dal Forno *et al.*, 1992).

In these studies its estimated affinity (pK_B) was similar to that reported in binding studies using brain homogenates (Bock *et al.*, 1989). Thus, in this study L-365,260 has been taken as the standard antagonist against which to test virginiamycin.

The structure of virginiamycin is considerably different from other known CCK_B receptor ligands. This raises the questions as to the nature of its interaction with the receptor (agonist or antagonist) and whether virginiamycin might discriminate between central and peripheral CCK_B receptors. These questions do not appear to have been addressed previously, as until now, only binding data have been reported.

In the LMMP, virginiamycin showed antagonistic activity for CCK-4, in conditions where CCK_A receptor activation had been blocked. No agonism was observed. However, the antagonism was not of a simple competitive nature as at 10 μM a significant reduction of the maximal response was seen. To evaluate further whether this depression is related to another mechanism of action of virginiamycin, combined dose ratio analysis was applied. This was justified since L-365,260 was available as a reliable standard antagonist. Combined dose-ratio analysis has become a powerful method for disecting out complex antagonism. In this case the results are consistent with a model of L-365,260 and virginiamycin both acting on the same receptor. In fact, in experiments where L-365,260 and virginiamycin were applied in combination, a protection of the depressant effect was observed. Thus, the reduction of the maximal response seems to be related to an interaction of virginiamycin with the CCK_B receptor. This might indicate that virginiamycin at high concentrations has a relatively slow off rate, or that a mechanism similar to that described by Liu *et al.* (1992) is involved, where antagonists might change the efficacy of an agonist. Further experiments are needed to elucidate those possibilities. The pK_B estimated for virginiamycin from the combined dose-ratio analysis was 6.2 which seemed to reflect its true affinity for virginiamycin on the CCK_B receptor in LMMP as this value was not significantly different from the affinity estimated in binding assays.

This was calculated from the IC_{50} values reported by Lam *et al.* (1991) as 6.24 using a concentration of radioligand equal to or less than its K_A value. The pA_2 calculated for virginiamycin using Schild analysis, where the depressant property has been ignored, gives a larger value of 6.64. The difference between this and the estimated pK_B is significant ($P<0.05$) using the standard error on the pA_2 estimation. It is not possible to define the standard error from the combined dose-ratio analysis as all of the data was pooled to perform the analysis. The difference probably reflects the influence of the depressant effect.

A comparison between the pK_B estimated in our study with the pK_i obtained from binding data, as decribed above (Lam *et al.*, 1991), suggests that virginiamycin does not discriminate between peripheral and central CCK_B receptors. The close convergence between the two estimates (6.2 and 6.24, respectively), however, does rely on the assumption that the pK_i can be obtained from the IC_{50} using the Cheng & Prusoff equation (1973). This itself requires that the antagonist–receptor interaction follows the law of mass-action.

In conclusion, these results have shown, for the first time, that virginiamycin acts as CCK_B receptor antagonist in a functional assay.

References

BLACK, J.W., GERSKOWITCH, V.P., LEFF, P. & SHANKLEY, N.P. (1986). Analysis of competitive antagonism when this property occurs as part of a pharmacological resultant. *Br. J. Pharmacol.*, **89**, 547–555.

BOCK, M. (1991). Development of non peptide cholecystokinin type B receptor antagonists. *Drugs of the Future*, **16**, 631–640.

BOCK, M.H., DIPARDO, R.M., EVANS, B.E., RITTLE, K.E., WHITTER, W.L., VEBER, D.F., ANDERSON, P.S. & FREIDINGER, R.M. (1989). Benzodiazepine gastrin and brain cholecystokinin receptor ligands: L-365,260. *J. Med. Chem.*, **89**, 13–16.

BOTELLA, A., DELVAUX, M., BERRY, P., FREXINOS, J. & BUENO, L. (1992). Cholecystokinin and gastrin induced cell contraction in pig ileum by interacting with different receptor subtypes. *Gastroenterology*, **102/3**, 779–786.

CHANG, R.S.L. & LOTTI, V.J. (1986). Biochemical and pharmacological characterisation of an extremely potent and selective non-peptide cholecystokinin antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 4923–4926.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (Ki) and the concentration of inhibitor that causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

DAL FORNO, G., PIETRA, C., URCIUOLI, M., VAN AMSTERDAM, F., TOSON, G., GAVIRAGHI, G. & TRIST, D. (1992). Evidence for two cholecystokinin receptors mediating the contraction of the guinea pig isolated ileum longitudinal-myenteric plexus. *J. Pharmacol. Exp. Ther.*, **261**, 1056–1063.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.*, **235**, 97–102.

KERVIN, J.F. Jr. (1991). Further developments in cholecystokinin antagonist research. *Drugs of the Future*, **16**, 1111–1119.

LAM, Y.K.T., BOGEN, D., CHANG, R.S., FAUST, K.A., HENSENS, O.D., ZINC, D.L., SCHWARTZ, C.D., ZITANO, L., GARRITY, G.M., GAGLIARDI, M.M., CURRIE, S.A. & WOODRUFF, H.B. (1991). Novel and potent gastrin and brain cholecystokinin antagonists from streptomyces olivaceus. Taxonomy, fermentation, isolation, chemical conversions, and physico-chemical and biochemical properties. *J. Antibiotics*, **44**, 613–634.

LEFF, P. & MORSE, J.M. (1987). Resultant pharmacological actions of verapamile: quantification of competitive 5-hydroxytryptamine antagonism in combination with calcium antagonism. *J. Pharmacol. Exp. Ther.*, **240**, 284–287.

LIU, Y.J., SHANKLEY, N.P., WELSH, N.J. & BLACK, J.W. (1992). Evidence that the apparent complexity of receptor antagonism by angiotensin II analogues is due to a reversible and syntopic action. *Br. J. Pharmacol.*, **106**, 233–241.

LOTTI, V.J. & CHANG, R.S.L. (1989). A new potent and selective non-peptide gastrin antagonist and brain cholecystokinin receptor (CCK-B) ligand: L-365,260. *Eur. J. Pharmacol.*, **162**, 273–280.

LUCAITES, V.L., MENDELSOHN, L.G., MASON, N.R. & COHEN, M.L. (1991). CCK-8, CCK-4 and gastrin-induced contraction of guinea pig ileum: evidence for differential release of acetylcholine and substance P by CCK-A and CCK-B receptors. *J. Pharmacol. Exp. Ther.*, **256,** 695–703.

PATEL, M. & SPRAGGS, C.F. (1992). Functional comparison of gastrin/cholecystokinin receptors in isolated preparations of gastric mucosa and ileum. *Br. J. Pharmacol.*, **106,** 275–282.

SHANKLEY, N.P., BLACK, J.W., GANELLIN, C.R. & MITCHELL, R.C. (1988). Correlation between log P_{OCT} H_2O and pK_B estimates for a series of muscarinic and histamine H_2-receptor antagonists. *Br. J. Pharmacol.*, **94,** 264–274.

TRIST, D.G., LEFF, P., BLACK, J.W., GERSKOWITCH, V.P. & SHANKLEY, N.P. (1987). Resultant action of cimetidine in a cardiac adenylate cyclase assay; its elucidation by concentration-ratio analysis. *J. Pharmacol. Exp. Ther.*, **243/3,** 1043–1047.

WOODRUFF, G.N. & HUGHES, J. (1991). Cholecystokinin antagonists. *Annu. Rev. Pharmacol. Toxicol.*, **31,** 469–501.

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   —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.

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Failure to comply with *Instructions to Authors* may lead to substantial delays in processing, review and publication and may even jeopardize acceptance of the manuscript.

NOMENCLATURE

Authors are reminded that accepted receptor and associated terminology is laid out in *Nomenclature Guidelines for Authors*, as published in the *British Journal of Pharmacology, Br. J. Pharmacol.*, 1993, **108,** 282–284.

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